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Dual Role of Osteoblastic Proenkephalin Derived Peptides in Skeletal Tissues

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Abstract Proenkephalin encodes a group of small peptides with opiate-like activity, the endogenous opioids, known to function as neurohormones, neuromodulators, and neurotransmitters. Recently, we have demonstrated that in addition to its abundance in fetal brain tissue, proenkephalin is highly expressed in nondifferentiated mesodermal cells of developing fetuses. We identified the skeletal tissues, bone, and cartilage as major sites of proenkephalin expression. To examine the possibility that proenkephalin is involved in bone development we have studied the expression of this gene in bone-derived cells, its modulation by bone active hormones, and the effects of enkephalin-derived peptides on osteoblastic phenotype. Our studies revealed that osteoblastic cells synthesize high levels of proenkephalin mRNA which are translated, and the derived peptides are secreted. Reciprocal interrelationships between osteoblast maturation and proenkephalin expression were established. These results together with our observations demonstrating inhibitory effects of proenkephalin-derived peptides on osteoblastic alkaline phosphatase activity, strongly support the notion that proenkephalin is involved in bone development. A different direction of research by other investigators has established the capability of the opioid system in the periphery to participate in the control of pain. On the basis of these two lines of observation, we would like to present the following hypothesis: The potential of embryonic skeletal tissue to synthesize proenkephalin-derived peptides is retained in the adult in small defined undifferentiated cell populations. This potential is realized in certain situations requiring rapid growth, such as remodeling or fracture repair. We suggest that in these processes, similarly to the situation in the embryo, the undifferentiated dividing cells produce the endogenous opioids. In the adult these peptides may have a dual function, namely participating in the control of tissue regeneration and in the control of pain.

Key words: analgesia, bone development, gene expression, opioids, tissue regeneration

A large number of small regulatory peptides have been discovered in neural and neuroendocrine mammalian cells in the last two decades. One of the best characterized families of those peptides is the opioid family which was discovered in the mid-1970s [Brownstein, 1993]. Opioid peptides, originally isolated from brain tissue, possess opiate-like activity that resides within the N-terminal tetrapeptide sequence (Tyr-Gly-Gly-Phe), which is common to these bioactive peptides. All endogenous opioids isolated to date, more than 20, are derived from one of three independent genes [Akil et al., 1984; Douglass et al., 1984; Holt, 1991]. Proopiomelanocortin (POMC) is the precursor of β-endorphin as well as of several nonopioid bioactive peptides: ACTH and α-, β-, and γ-MSH. Proenkephalin (PENK) is the precursor of Leu- and Met-enkephalin, Met-enkephalin-Arg-Phe, Met-enkephalin-Arg-Gly-Leu, peptides B, E, and F, and the nonopioid peptides synkephalin and amidorphin 8-26. Prodynorphin (PDYN) is the precursor of Leu-enkephalin, dynorphin A and B, α- and β-neoendorphin. The primary products of the opioid encoding genes are three large inactive polypeptide precursors. Tissue specific proteolytic processing of these precursors gives rise to the variety of opioids. Therefore, not all cells making a given precursor polypeptide, store and release a similar range of opioids [Douglass et al., 1984; Holt, 1991].

Today it is well recognized that the precursor molecules and the peptides derived therefrom are not limited to the central nervous system (CNS) or the pituitary, but are more widely distributed [Imura et al., 1985; Schafer et al.,

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1991; Holt, 1991]. The expression of the opioid genes was investigated using a large variety of approaches, such as in situ hybridization and semi-quantitative Northern blot analyses as well as immunohistochemical and radioimmunoassay studies. These studies revealed that the opioid genes are also expressed in the adrenal gland, the heart, the gastrointestinal tract, the reproductive and the immune systems. The predominant opioid gene expressed in those tissues is PENK, and the proteolytic processing of the precursor protein is different from its processing within the brain.

Opiate-like activity of both exogenous opiates and endogenous opioids is mediated through specific interactions between ligands and opiate receptors [Brownstein, 1993]. Based on numerous pharmacological and physiological studies these have been classified into 3 main groups: δ-opioid receptor with high affinity to enkephalins; μ-opioid receptor with high affinity to morphine, β-endorphin, and some extended forms of Met-enkephalin; and κ-opioid receptor with high affinity to the dynorphins [Holt, 1991; Simon, 1991]. All of them belong to the family of G-protein coupled receptors containing 7 putative transmembrane domains. They interact with guanine nucleotide regulatory protein, thereby modifying functions of membrane enzymes or ion channels [Simon, 1991; Brownstein, 1993]. Recent cloning of opiate receptor cDNAs suggests that the various types (δ, μ, and κ) are derived from different genes [Kieffer et al., 1992; Evans et al., 1992; Yasuda et al., 1993].

In the nervous and neuroendocrine systems the opioids function as neurohormones, neuromodulators, and neuropeptides. It is well recognized that they are involved in the modulation of stress response, sexual behavior, water balance, pain, anxiety, and addictive processes [Akil et al., 1984]. The distribution of endogenous opioids and opiate receptors throughout the body suggests a large variety of physiological activities to this system. However, in contrast to the relative good knowledge on the opioid functions in the nervous and neuroendocrine systems, the physiological role of opioids in the periphery is just beginning to emerge.

