Bone Development and the Nervous System

GREGOR SISASK
Dissertation presented at Uppsala University to be publicly examined in Rosénalen, ingång 95/96, Akademiska barnsjukhuset, Uppsala, Friday, April 24, 2009 at 09:00 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in Swedish.

Abstract

Innervation of bone influence bone modeling, growth and remodeling. Pro-inflammatory cytokines released after tissue trauma are recognized as neurotrophic factors as well as factors influencing bone formation. The Wnt signaling pathway, essential for cell migration during embryogenesis is found to influence bone formation during fracture healing. Alterations in growth and bone formation are seen in denervating disorders and in manipulated Wnt signaling. The aim of the present thesis was to study; sensory and autonomic innervation in the developing skeleton in rats and mice, a possible influence on bone formation in IL-4 and IL-13 depleted mice, and fracture healing in altered Wnt signaling by glycogen synthase-3β inhibition in rats.

Bone innervation with sensory and autonomic nerves in modeling and growth follows a predictable and reproducible pattern both in the rat and in the mouse with sensory nerves occurring prior to autonomic nerves in areas with high chondrogenic and osteogenic activity. The time lag in occurrence between sensory and autonomic nerves indicates the importance of developmental timing between different nerve qualities in skeletal ontogeny. These findings give substantial morphologic support for important regulatory effects by the nervous system on bone development.

Depletion of the anti-inflammatory cytokines IL-4 and IL-13 production in mice resulted in an inhibited autonomic innervation and lack of implant capillary ingrowth, studied by DXBM implants. In fracture healing no differences between IL-4/13 knockout mice and wild type mice were found concerning fracture callus parameters, biomechanical properties or histology except that sensory and autonomic nerves were found in the bone marrow in knockout mice but not in wild type mice.

An altered canonical Wnt signaling was achieved by the GSK-3β inhibitor AR28. The increase in cytoplasmic β-catenin, due to inhibited degradation, resulted in a remarkable anabolic effect both on the fractured bone and on fracture healing. The histological analysis showed that the fractures healed without the usual formation of fibro-cartilage callus. This finding suggests that inhibition of GSK-3β inhibits the differentiation of chondrocytes and instead promotes the differentiation of mesenchymal progenitor cells into osteogenic cells.

Keywords: Neuropeptides, Bone innervation, Growth, Cytokines, Fracture healing, Wnt signaling

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ISSN 1651-6206
urn:nbn:se:uu:diva-99443 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-99443)
To Eva, Johanna and Fredrik
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


Reprints were made with permission from the respective publishers.
Front page: free interpretation of the close relationship between bone and brain.
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<tr>
<td>AR28</td>
<td>GSK-3β inhibitory drug</td>
</tr>
<tr>
<td>BMC</td>
<td>Bone mineral content</td>
</tr>
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<td>BMD</td>
<td>Bone mineral density</td>
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<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>cAMP</td>
<td>Adenosine 3’,5’ cyclic Mono Phosphate</td>
</tr>
<tr>
<td>Cbfa1</td>
<td>Core-binding factor family</td>
</tr>
<tr>
<td>CGRP</td>
<td>Calcitonin gene-related peptide</td>
</tr>
<tr>
<td>DRG</td>
<td>Dorsal root ganglion</td>
</tr>
<tr>
<td>DXBM</td>
<td>Demineralized xenogeneic bonematrix</td>
</tr>
<tr>
<td>ECM</td>
<td>Extra cellular matrix</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular-regulated protein kinase</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>GAP-43</td>
<td>Growth associated protein-43</td>
</tr>
<tr>
<td>GH</td>
<td>Growth hormone</td>
</tr>
<tr>
<td>GSK-3β</td>
<td>Glykogen synthase kinase-3β</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin and eosin</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IGF-1/2</td>
<td>Insulin growth factor-1and 2</td>
</tr>
<tr>
<td>IGFBP</td>
<td>Insulin growth factor binding protein</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>LRP5</td>
<td>Low density lipoprotein receptor-related protein-5</td>
</tr>
<tr>
<td>lb</td>
<td>pound (1 lb = 0.453 kg)</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage-colony stimulating factor</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stem cell</td>
</tr>
<tr>
<td>NPY</td>
<td>Neuropeptide Y</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PECAM-1</td>
<td>Platelet endothelium cell adhesion molecule-1</td>
</tr>
<tr>
<td>PGP 9.5</td>
<td>Protein gene product 9.5</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>POC</td>
<td>Primary ossification centre</td>
</tr>
<tr>
<td>pQCT</td>
<td>Peripheral quantitative comput. tomography</td>
</tr>
<tr>
<td>PTH</td>
<td>Parathyroid hormone</td>
</tr>
<tr>
<td>PTHrP</td>
<td>Parathyroid hormone related protein</td>
</tr>
<tr>
<td>RANK</td>
<td>Receptor activator of nuclear factor-κB</td>
</tr>
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RANKL  Receptor activator of NF-κB ligand
Rab23  Ras oncogene family
Smad  C elegans protein + 'mothers against' decapentaplegic
Shh   Sonic hedgehog
SOC   Secondary ossification centre
Sox9  Sex determining region Y, -box9
SP    Substance-P
SYN   Synaptophysin
TGF-β Transforming growth factor-β
Th2   T-helper 2 lymphocytes
TNF-α Tumor necrosis factor-α
TRAP  Tartrate-resistant acid phosphatase
VEGF  Vascular endothelial growth factor
VIP   Vasoactive intestinal peptide
Wnt  Wingless + int-1 (oncoprotein)
WT    Wild-type
INTRODUCTION

Bone and cartilage tissue

In man and other mammals bone and cartilage tissue are developmentally and functionally linked together. Both tissues are essential for growth and mobility, and serve as protectors of vital organs. Bone and cartilage are both connective tissues with a mesodermal origin and with the mesenchymal stem cell (MSC) as the cell of origin similar to fibroblasts, adipocytes and muscle cells.

The cartilage, mostly avascular, has a low metabolic activity and is mainly nourished by diffusion with difficulties to adapt and regenerate, as seen in osteoarthritis and cartilage damage. In contrast bone is richly vascularized and has a high biological activity with ability to adapt to changing demands and needs, as seen in growth and fracture healing.

In the skeleton there are two main types of bone, compact bone (cortical) and trabecular bone (cancellous) (Fig. 1). Both types are made up of lamellar bone, i.e. mineralized bone matrix where the type I collagen is arranged in alternate direction in a dense flat bone construction or in a multilayer helical construction, an osteon (Fig. 1). Both types of lamellar construction strive for an optimal strength in response to functional demands. Woven bone is less organized non-lamellar bone, seen in early fracture healing or in the bone of the skull and ribs.

A prerequisite for bone formation is the osteoblast, a MSC-derived cell, synthesizing extracellular matrix, the osteoid that can be mineralized. Osteocytes are concentrically arranged between lamellae, in lacunas, both in osteons and trabecular lamellae (Fig. 1). They are inter-connected by cell processes in close relation to nutrient capillaries and nerves. The osteons are mainly arranged parallel to the long bone axis hosting blood vessel and nerves in the Haversian and Volkmann’s canals (Fig. 1) (Cooper et al. 1966).

In the compact bone the osteoblasts and osteoclasts are located in the Haversian and Volkmann’s canals, but in the cancellous bone, where there are no Haversian canals, the blood vessels and nerves are located in the endosteum, covering the trabeculae and the rest of the long bone interior surface.
The long bones of the extremities consist of a long central part, the diaphysis, a tube of cortical bone hosting the medullary cavity (Fig. 1). The diaphysis meets the metaphysis and the cortical bone becomes thinner although supporting the trabecular bone that fills out the epiphysis. Between the metaphysis and epiphysis the growth plate is situated. During growth this plate consists of cartilage arranged in a highly organised way, i.e. the physis being a well defined and clearly visible structure, radiologically. When growth has ceased it becomes a thin but visible line. The outer surface of the diaphysis and part of the metaphysis are covered by an osteogenic connective tissue abundant in blood vessels, nerves and pluripotent MSC, the periosteum. The inner surface is covered by the endosteum, a connective tissue consisting of stromal MSC, blood vessels and nerves. The endings of the bone organ consist of articular cartilage. Nerves and blood vessels from the periosteum enter the bone tissue through Volkmann’s canals connecting to the endosteum and the bone marrow. Branches from Volkmann’s canals connect to the Harversian canals (Cooper et al. 1966). Vessels supplying the hind limbs originate from the iliac artery, femoral artery branching to the deep femoral and popliteal artery. Branches from these vessels provide the bone organ with nutrient separately to the epiphysis, supplying the growth plate and the articular cartilage and to the diaphysis supplying the osteons, the trabecular bone and the bone marrow. The nerves follow the same route of entry.
Formation of cartilage and bone

The templates of the extremity skeleton during embryogenesis consist of cartilage. Cartilage is built by chondroblasts and chondrocytes synthesizing extracellular matrix (ECM), or chondroid, consisting of type II collagen, hosting proteoglycans and glycoproteins. The water content is high. Occasionally this matrix can mineralize, e.g., in growth plate and primary ossification centre. Transforming growth factor ß, (TGF-ß), hypoxia, bone morphogenetic proteins (BMP) 2 and 7, insulin growth factor-1 (IGF-1), Wnt and sonic hedgehog (Shh) are important regulatory factors for chondrogenesis that induce collagen II and proteoglycans. A number of interesting findings have clarified the regulation at the genetic level, the Rab23gene promotes chondrogenic differentiation, and the Sox9 gene controls the expression of type II collagen and the proteoglycan aggregate as well as the differentiation of chondroprogenitor cells to chondroblasts and chondrocytes (Yang et al. 2008).

The two main types of bone formation are endochondral, where a preformed cartilage template is mineralized and intramembranous where osteoblasts lay down an osteoid that is mineralized without a preformed chondrous model. In both bone forming processes pluripotent MSC are recruited locally or from the circulation, and these cells differentiate into osteoblasts. The differentiation of osteoblasts is governed by a number of factors, most importantly the BMP 2, 4 and 7 (Urist et al. 1983, Chang et al. 2001). These proteins are members of the TGF-ß family and are essential in bone morphogenesis, growth and repair. Proliferation is also governed by a number of other growth factors and cytokines in a complex interaction, among them IL-4/13 (Ura et al. 2000).

In bone tissue ECM is produced by the osteoblasts and consists of osteoid together with a type I collagen network to which proteoglycans, e.g., glycosaminoglycans (GAG), non-collagenous proteins, osteocalcin, osteopontin, osteonectin, bone sialoproteins and other bone related proteins adhere (Aukland and Reed 1993). Mineralization of the osteoid by deposition of calcium phosphate (hydroxyapatite) is dependent on sufficient blood supply to allow the presence of highly differentiated osteoblasts. This process is stimulated by vitamin D and its metabolites calcitriol (1,25(OH₂)D₃) and calcidiol (25OHD). Genetically the osteoblasts are governed by the Cbfa1-gene, induced by BMP7, to differentiate and synthesize the osteoid, to be mineralized and osteocalcin which initiates mineralization. In addition, the osteoblasts synthesize several factors involved in bone formation and remodeling as BMP, macrophage colony-stimulating factor (M-CSF), receptor for activation of nuclear factor kappa B (RANKL) and osteoprotegerin (OPG). Several osteoblast receptors bind ligands influencing bone formation; sex hormones, growth hormone (GH), Ca²⁺-regulating hormones such as, parathyroid hormone (PTH), parathyroid hormone - related protein (PTHRP), calcitonin, vitamin D₃, cytokines (IL-1, IL-4, IL-6, IL-13), neuropeptides (CGRP, SP, NPY, VIP), fibroblast growth factor (FGF), Wnt (Spencer et al. 2006) and leptin (Kwun et al. 2007).
Modeling and remodeling

The capability in bone to rebuild, *i.e.* to model and remodel, according to changing needs, mechanical as well as metabolic, is present throughout life (Buckwalter et al. 1996, Frost 2001). In modeling, the bone-forming osteoblasts work in an uncoupled manner to the bone-resorbing osteoclasts, while in remodeling the osteoblasts and osteoclasts function in a coupled manner resorbing “old bone” and building “new bone”. Modeling is first seen during the gestational period, in flat bone synthesis and in the inner layer of the perichondrium. Recruitment and differentiation of pluripotent cells, MSC to the osteoprogenitor lineage is governed by Wnt (Gaur et al. 2005), Cbfa1 induced by BMP7 (Tou et al. 2003) and TGFβ (Alliston et al. 2001) to form the bone collar in the long bone diaphyseal primary ossification centres. Modeling is also seen in appositional growth, *i.e.* the widening of the long bone metaphyses (Buckwalter et al. 1996). After growth has ceased modeling occurs during correction of deformities after fracture (Li et al. 2005) and at a slow rate in adaptation to changes in load (Frost et al. 1989, Parfitt 1994 and 2004).

Remodeling is first seen in the primary ossification centre, in the growth plate, and in the endosteal site of the long bones. The basic multicellular unit (BMU) consists of synthesizing osteoblasts and bone resorbing osteoclasts (Frost 1979). The osteoclast of hematopoietic origin, derived from the monocyte-macrophage progenitor cell lineage is recruited and activated by osteoblast-synthezised M-CSF and RANKL. After connecting to the bone surface, anchored by integrins (Duong 2000) the osteoclast resorbs bone by acidification by tartrate-resistant acid phosphatase (TRAP), and proteolysis (Teitelbaum 2000).

Fracture healing

An inflammatory response is seen as a general reaction to trauma, (Lenz et al. 2007). In the fracture site there is an invasion of MSC, fibroblasts and inflammatory cells secreting pro-inflammatory cytokines (Zellweger et al. 1995, Mack et al. 1996, Meert et al. 1998). Peptide signaling molecules, such as BMP and platelet derived growth factor (PDGF), add to the fracture hematoma initiating triggering events such as an early vascularization (Singh and Nigam 1983) providing necessary nutrients to the growing callus tissue (Folkman and Shing 1992). Also relative tissue hypoxia stimulates hypoxia inducible factor 1-α (Hif1-α) which induces the expression of vascular endothelial growth factor (VEGF). Pluripotent MSC are recruited from the peri- and endosteum and from the circulation. The fracture hematoma is also invaded by regenerating nerve fibres already two days after fracture (Li et al. 2001) indicating a neuronal influence. The initial pro-inflammatory response
is followed by an anti-inflammatory response. In the fibro-cartilage callus tissue intramembranous and endochondral bone formation is initiated, endochondral bone formation occurs in the central part and intramembranous bone formation in the periphery in close relation to the endosteum and peristeum. After surgery with fracture reduction and stabilizing fixation, the bone forming process is almost exclusively intramembranous, i.e. direct fracture healing. The initially produced woven bone is transformed to lamellar bone by remodeling. To restore the original shape of the fractured bone modeling during one or more years is necessary.

Neuropeptides

Neuropeptides are substances synthesized by nerve cells with a number of biological effects, most importantly as signaling molecules.

In addition, neuropeptides have been shown to exert important effects on a great variety of biological functions. They are involved in regulation of sensory modalities, vasoregulation (CGRP, SP, VIP and NPY) (Uddman et al. 1986, von Euler and Gaddum 1931, Said and Mutt 1970, Lundberg et al. 1982), or in indirect and direct endocrine effects in nerve terminals of the adrenal medulla (Schultzberg et al. 1978), in the pituitary gland (Morel et al. 1982), moreover, they have direct effects in the kidney (Barajas et al. 1983) and liver (Felú et al. 1983). In addition, influence on the immune system (Stanisz et al. 1986, Ottaway and Greenberg 1984, Ahmed et al. 1994 and 1995) and other essential biological processes including inflammation (Ma and Eisenach 2003), angiogenesis (Haegerstrand et al. 1990, Hu et al. 1996) and cell proliferation (Trantor et al. 1995, Villa et al. 2003) is in part mediated via neuropeptides.

While classical neurotransmitters are synthesized in the nerve endings, neuropeptides are synthesized in the soma, condensed in vesicles and transported distally by anterograde axonal transport (Ochs et al. 1972). The axons are often thin and unmyelinated, free nerve-endings are common (Kruger et al. 1989, Pernow 1983). More than 50 neuropeptides are known in the mammalian nervous system (Strand 1999) of which several are distributed throughout the central- and peripheral nervous system (Zaidi et al. 1987, Otsuka and Yoshioka 1993). Neuropeptides are often co-localized in neurons with other neuropeptides, or classical transmitters such as norepinephrine (Hokfelt et al. 1986, Morris and Gibbins 1989).

Neuropeptides exert their action in a paracrine way, when released from nerve-endings (Offley et al. 2005), or in an autocrine way (Drissi et al. 1997) and also in an endocrine manner when released into the circulation (Morris and Gibbins 1989, Pernow 1986, Onuoha and Alpar 2000). Many neuropeptides are expressed in cells throughout the body.
The first report on a nerve supply to bone was published by Gros in 1846. In 1936 Schartau reported on nerve supply to bone with nerve endings in direct relation to osteoblasts. In 1937 Hurrel confirmed this finding by showing small nerve bundles in Haversian canals and in 1965 Milgram described unmyelinated nerves in Haversian canals by electron microscopy. When the methodological problems with handling and staining mineralized tissue to determine nerve fibre content were solved, in the late 80’s (Bjurholm et al. 1988), nerve fibres, e.g., autonomic and sensory nerves could be identified in bone according to their neuropeptide content by immunohistochemistry. This methodological break-through made it possible to correlate morphological findings to physiological effects.

CGRP and SP are well recognized as sensory neuropeptides. CGRP, a peptide produced in the CNS and PNS, was discovered in 1983 by Rosenfeld, and SP was discovered in 1931 by von Euler and Gaddum. SP co-exists with CGRP in sensory neurons (Hokfelt et al. 1975, Ju et al. 1987). Almost all (80-90%) of these peptides produced in the dorsal root ganglion (DRG) neurons are transported distally (Kashihara et al. 1989, Brimijoin et al. 1980). Release of CGRP and SP is calcium-dependent (Holzer 1988, Bevan and Geppetti 1994). Both are recognized as potent vasodilators (Brain and Cambridge 1996, Holzer 1988). New and Mudge (1986), and Miles et al. (1989) have described CGRP induction and regulation of nicotinic receptors at the neuromuscular junction.

CGRP and SP receptors are expressed in a majority of the cells involved in skeletal development, bone formation, bone modeling and remodeling (Edoff et al. 2003, Bjurholm et al. 1992, Shih et al. 1997, Nakamura et al. 2005, Goto et al. 2001). CGRP stimulation of osteoblasts in vitro increases cAMP level (Michelangeli et al. 1989, Bjurholm et al. 1992). In osteoblasts, CGRP stimulate proliferation through receptor binding and activation of the cAMP/protein kinase C (PKC)-pathway (Cornish et al. 1999, Villa et al. 2003). Interestingly, CGRP stimulation dose-dependently inhibits the OPG production in human osteoblast-like cells through cAMP/ protein kinase A (PKA)-pathway in favour of osteoclastogenesis (Villa et al. 2006) thus giving the osteoblast the possibility to modulate both modeling and remodeling. Perichondrial cell proliferation, important for the epiphyseal growth, are stimulated by CGRP through cAMP activation of chondrocytes (Edoff and Hildebrand 2003).

CGRP also has proliferative effects in cell cultures, on endothelial cells, fibroblasts and osteoblasts (Haegerstrand et al. 1990, Trantor et al. 1995, Villa et al. 2000) and has the capacity to influence other factors involved in bone formation, i.e., growth. IGF-1 is markedly increased after CGRP-receptor activation in vitro in foetal rat osteoblasts (Vignery and McCarthy 1996).

SP is a peptide widely distributed in the CNS and PNS, (Hokfelt et al. 1975). SP receptors are richly expressed in the hypothalamus, (Yip and Chahl 2001). An increased vascular permeability in inflammation is related to SP (Holzer 1988). SP activation of mast cells has been described
(McCormack et al. 1996), as well as mitogenic effects on endothelial cells, fibroblasts and macrophages (Moore et al. 1988, Kähler et al. 1993, Reid et al. 1993). SP receptors (NK-1) are expressed on osteoclasts, and stimulation activates osteoclastogenesis and bone-resorption (Goto et al. 2001). SP stimulates osteogenesis by activating receptors on osteoblasts in vitro, (Shih and Bernard 1997, Goto et al. 1998).

NPY is a neuropeptide that is found in the CNS and in autonomic nerves. It was first isolated from the porcine hypothalamus (Tatemoto et al. 1982). NPY is co-localized with noradrenaline in autonomic neurons (Fried et al. 1985). The peptide is associated with energy balance, food intake, regulation of nociception and vasoconstriction. NPY neurons in the arcuate nuclei (ARC) in the hypothalamus, mediate the feedback effect of GH on the hypothalamus (Kamegai et al. 1996). Adipocytes express NPY and secretion is stimulated by insulin. In addition this neuropeptide has a number of interesting effects. It mediates reduction of leptin secretion in adipocytes (Kos et al. 2007). In vitro T-cell cytokine secretion is affected; Th2 alfa-CD3 is stimulated, IL-4 increased and IFN-gamma decreased (Levite 1998). NPY act directly on NPY receptors, named NPY1R to NPY5R or Y1-Y5 but can also modulate, potentiate, or inhibit adrenergic receptor signaling (Bjurholm et al. 1992, Pellieux et al. 2000). Apart from neuronal signaling NPY also act as an endocrine peptide, activating Y1 and Y2 receptors (Lundberg and Modin 1995). In bone tissue NPY exerts an inhibitory effect on osteoprogenitor cells and osteoblasts (Baldock et al. 2007). In vitro NPY inhibit stimulation of cAMP in response to PTH and noradrenalin through receptor-receptor interaction (Bjurholm et al. 1992). NPY also influences osteoclasts inhibiting isoprenaline-induced osteoclastogenesis in mouse bone marrow cells (Amano et al. 2007).

Leptin, a cytokine-family hormone produced by white and brown adipose tissue (Friedman et al. 1994 and 1997) and also expressed by other cells such as mesenchymal stem cells and osteoblasts (Whitfield 2001, Bassiliana et al. 2000) acts in the CNS on hypothalamic NPY-neurons. Baldock et al. demonstrated in 2002 a hypothalamic Y2-mediated and leptin-mediated anabolic control of bone formation. In a study from 2007, Allison et al. showed that ablation of Y2-receptors, increased osteoblast activity independent of leptin, while leptin-deficient mice showed reduced cortical bone mass and density. This effect was conducted via autonomic nerves by two independent, Y2- and leptin-pathways. Human osteoblasts express leptin-receptors, and in vitro continuous leptin exposure significantly increases cell proliferation. Also the expression of TGFβ, IGF-1, type I collagen, ALP and mRNA’s osteocalcin, IL-6 and OPG are all increased by leptin (Gordeladze et al. 2002).

VIP, a peptide produced in the gut, pancreas and hypothalamus, is found in autonomic nerves. In addition to effects in the digestive system, VIP like NPY is implicated in the circadian timekeeping system in the body. Immunocompetent cells have been found to contain VIP. Studies indicate that VIP acts like an endogenous anti-inflammatory mediator much like T-helper-2 cytokines
(Pozo 2003). VIP promotes Th2-type responses, and reduce pro-inflammatory Th1-type responses (Gonzalez-Rey et al. 2007 and 2007) interfering directly with the IL-12Jak2/STAT4 signaling pathway (Liu et al. 2007). In bone tissue VIP stimulates bone resorption (Hohmann et al. 1983). VIP increases IL-6 production in osteoblasts in vitro through activation of the VPAC2 receptor. VIP also increases the expression of RANKL and decreases the expression of OPG and M-CSF in osteoblasts (Persson and Lerner 2005).

Skeletal development and innervation

The gestational time, i.e. the period from conception to delivery, partum, is 21 -23 days in the rat and 19 - 21 days in the mouse (Hebel and Stromberg 1982, Kaufman 1992). The fertilized egg, the zygote, develops influenced by the maternal, autocrine and paracrine growth factors, insulin, IGF-1, IGF-2 and TGF-α in the uterus before implantation (Kaye 1997). After implantation of the blastocyst at day 5 - 6 after fertilization, the maternal fetal circulation adds growth factors and nutrients to the embryo (Harigaya et al. 1997, Kaku et al. 2007). A key-event on the way to skeletal innervation is when the neuronal plate becomes a tube forming the neural crest, at GD 10 in the rat and at GD 8 in the mouse. Neural crest cells (NCC) governed by the Wnt protein undergo an epithelial to mesenchymal transition allowing disconnection and migration to form the peripheral sensory and autonomic nervous systems (Schmidt and Patel 2005). ECM components as laminin, fibronectin and tenascin work as substrates for cell migration and as path finders or barriers (Newgreen and Erickson 1986). In addition, different Wnt signalling pathways, i.e. Fz/LIN-17 and Ryk/LIN-18, which control β-catenin, are important in controlling cell-polarity and orientation (Green et al. 2008).

At GD 8 and 10 blood flow occurs and heartbeat is detected in mice and rats respectively (Ji et al. 2003). The limb buds, consisting of a mesodermal core covered by an epithelial layer of ectodermal cells, are recognized at gestational day (GD) 9 in mice and GD 11 in rats. The handplate with fingers is seen at GD 13.5 and the first signs of ossification in the hind limb long bones are seen at GD 19 in rat and at GD 14 in mice. The mesodermal cells condense and the MSC differentiate to chondrocytes. At this timepoint nerves and capillaries occur in the limb. A capillary network is a necessity for a humoral influence and recruitment of circulating pluripotent cells. At the tip of the limb bud a thickening, the apical ectodermal ridge, is seen. The majority of DRG neurons have become bipolar in the rat embryo at GD 14, (Matsuda et al. 2005). The first signs of ossification in the hind limb long bones are seen at GD 14 in mice and GD 17 - 19 in rats (Kaufman 1992, Hebel and Stromberg 1986). The mineralization of the cartilage extremity template is dependent on blood flow distributed by invading capillaries and invading pluripotent MSC. Mineralization starts together with intramembra-
nous bone formation in the disphyseal perichondrium and endochondral bone formation in the centre of the template.


In humans the epiphyseal zones of growth disappears when the skeleton is fully grown whereas in the rat and mouse the growth plate stays open lifelong (Savendal 2005).

Heterotopic bone formation

Bone formation in locations outside the normal skeleton is called heterotopic ossification. This phenomenon is most often seen in traumatized tissue; after soft tissue trauma as myositis ossificans (Cushner and Morewessel 1992), after joint replacement (Warren 1990) and secondary to neurological injury or neoplasm (Puzas et al. 1989). Heterotopic bone formation closely mimics bone formation during embryogenesis and fracture healing. Angiogenesis together with proliferation, migration and differentiation of pluripotent mesenchymal stem cells to chondrocytes and bone forming osteoblasts is seen (Huggins 1953, Urist 1983, Albrektsson and Johansson 2001). Heterotopic bone formation can be experimentally induced in rodents by the implantation of demineralized allogeneic bone matrix (DABM) or demineralized xenogeneic bone matrix (DXBM) into the muscle tissue (Urist 1965, Urist et al. 1967). Bone formation within the implants follows a well defined sequence of events resulting in the formation of a complete bone organ containing bone marrow. Cytokines are important regulators in all types of bone formation, especially BMPs (Urist et al. 1983, Linkhart et al. 1996). It is also well recognized that pro-inflammatory (e.g. IL-1 and TNF-α) and anti-inflammatory (e.g. IL-4 and IL-13) cytokines affect bone formation in different aspects (Olmedo et al. 1999, Kon et al. 2001, Frost et al. 1997). Non-steroidal anti-inflammatory drugs (NSAID) are recognized to inhibit heterotopic bone formation (Nilsson et al. 1986). In 1989 Bjurholm et al. de-
scribed sensory and autonomic (CGRP, SP, NPY and VIP) innervation of DBM implants in rat. These highly interesting findings point towards an important influence by the nervous system on the development and maintenance of bone homeostasis.
AIMS

- To study skeletal sensory and autonomic innervation during fetal and neonatal long bone development and growth in the rat.

- To study skeletal sensory and autonomic innervation and angiogenesis, during fetal and neonatal long bone development and growth in the mouse.

- To correlate the development of skeletal sensory and autonomic innervation and angiogenesis to morphological events during bone ontogeny and growth.

- To study effects of absence of IL-4 and IL-13 on innervation during bone formation in DXBM-induced heterotopic bone formation and fracture healing in mice.

- To study the influence of GSK-3β inhibition by the drug AR28 on fracture healing.
MATERIALS AND METHODS

Animals (Paper I and II)
A total of 6 adult male and 12 female Sprague Dawley rats were used for breeding. The pregnant rats were housed one in a cage and with free access to standard pellets and tap water. A total of 16 rat fetuses 2 from each of 8 females were used. In total 48 neonatal rats, 2 from each of two litters were collected ND 1 and ND 4 and thereafter every second day until ND 24. The adult and neonatal rats were killed by carbon dioxide inhalation and the fetuses by decapitation.

The experiments were approved by the Ethical Committee for Animal Research, in Uppsala, (C 67/93).

Animals (Paper III)
A total of 24 mice fetuses, 4 from each of 6 females, and 44 newborn mice were used. The mice were housed singular in a cage with free access to standard laboratory pellets and tap water. The adult mice were killed by carbon dioxide and the fetuses by decapitation.

The experiments were approved by the Ethical Committee for Animal Research, in Uppsala, (C155/5).

Animals (Paper IV)
In the heterotopic bone-induction model investigation 48 adult mice i.e. 12 male and 12 female wild type (WT) mice and 12 male and 12 female IL-4/13 knockout (KO) mice were used. The mice were obtained by interbreeding homozygotes (WT + WT and KO + KO).

In the fracture investigation 24 adult male mice, i.e., 12 WT and 12 IL-4/-13 KO, These mice were obtained by interbreeding heterozygotes. Tail DNA for genotyping by PCR was collected at three weeks of age. All mice were housed with free access to tap water and food pellets consisting of wheat, barley, soy, wheat-germ, wheat meal flour, wheat-bran, potato protein, minerals, vegetable fat, vitamins and trace elements. All animals were killed by carbon dioxide inhalation.
The study was approved by the approved by the Ethical Committee for Animal Research, in Uppsala, (C110/5).

Animals (Paper V)

A total of 40 adult female Sprague-Dawley rats, eight weeks of age weighing from 210 to 240 g were used. The animals were housed one in each cage on a 12-h light/12-h dark cycle with controlled temperature and with free access to standard certified pelleted laboratory diet and tap water. An acclimation period of 14 days was applied prior to the experiment. All animals were anaesthetized by fentanyl fluanisone (Hypnorm® Janssen Pharmaceutical, Beerse, Belgium) and killed by decapitation and collection of blood samples. The study was approved by the Ethics Committee of Animal Research in Uppsala, (C101/6).

Surgery (Paper IV)

**DXBM:** The mice were anaesthetized by an intraperitoneal injection of 0.2 mL of a mixture of Dormitor® Vet. 1 mg/mL (Orion Pharma Animal Health Care AB, Sollentuna, Sweden) 1 mL and Ketalar® 50mg/mL (Pfizer AB, Täby, Sweden) 1.5mL suspended in 2.5 mL NaCl. The implants of demineralized xenogeneic bone matrix (DXBM), prepared from Sprague-Dawley rat long bones (Nilsson et al. 1990), weighing 2 mg was implanted in a muscle pouch, one in the rectus femoris muscle on each side. After closing of the fascia and skin, the mice showing slow recovery were given an antidote of 0.1 mL of Antisedan® Vet. 5 mg/mL (Orion Pharma Animal Health Care, Sollentuna, Sweden) suspended in NaCl in a 1:4 ratio. Immediate weight bearing was allowed and most of the animals started to use their legs within 2 days. Five weeks after surgery the mice were killed by carbon dioxide inhalation (Fig. 2 A).

![Figure 2 A. DXBM implants and X-ray five weeks after implantation surgery showing radiological signs of mineralization.](image_url)
Fractures: The animals were anaesthetized by an intra-peritoneal injection of Dormitor® Vet. as described in the heterotopic bone model. Under sterile conditions a femur fracture was produced in a standardized manner as described by Skoglund et al. (2002). A lateral knee-arthrotomy was performed. The patella was medially dislocated and the femoral condyles exposed. A cannulated needle (Ø 0.6mm) was drilled into the femoral marrow cavity to the level of the major trochanter. After 1 mm retraction the needle was cut and reinserted, to minimize injury to the knee joint cartilage. A femoral diaphysial fracture was created in a standardized manner by a special tongs sparing the needle. After adapting the soft tissues by sutures the mice were given an anaesthetic antidote as in the heterotopic bone model mentioned above. Full weight bearing was allowed immediately after surgery. The operated leg was in use after 1-3 days. After 5 weeks the animals were killed by carbon dioxide inhalation. An X-ray was performed before dissection of the femora (Fig. 2 B). The right femur was kept frozen and the fractured left was fixed in Zamboni´s solution (Zamboni and De Martino 1967).

Surgery (Paper V)

A total of 40 adult female Sprague-Dawley rats, 8 weeks of age weighing from 210 to 240 g, were housed as detailed above under Animals (Paper V) and randomly assigned to treatment groups. In general anaesthesia by fentanyl-fluanisone (Hypnorm® Janssen Pharmaceutica, Beerse, Belgium) 1.0 mL/kg body weight, a lateral knee arthrotomy was performed. A cannulated needle (Ø 1.2mm, Venflon™, BOC Ohmeda, Halsingborg, Sweden) was drilled into the femoral marrow cavity to the level of major trochanter and a fracture was created as mentioned above, 1 cm proximal to the femoral condyles. A postoperative Faxitrone X-ray confirmed the position of the needle. Full weight bearing was allowed immediately after surgery. One rat
died in surgery. In total, 20 rats were treated once daily with AR28 (30μmol/kg) and the remaining 19 were given vehicle (MQ water ph 3.5). The vehicle or AR28 was given orally as solution using a plastic gavage. The dose volume was 10 mL/kg. At one week 2 AR28 rats and 2 vehicle rats were killed, at two weeks 9 rats from the AR28 group and 8 from the vehicle group were killed and at three weeks 9 rats from each group were killed. The femurs were dissected and kept frozen at -20°C.

Tissue collection (Paper I-II)
The hind limbs in fetal rats were dissected out at gestational day (GD) 15, 17, 19 and 21 and fixed by immersion in Zamboni’s solution (Zamboni and De Martino 1967) for 12 h. In the neonatal animals the hind limbs were dissected day at ND 1 and 4 and then every second day until ND 24. The specimens were fixed by immersion in Zamboni’s solution for 24 h. All fixed specimens were rinsed in 10% sucrose with Sörensen phosphate buffer, containing 0.01% sodium azide and 0.02% Bacitracin (Sigma Chemicals, St Louis, MO) and stored in the same solution before sectioning. The neonatal specimens were demineralized, in a 4% EDTA solution for 3 weeks, as described by Bjurholm et al. (1989) before sectioning while the fetal specimens were sectioned without previous demineralization. The sectioning was performed on a Leitz cryostat with sectional thickness 15μm. The frozen sections were mounted directly on chrome-alum-treated slides and kept in -70°C until staining with anti-bodies for indirect immunofluorescence staining (Coons 1958).

Tissue collection (Paper III)
The hind limbs together with the pelvis in fetal mice were dissected out at GD 15, 17 and 19 and fixed by immersion in Zamboni’s solution for 12 h. In the neonatal animals the hindlimbs were dissected at ND 1 and 2 and subsequently every second day until ND 20. After dissection the hind limbs were fixed by immersion in Zamboni’s solution for 24 h. All fixed specimens were rinsed in 10% sucrose with Sörensen phosphate buffer, containing 0.01% sodium azide and 0.02% Bacitracin (Sigma Chemicals, St Louis, USA), then rinsed in 10% sucrose buffer solution and stored in the same solution. The specimens from the neonatal mice were demineralized, in a 4% EDTA solution for 3 weeks, as described by Bjurholm et al. (1989) before sectioning while the specimens from the fetal animals were sectioned without previous demineralization. The sectioning was performed on a Leica cryostat with sectional thickness 14μm. The frozen sections were mounted directly on SuperFrost® Plus slides (Menzel-Glaser, Germany) and kept in -70°C until staining with antibodies for indirect immunofluorescence staining (Coons 1958).
**Tissue collection (Paper IV)**

DXBM: After X-ray examination by Faxitrone the implants were dissected and collected one for ash weight and one for histological analysis. The later was fixated by immersion in Zamboni’s solution for 12 h followed by rinsing and storage as mentioned above.

Fracture: After X-ray and dissection the right femurs were kept frozen at -20°C and the fractured left femurs were fixated in Zamboni’s solution for 24 h and after rinsing in 20% buffered sucrose stored in the same solution before further analysis, pQCT and immunohistochemistry.

**Tissue collection (Paper V)**

A total of 40 adult female Sprague-Dawley rats eight weeks of age and in weight from 210 to 240 g were housed as mentioned under Animals (Paper V). One rat died during surgery. One week after surgery 2 AR28 rats and 2 vehicle rats were killed, at two weeks 9 rats from the AR28 rats and 8 vehicle rats were killed and at three weeks 9 AR28 rats and 9 vehicle rats were killed. For histological analysis the 11 femurs, i.e. 6 AR28 and 5 vehicles, were fixed in 4% formalin at +4°C overnight. The hind limbs were demineralized in 10% formalin/20% EDTA in PBS for 3 weeks, and then paraffin blocks were prepared by standard histological procedures. The remaining femurs were dissected and kept frozen at -20°C for further analysis.

**Immunohistochemistry (Paper I, II)**

The frozen sections were hydrated in phosphate buffer saline (PBS) before they were incubated overnight in +4°C, in a humid atmosphere, with the primary antibodies, (Table 1). After rinsing in PBS, 3 x 10 min at room temperature (21°C) the sections were incubated for 30 min in darkness and in a humid atmosphere with the fluorescein isothiocyanate (FITC)-conjugated secondary antibodies. After a final rinse in PBS (15 min) the sections were mounted in a mixture of glycerol and p-phenylenediamine as anti-fader (Johnson and Nogueira-Araujo 1981).

**Immunohistochemistry and H&E (Paper III, IV)**

The frozen sections were hydrated in PBS and sequentially incubated with the five different antibodies (Table 1), one for each section and haematoxylin and eosin (H&E) and subsequently incubated with the secondary antibodies as described above. After rinsing in PBS, 3 x 10 min at room temperature
(21°C) the sections were incubated with the FITC-conjugated secondary antibody for 30 min at +37°C in a dark and humid atmosphere. After a final rinse in PBS the sections were mounted in anti-fading medium (Vectorshield® Vector Laboratories, USA) described by Florijn et al. (1995). A Leica epifluorescence- and light-microscope was used to analyze the sections for morphology and immunoreactivity. Digital images were stored on a Dell™ computer.

Microscopy was performed by two independent observers. For each specimen the whole hind limb was analysed. For each antibody there was a minimum of three sections for each specimen. The sections were examined for occurrence of specific immunostaining for the antibody in the following compartments (when applicable); periosteum, endosteum, primary ossification centre, secondary ossification centre, perichondrium, groove of Ranvier, cartilage, and the physis, i.e. the epiphyseal plate.

Table 1. Antibodies (Dk - Denmark, UK - United Kingdom).

<table>
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<tr>
<th>Primary antibodies</th>
<th>Company, Country</th>
<th>Raised in</th>
<th>Diluted</th>
<th>Papers</th>
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<tr>
<td>CGRP</td>
<td>Peninsula Lab. St Helenius, UK</td>
<td>Rabbit</td>
<td>1:200</td>
<td>I, II</td>
</tr>
<tr>
<td>CGRP</td>
<td>Peninsula Lab., San Carlos, USA</td>
<td>Rabbit</td>
<td>1:200</td>
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<td>Peninsula Lab. St Helenius, UK</td>
<td>Rabbit</td>
<td>1:200</td>
<td>I, II</td>
</tr>
<tr>
<td>NPY</td>
<td>Peninsula Lab. St Helenius, UK</td>
<td>Rabbit</td>
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<td>I, II</td>
</tr>
<tr>
<td>NPY</td>
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<tr>
<td>VIP</td>
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<td>IV</td>
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<tr>
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<td>1:200</td>
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<td>1:200</td>
<td>III, IV</td>
</tr>
<tr>
<td>SYN</td>
<td>Dakopatts AB, Sweden</td>
<td>Rabbit</td>
<td>1:200</td>
<td>I, II</td>
</tr>
</tbody>
</table>

Secondary antibodies

FITC-conjugated Amersham, Sweden | Goat | 1:10 | I, II |
FITC-conjugated DakoCytomation, Glostrup, Dk | Swine | 1:20 | III, IV |
FITC-conjugated Jackson, West Grove, USA | Donkey | 1:20 | III, IV |

Normal serum

Swine DakoCytomation, Glostrup, Dk | 1:10 | III, IV |
Donkey DakoCytomation, Glostrup, Dk | 1:10 | III, IV |

Radiology (Paper IV)

In the fracture-model and in the heterotopic bone-model, the animals were killed 5 weeks after surgery and a single projection Faxitron X-ray was taken before dissection of femurs and implants.
Radiology (Paper V)
A postoperative Faxitrone X-ray was taken to confirm the position of the needle and configuration of the fracture. After dissection of the fractured femurs the specimens were examined by ordinary X-ray at the Radiology Unit at Uppsala University Hospital, Uppsala, Sweden.

Peripheral quantitative computerized tomography (Paper IV)
Fractured left femurs from 8 IL-4/13 knockout mice and 8 WT were dissected and analysed by a peripheral quantitative computerized tomography (pQCT, Strattec™, XCT Research M, Norland, Germany). The reference line was placed in the centre of the callus from where 5 consecutive pQCT scans were performed. The analysis of the callus included average cross-sectional area, callus volume, bone mineral content (BMC) and bone mineral density (BMD).

Peripheral quantitative computerized tomography (Paper V)
Left fractured femurs from 2 AR28 rats and 2 vehicle rats (one week after surgery), 8 AR28 rats and 7 vehicle rats (2 weeks after surgery) and 8 AR28 and 8 vehicle rats 3 weeks after surgery were analysed by pQCT using an XCT Research SA-bone scanner. The reference line was places at the fracture site and three scans on each side of the line were acquired. Bony-callus was defined as tissue density above 240 mg/cm³. The cross-sectional callus area, BMC and BMD were calculated.

Biomechanical testing (Paper IV)
In total 6 fractured femurs from IL-4/-13 knockout mice and 10 femurs from WT mice were tested to failure by a three-point bending test. The test was performed at room temperature (21°C) on an electromechanical testing machine (Avalon Technologies, Rochester, MN, USA). The specimens were placed with the posterior cortex resting against two end supports placed with a span length of 8mm. An indenter connected to an axial load cell (Transducer Techniques Inc, California, USA) with range 0-25 lb. was used to apply a load, at the speed of 1 mm/s, to the center of the callus. Data for load and displacement were collected 50 times per second using software pro-
vided with the testing machine (Testware II). The collected data were stored as data-files including the variables time, load and displacement. The bending stiffness was calculated by using the load and displacement curve defining the slope and its tangent.

Biomechanical testing (Paper V)
A total of 6 fractured femurs from AR28 rats and 6 from vehicle rats were tested to failure by a four-point bending test. The test was performed on the same testing machine as described above. The specimens were placed with the posterior cortex resting against two end supports placed with a span length of 17mm. A fork-indentor, connected to an axial load cell (Sensotec, Columbus, OH, USA) with range 0-250 lb. was centred over the fracture site. At the speed of 1 mm/sec the indentor applied a load to the bone. Data for load and displacement were collected and stored as previously described.

Ash weight (Paper IV)
A total of 41 DXBM implants, i.e. 10 male and 11 female IL-4/-13 KO mice and 8 male and 12 female WT mice were collected from the femoral muscle pouch in the left thigh. After dissection the implants were ashed in a muffle furnace (Furnace Type 48000, Barnstead/Thermolyne Corp. Essex, UK) at 600°C for 24 h and weighed.

Statistics (Paper IV and V)
In Paper IV Two-tailed Student’s t-test for independent groups was used for statistical evaluation of ash weight and pQCT-data (BMD, BMC and callus Volume). Biomechanical data in Paper IV and V was evaluated by Mann-Whitney U-test and with the Bonferroni correction.
RESULTS AND DISCUSSION

Development of sensory and autonomic nerves during bone modeling and growth

Studies during the last two decades have increased our understanding of the complex interplay between the nervous system and bone, as a tissue as well as an organ. However, little is known about the skeletal innervation during embryonic and neonatal growth. The increasing knowledge of neuropeptides and their regulatory effects in physiological processes and on bone formation motivated the present studies to investigate the sensory and autonomic innervation in the rat and in the mouse during embryonic and neonatal development and growth and to correlate the development of innervation to morphologic events.

In Paper I, II and III we studied sensory and autonomic innervation in the developing hind limb skeleton. In Paper I and II Sprague Dawley rats were used and in Paper III mice. Embryos from the two species were collected every second day from gestational day 15 (GD), 17 to 19 in mice and GD 15, 17, 19 and 21 in rats. Neonates were collected from neonatal day 1 (ND), i.e. during the first 24 hours after partus, and ND 2 in mice and ND 1 and 4 in rats and then every second day until ND 20 (mice) and ND 24 (rats). The starting point was chosen since before GD 15 the tissues are gelatinous and very difficult to handle. In addition, it is difficult to define anatomic structures prior to this timepoint in fetal development. We adopted the indirect immunofluorescence staining-technique previously described by Coons (1958) and used by Bjurholm et al. in (1988). Bone from neonatal rats was demineralized in 4% EDTA, (Bjurholm et al. 1989) before cryo-sectioning.

In Paper I and II calcitonin gene-related peptide (CGRP) and substance P (SP), two well recognized sensory neuropeptides, both with receptors expressed in bone-related cells (Edoff et al. 2003, Bjurholm et al. 1988, Shih et al. 1997, Nakamura et al. 2005 Goto et al. 2001) were selected as markers of sensory nerves. CGRP and SP are co-localized in the CNS and the PNS (Hokfelt et al. 1975, Ju et al. 1987). Neuropeptide Y (NPY) and vasoactive intestinal peptide (VIP) were selected as markers of autonomic nerves. Both are distributed throughout the CNS and PNS. NPY is co-localized with noradrenalin in autonomic neurons (Fried et al. 1985) and VIP is co-localized with acetylcholin in cholinergic nerves (Lundberg et al. 1980), both with receptors expressed in bone-related cells (Bjurholm et al. 1992,
Baldock et al. 2007, Amano et al. 2007). As markers of neurofilaments, protein gene product 9.5 (PGP 9.5) (Lundberg et al. 1988, Thompson et al. 1983) and synaptophysin (SYN) (Gould et al. 1986) were chosen. In a study from 1994 Johnson et al. show excellent correlations between PGP 9.5-staining in unmyelinated nerve fibres and quantifications by electron microscopy. SYN staining of vesicles in the synapses is consistent with sensory and secretory function in mature nerve endings (Scarfone et al. 1991).

The limb buds occur at GD 11 (Hebel and Stromberg 1986) and footplates are seen at GD 13-14, in the rat. At GD 15, two days before the first signs of the primary ossification in middle part of the diaphyses, PGP 9.5-immunoreactive nerves were identified in the diaphyseal perichondrium of femur and tibia (Fig. 3 A). At GD 19 CGRP- and SP-containing nerves occurred in the diaphyseal periosteum and in the epiphyseal perichondrium while NPY-containing nerves occurred at GD 21, just prior to partus. The nerves were thin and rich in varicosities. Ramifications and sprouting were seen. Four days after partus, i.e. on ND 4, sensory and autonomic nerves were seen in the intramedullary cavity, CGRP close to the osteo-chondral junction and NPY in the bone marrow, often adjacent to blood vessels. At ND 6 the primary ossification centres, i.e. the chondro-osseous junction had reached to the metaphyses. In the epi-metaphyseal junction in the Ranvier groove a highly chondrogenic location (Shapiro et al. 1977) the first sensory nerves occurred at ND 4 and autonomic nerves at ND 6. Nerves embracing single cells or groups of cells, in chondro- and osteogenic areas and in cartilage canals (Fig. 3 B) indicates a nerve to cell influence.

Cartilage canals or fibrous strands, growing from the epiphyseal perichondrium towards the centre of the epiphyses, preceded mineralization in the secondary ossification centers. CGRP-containing free nerve endings, with ramifications adjacent to group of cells (Fig. 3 C), occurred in the cartilage canals at ND 8. NPY-containing nerves occurred in the cartilage canals at ND 14. In the secondary ossification centres sensory nerves were found at ND 10 and autonomic nerves at ND 24 (Fig. 3 D).
Figure 3: A. PGP 9.5-staining nerves in the perichondrium (pc) in the proximal femur, at GD 17. B. CGRP-staining nerves in the groove of Ranvier (RG) in the proximal tibia, at ND 10. C. CGRP-staining nerves in cartilage canal/fibrous strands (fs) in the epiphyseal cartilage (ec) in the distal femur, at ND 8. D. NPY-staining nerves in the secondary ossification centre, in the proximal tibia, at ND 24. (Scale bars 50 micrometer).
This time lag in appearance between sensory and autonomic nerves may reflect differences in action exerted by these peptides. CGRP stimulates proliferation in chondrocytes, perichondrial cells and osteoblasts (Edoff et al. 2003, Villa et al. 2000, Cornish et al. 1999) essential for growth, both appositional and in length, indicating a functional role for sensory nerves in the chondro- and osteogenic locations.

CGRP and SP also act in a pro-inflammatory way (Lotz et al. 1987, Foreman et al. 1982, Yamaguchi et al. 2004, Calland et al. 1997) mobilizing different growth promoting cytokines such as interleukin-1 (IL-1), tumour necrosis factor (TNF) and bone morphogenetic proteins (BMP). IL-1 and TNF can act as endogenous mitogens for human osteoblasts in vitro (Modrowski et al. 1995) and also as exogenous mitogens (Frost et al. 1997). In contrast, NPY and VIP act in an anti-inflammatory way, (Pozo 2003, Gonzales-Rey et al. 2007) mimicking the effects of the anti-inflammatory cytokines IL-4 and IL-13. NPY in vitro stimulates IL-4 production in T-helper-2 cells (Th2), (Levite 1998) and IL-4 inhibits osteoblast proliferation (Riancho et al. 1993, Frost et al. 1998). Osteoclast development is also inhibited by IL-4 (Riancho et al. 1993, Wei et al. 2002).

A well developed capillary network during early skeletal growth is a necessity to provide the tissues with nutrients. CGRP and SP stimulate angiogenesis (Haegerstrand et al. 1990, Reid et al. 1993) and are potent vasodilators (Brain and Cambridge 1996, Holzer 1988).

Autonomic nerves were mostly localized close to capillaries indicating mainly a vasoregulatory task. NPY is recognized as a vasoconstrictor (Lundberg et al. 1982, Lundberg and Modin 1995) and has an inhibitory effect on osteoprogenitor cells, osteoblasts and osteoclasts (Baldock et al. 2007, Amano et al. 2007) which may explain the late occurrence in the rat embryos.

In Paper I and II we found that the developing bone is supplied with sensory CGRP and SP-containing nerves from GD 19 and autonomic NPY-containing nerves from GD 21, while cholinergic VIP nerves emerge first at 4 days after partus. Nerves identified by neurofilaments were present already at GD 15. Their morphology indicated a newly established innervation since the neuropeptides, axonally transported from the dorsal root ganglion and distally, were lacking. The functional importance of this finding is substantiated by the fact that receptors to all four neuropeptides are expressed in a majority of cells involved in skeletal development, bone formation, remodeling and modeling (Edoff et al. 2007, Bjurholm et al. 1988, Shih et al. 1997, Nakamura et al. 2005, Goto et al. 2001, Bjurholm et al. 1992, Pellieux et al. 2000, Persson and Lerner 2005).

In Paper III we also used CGRP and NPY as markers of sensory and autonomic nerves respectively. To study the angiogenesis we added staining to vascular endothelium using platelet endothelium cell adhesion molecule-1 (PECAM-1). Growth associated protein-43 (GAP-43) was chosen as a second marker of neurofilaments. Hematoxylin-eosin (H&E) - staining was
added as a complement in the morphological analysis. Development in the mouse is almost two days prior to that in the rat. The limb buds occur at GD 9-10 (Kaufman 1992) and the footplate is seen at GD 11-12, in the mouse. Neurofilaments and CGRP-containing nerves were present in the peristome, perichondrium and Ranvier groove at GD 15 and in the primary ossification centre at GD 19 (Fig. 4 A). NPY-containing nerves were seen in the peristome at ND 4 and in the primary ossification centre at ND 6, (Table 2). The primary ossification in mice starts at GD 14 (Kaufman 1992). A dense and covering capillary network was present outside and inside the bone organ at GD 17-19 (Fig. 4 B). CGRP-nerves and neurofilaments were found in the cartilage canals at ND 6 and in the secondary ossification centres at ND 8. NPY occurred in the cartilage canals and the secondary ossification centres at the same time, at ND 12 (Fig. 4 C). During the rest of the observation period until ND 20 the secondary ossification centres became larger containing bone marrow. The cartilage canals persisted and the growth-plate became wave-shaped. The findings are in accordance with those in Paper I and II.

Figure 4 A. CGRP-staining nerves in the groove of Ranvier, the latter bulging into the epiphyseal cartilage (ec) of the proximal tibia, at GD 15.
Figure 4 B. PECAM-1-staining periosteal (po) and endosteal capillaries in the proximal tibia, at GD 19. Bone marrow (bm).

Figure 4 C. NPY-staining nerves in cartilage canals (cc) extending from the perichondrium (po) through the epiphyseal cartilage (ec) towards the secondary ossification centre, in the proximal tibia at ND 10.
Table 2. The temporal occurrence of different neurofilament-staining nerve fibres (GAP-43, and PGP 9.5) and CGRP and NPY-containing nerves in the fetal (GD) and newborn mouse (ND). Inserted pictures distal showing femur at GD 17, ND 6 and ND 18. P.O.C = primary ossification center ; S.O.C = secondary ossification center.

<table>
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<tr>
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<th>Neonatal day</th>
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<tr>
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</tr>
<tr>
<td>S.O.C</td>
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<td>NPY</td>
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In Paper I-III positive immunofluorescence in the surrounding muscular tissue was used as primary anti-body control.

Comments
Bone innervation with sensory and autonomic nerves in modeling and growth follows a predictable and reproducible pattern both in the rat and in the mouse with sensory nerves occurring prior to autonomic nerves in areas with high chondrogenic and osteogenic activity. The time lag in occurrence between sensory and autonomic nerves indicates the importance of developmental timing between different nerve qualities in skeletal ontogeny. These findings give substantial morphologic support for important regulatory effects by the nervous system on bone development. However, to draw firm conclusions about functional associations it is necessary to intervene in the bone forming processes. This can be done in a number of ways, e.g., by introducing genetic modifications to alter the bone phenotype, or by introducing treatments that affect bone physiology. By adoption of the techniques for immunostaining from rats to mice it has become feasible to study the occurrence of neuropeptides and vascular peptides, in genetically modified animals.

Development of sensory and autonomic nerves during fracture healing and bone induction in IL-4/13 depleted mice
In Paper IV we studied the effects of absence of interleukin-4 (IL-4) and IL-13 on innervation during bone formation in demineralized xenogeneic bone matrix (DXBM)-induced heterotopic bone formation and fracture healing in mice. This study was performed as a result of a previous study that reported on reduced cortical bone mass in IL-4/13 depleted, knockout (KO), male mice (Silfverswärd et al. 2007). We considered this an interesting model to analyze the interaction between the nervous system and bone in the light of genetic alterations. To specifically study the effects of IL-4 and IL-13 depletion on bone formation we used two well defined pathophysiological models of increased bone formation, i.e., heterotopic bone formation induced by DXBM (Urist et al. 1990) and fracture healing. Both fracture healing and heterotopic ossification in many ways mimic bone formation during skeletal development and growth. In addition, an early sensory re-innervation during fracture healing and modeling, indicating a nervous influence, has been described, by Hukkanen et al. (1995) and Li et al. (2001). In experimental denervation and in neurological disorders an altered fracture healing and growth are seen (Aro et al. 1981, Freehafer and Mast 1965,

In fracture study we compared adult male wild type (WT) mice to IL-4/13-knockout (KO) mice. A diaphyseal fracture over an intramedullary nail was performed in the left femur, in WT and in IL-4/13-KO. After 5 weeks the animals were killed and radiological healing with mineralized callus was present in all animals. No significant differences in BMD and BMC were evident by pQCT. Biomechanical testing, by a four-point bending test, revealed no significant differences between WT and IL-4/13-KO in biomechanical properties.

Histological analyses showed sparse fracture callus in all femurs. Immunohistochemistry after decalcification and cryo-sectioning, as described in Paper I, II and III showed no differences in neurofilament (PGP 9.5 and GAP-43)-staining between WT and IL-4/13-KO mice. Sensory (CGRP) and autonomic (NPY) nerves were found in the periosteum and surrounding muscular tissue but not in the callus tissue, with the same findings in both groups. In the intramedullary cavity NPY and CGRP-containing nerves were found in IL-4/13-KO mice but, not in WT mice. This difference might be explained by an increased pro-inflammatory neurotrophic effect in the KO group.

DXBM implanted intramuscularly in both thighs, in WT and IL-4/13-KO mice of both genders were collected after 5 weeks. A mineralized ossicle was detected radiologically in all implants. Half of the implants were analyzed according to ash weight and no differences in mineral content were found between WT and KO-mice, although there was a tendency towards less bone in the IL 4/13 deficient mice. H&E-staining showed an ossicle surrounded by a periosteum-like fibrous capsule. Immunohistochemistry was performed without decalcification. Neurofilament-staining (PGP 9.5 and GAP-43) showed no differences between WT and KO-mice or between the genders. Autonomic (NPY) innervation of the capsular tissue was absent in male and female KO-mice but present in WT. A capillary, PECAM-1-positive network was found in WT marrow and matrix of the implants but was found in the marrow but not the matrix of the KO-mice, in both males and females.

Comments

A number of studies have shown an interaction between the nervous system and the immune system (Ahmed et al. 1994, Kawamura et al. 1998). The sensory neuropeptides CGRP and SP act in a pro-inflammatory mode by stimulating the synthesis of pro-inflammatory cytokines in immune competent cells, monocytes, macrophages, and T-lymphocyte (Lotz et al. 1987, Foreman et al. 1982, Yamaguchi et al. 2004, Calland et al. 1997). The autonomic neuropeptides NPY and VIP act in an anti-inflammatory way by
stimulating synthesis and release of anti-inflammatory cytokines in immune-competent cells and by inhibition of pro-inflammatory cytokine production (Pozo 2003, Gonzales-Rey et al. 2007). Thus, an effect by the combined depletion of the two anti-inflammatory cytokines IL-4 and IL-13 on the nervous system would not be surprising since the pro-inflammatory cytokines have a neurotropic effect (Kannan et al. 1996, Satoh et al. 1988, Wu and Bradshaw 1996). In addition, osteoblasts express receptors for NPY indicating a role in bone formation (Bjurholm et al. 1992) and NPY has also been shown to inhibit the parathyroid hormone (PTH) effects on osteoblastic cells (Bjurholm et al. 1988) thus possibly explaining the reduction in cortical bone mass in IL-4/13 depleted male mice as reported by Silfversward et al. (2007).

Depletion of IL-4 and IL-13 production in mice resulted in subtle differences in the skeletal phenotype with less cortical bone, but no definite effects in fracture healing or net amount of heterotopic bone formation. However, there was a tendency towards less callus in the fractures and less bone induction in the IL-4/13-deficient mice compared to controls. The small but reproducible phenotypic changes were accompanied by a similarly subtle alteration in the development of NPY and CGRP-containing nerve fibres. When considering these effects it is important to realize that the altered cortical bone phenotype is a result of the inhibition of IL-4/13 on the combined skeletal development and growth until 20 weeks of age, while the present experimental model addresses two different physiological processes – bone induction and fracture healing at maturity. Thus, these differences in outcomes are not surprising.

Effects on fracture healing by GSK-3β inhibition

In Paper V we investigated fracture healing in altered canonical Wnt signaling by drug-mediated inhibition of the β-catenin phosphorylation and degrading by glycogen synthase kinase 3β (GSK-3β). The Wnt family molecules regulate processes during development, and later in life. The consequences of Wnt signaling range from cell adhesion, cell-polarity and orientation during embryonic growth to mitogenic stimulation and differentiation (Brannon et al. 1997, Gordon and Nusse 2006). Mutations in the Wnt-binding Frizzled/LRP5 receptor have shown that Wnt signaling is important for the regulation of bone mass. Activating mutation induces a high bone mass phenotype and an inactivating mutation induces osteoporosis (Little et al. 2002, Kugiyama et al. 2007, Van Wesenbeeck et al. 2003).

The hallmark of the Wnt signaling pathway is an increase in cytoplasmic β-catenin which leads to a nuclear accumulation and transcription. Phosphorylation of β-catenin at a certain binding-site results in degradation and elimination of β-catenin while phosphorylation of other binding-sites leads
to an increase and accumulation of β-catenin (Hino et al. 2005, Norvell et al. 2004). Kinases, PKA and PKB and other factors, i.e. ATP, IGF-1 and PTH interact in these processes governing the nuclear response to signaling. Thus, there are a number of potential common pathways and interactions between neuropeptides and Wnt-signaling. Moreover, the implication of Wnt-signaling (AR28) in psychiatric and neurodegenerative disorders further supports such a conclusion (Sinha et al. 2005, De Ferrari and Inestrosa 2000).

In 40 female Sprague Dawley rats the left femur was fractured over an intramedullary nail, as described in Paper IV. The animals were given the AR28-drug or placebo orally. Both femurs were collected 1, 2 and 3 weeks after surgery. The fractured femurs showed radiological healing after 2 weeks. Peripheral quantitative computerized tomography (pQCT) showed increase in BMD (53%) and BMC (66%) after two weeks and BMD (77%) and BMC (97%) after three weeks. In the diaphysis, BMD and BMC were increased by 10% after three weeks. The callus area was increased 39 - 36% two and three weeks respectively. Callus BMC was increased 81 - 93% after two and three weeks.

Biomechanical testing by four-point bending test in the fractured femurs and three point bending in the unfractured femurs showed an increase in strength and stiffness in the AR28 treated animals, after three weeks load at failure was up 270%, energy at failure by 590% and stiffness by 103%. Thus, the treatment resulted in a remarkable anabolic effect both on the fractured bone and on fracture healing.

Histology was performed by H&E. Some isolated chondral inclusions were found in the callus tissue one, two or three weeks after surgery, indicating almost exclusively intramembranous non-chondral fracture healing. By inhibiting GSK-3β phosphorylation which in turn increases β-catenin, pluripotent MSC are recruited and differentiated only to osteoblasts. In this paper we found that AR28 enhances fracture healing by stimulating ossification and thus improving the biomechanical properties of the healing bones.

Comments

Wnt signaling seems to play an important role in fracture healing and for the regulation in bone mass; this pathway is activated during fracture healing, and activation of this pathway results in increased bone mass while inhibition results in osteoporosis. Canonical Wnt signaling is propagated through GSK-3β. Inhibition of β-catenin degradation by GSK inhibition resulted in a remarkable anabolic effect both on the fractured bone and on fracture healing. Interestingly, the histological analysis showed that the fractures heal without formation of cartilage in the callus except for some minor inclusions. This finding suggests that inhibition of GSK-3β inhibits the differentiation of chondrocytes and instead promotes the differentiation of mesenchymal progenitor cells into osteogenic cells. This is probably a Wnt path-
way-dependent effect since previous investigations have showed that stimu-
lation of this pathway leads to a preference of mesenchymal progenitors for
osteogenic differentiation. We found that the callus is rapidly filled with a
cell-rich fibrous tissue that is mineralized to bone at three weeks by intram-
embranous bone formation. CGRP is recognized to stimulate osteoblast-like
cells through PKA-phosphorylation of β-catenin, a non-canonical pathway,
with elevated β-catenin levels in the cytoplasm supporting the idea of a neu-
ronal influence on growth and bone formation.
CONCLUSIONS

- The skeletal sensory and autonomic innervation, during fetal and neonatal long bone development and growth in the rat follows a highly reproducible and predictable pattern with sensory innervation slightly proceeding autonomous innervation. Neurofilaments are present very early in bone development, already at gestational day 15.

- The skeletal sensory and autonomic innervation and angiogenesis, during fetal and neonatal long bone development and growth in the mouse, follows a pattern very similar to that in the rat, although slightly earlier after fertilization, thus corresponding to a shorter gestational time.

- The sensory and autonomic innervation during bone ontogeny and growth is co-localized with development of cartilage and bone of the peripheral skeleton. Sensory innervation is especially rich in regions with high bone-forming activity, while autonomous innervation seems to have a modulating effect. Angiogenesis occurs early in bone formation and is correlated to the development of the differentiated bone tissue.

- Absence of IL-4 and IL-13 in mice resulted in slightly less cortical bone, but no definite effects on fracture healing or net amount of heterotopic bone formation. The small but reproducible phenotypic changes were accompanied by a similarly subtle alteration in the development of NPY and CGRP-containing nerve fibres.

- GSK-3β inhibition by the drug AR28 resulted in a pronounced increase in amount of callus and in strength of healed fractures. The fractures healed by intramembranous bone formation, without cartilage formation.
ACKNOWLEDGEMENTS

For the support that made this thesis possible, I would like to express my gratitude and thanks especially to:

Anders Bjurholm, my tutor, fellow-student and friend since the 70’s, who opened the door to the amazing world of neuropeptides, introduced me to the art of hunting and provided encouragement throughout the years.

Olle Nilsson my tutor, mentor and friend, who made it possible for me to take a second step amongst bones and peptides. For being an unflailing optimist during my periods of doubt. The humble and wise long distance runner, bare-headed finding his way through the snowstorm.

Kenneth B Jonsson my co-tutor and colleague. A visionary young man from Västerbotten, who with his frank attitude wins everybody’s heart.

Sune Larsson for kindly introducing me to the art of biomechanics, for teaching me statistics necessary on the subject, and for his never ending support and encouragement.

Carl-Johan Silfverswärd research pal and friend, who with vast amounts of energy and brilliant solutions makes things happen. Admirably living in the present.

Anders Frost, co-author who brought the IL-4 and IL-13 knockout mice home, a prerequisite for the subsequent research.

Östen Ljunggren, advisor and co-author.

Andris Kreicbergs, visionary co-author.

Mahmood Ahmed, co-author.
Lars Grimelius who advised me on neurofilament staining, and allowed me to practice cryosectioning at the Department of Pathology.

Håkan Ström, in memoriam, a room-mate with an inspiring originality and attitude - I will always remember you.

Monika Nygren for skillful surgical assistance in major pilot projects.

Mari-Anne Karlsson for giving good advice and great H&E stainings.

Britt-Marie Andersson for skillful surgical assistance and biomechanical laboratory work.

The staff at Rudbeck Laboratory, Uppsala, for taking care of our mice.

Gunnar Grotte and Staffan Meurling for their lasting encouragement and generous support.

My fellow-colleagues and other staff members at the Department of Orthopeadic surgery for providing a pleasant, supportive working atmosphere.

My dear parents, Eva and Guido for their never ending support and encouragement.

My brothers, Peter and Artur, for brotherly love, inspiring talks and laughter over the years.

And last but not least, my beloved wife Eva for her support and love, and our wonderful children Johanna and Fredrik, for giving me so much joy and inspiration.
REFERENCES


Alliston T., Choy L., Ducy P., et al. TGF-β-induced repression of CBFA1 by Smads3 decreases Cbfal and osteocalcin expression and inhibits osteoblast differentiation.


Kahler C.M., Herold M. and Wiedermann C.J. Substance P:a competence factor


Linkhart T.A., Mohan S. and Baylink D.J. Growth factors for bone growth and repair:IGF, TGF beta and BMP. Bone 1996;19(1 Suppl):1S-12S.


Liu L., Yen J.H. and Ganea D. A novel signaling pathway in T cells cAMP>protein tyrosine phosphatase (SHP-2?)>JAK2/STAT4>Th1 differentiation.


Persson E. and Lerner U.H. The neuropeptide VIP potentiate IL-6 production induced by proinflammatory osteotropic cytokines in calvarial osteoblasts and the osteoblastic cell line MC3T3-E1 Biochem Biophys Res Commun 2005;335(3):705-11.


Acta Universitatis Upsaliensis

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Editor: The Dean of the Faculty of Medicine

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urn:nbn:se:uu:diva-99443

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