ENDOGENOUS OPIOIDS AND BONE DEVELOPMENT

Several lines of evidence suggest that endogenous opioid systems may also act as important signalling molecules during development of the nervous system. First, numerous studies have confirmed that opiates such as morphine, heroin, and methadone, when given to pregnant animals or human subjects, produce dependence in the newborns [Tylden et al., 1987]. In addition, opiates are known to affect brain weight and to produce a variety of long-term effects on somatic and neurobiological development of newborn rats and children [Zagon and McLaughlin, 1981; Zuckerman, 1985; Handelmann and Dow-Edwards, 1985; Erikson and Ronnback, 1989]. These drug effects are stereospecific, and can be blocked by opioid receptor antagonists, suggesting that they are mediated by opioid receptors. The notion of linkage between the endogenous opioid systems and neural development was also supported by the demonstration that PENK is widely and transiently expressed during brain development [Schwartz and Simantov, 1988; Rosen and Polakiewicz, 1989; Vilijn et al., 1988; Melner et al., 1990]. A three-fold difference in PENK mRNA levels between brain astrocytes cultured from embryonic and neonatal brains was demonstrated [Vilijn et al., 1988]. On the basis of these findings it was suggested that PENK may be down-regulated during brain development. Furthermore, cell culture and whole animal studies have demonstrated that opioid peptides profoundly influence the proliferation of neuroblastoma as well as normal brain cells [Zagon and McLaughlin, 1987, 1989; Isayama et al., 1991]. PENK-derived peptides, but not peptides derived from PDYN or POMC, exhibited inhibitory effects on the proliferation of the cells which were blocked by the opiate antagonist, naloxone.

To determine whether the involvement of PENK is limited to developmental processes in the brain, or whether there are other developmental processes in which the opioid might be involved, we decided to examine PENK expression in a large number of tissues during embryonic development. Using in situ hybridization we were able to demonstrate that in addition to its abundance in brain, PENK mRNA is highly expressed in embryonic mesenchymal tissues of cartilage, bone, dermis, muscle, kidney, and choroid of the eye [Keshet et al., 1989; Polakiewicz and Rosen, 1990]. We were unable to detect POMC or PDYN mRNAs in those sections. The expression was restricted to and correlated with organogenesis. Maximal mRNA levels were observed just prior to commitment of specific cell lineages and dropped to undetectable levels upon terminal differentiation of these tissues. The

transient PENK expression and the correlation between this expression and maturation of mesodermal tissues, strongly support the hypothesis that PENK is involved in as yet undefined regulatory processes during normal development. Of special relevance to this essay are our observations that bone and cartilage are major sites of PENK expression and a study reporting that a group of infants, born to mothers on well-controlled low dose methadone maintenance, exhibited significantly smaller bones (both length of body and head circumference were affected) [Chasnoff et al., 1982]. These observations prompted us to examine PENK expression in bone cells. To this end, we have analyzed PENK expression in primary bone-derived cells and transformed osteoblastic cell lines. We found that they express high levels of PENK mRNA, but not POMC or PDYN [Rosen et al., 1991]. In situ hybridization examinations demonstrated that high levels of PENK mRNA are present in >85% of authentic calvaria-derived preosteoblasts making this model system suitable for molecular examination of PENK expression in osteogenesis. To explore the biological significance of the osteoblastic PENK expression, it is crucial to investigate the synthesis, processing, and secretion of PENK-derived peptides by osteoblastic cells. Radioimmunoassay of free and cryptic Met-enkephalin was performed and we found significant amounts of enkephalin-containing peptides in cell extracts and culture medium, whereas free Met-enkephalin was found only in cell extracts. To determine whether PENK expression is regulated by factors affecting osteoblastic phenotype we have used ROS 17/2.8 cells. A decrease in PENK mRNA levels which was associated with enhancement of alkaline phosphatase activity, was seen after treatment of cells with osteogenin or the active metabolite of vitamin D, $1,25(OH)_2D_3$. The use of in vitro differentiating fetal rat calvarial cells also revealed reciprocal interrelationships between PENK expression and osteoblastic maturation [Rosen et al., 1993]. The possibility that PENK-derived peptides affect osteoblast physiology was investigated by examination of the effects of characterized products of PENK (Met- and Leu-enkephalin and Met-enkephalin Arg-Phe) on alkaline phosphatase activity in rat osteosarcoma-derived osteoblast-like cells (ROS 17/2.8). Up to 50% decrease was observed. Significant effects were already observed with 10^{-10} M of the peptides. In this context, it is worth noting that the neutral endopeptidase EC 3.4.24.11, also known as enkephalinase (ENKase), is highly expressed in osteoblastic cells and its activity is modulated by $1,25(OH)_2D_3$ [Indig et al., 1990]. The abundance of PENK mRNA and peptides in osteoblastic cells, the modulation of PENK expression in osteoblasts, the effects of PENK derived peptides on these cells, together with the in vivo transient opioid expression, strongly support the hypothesis that the PENK gene expression plays a significant role in bone development. We further propose that PENK-derived peptides may act in an autocrine and/or paracrine manner (by interacting with neighboring osteoblasts or other cells like osteoclasts, macrophages, and fibroblasts) to modulate bone cell maturation. This activity is attenuated by osteoblast-derived proteolytic enzyme(s).

OPIOID SYSTEM IN THE PERIPHERY AND THE CONTROL OF PAIN

The recognition of the presence of both multiple opioid receptors and their endogenous ligands (the opioid peptides) promoted studies regarding its relevance to response to pain. Traditionally, the opiates' analgesic effects were thought to be mediated through actions within the CNS [Stein, 1991]. It was recognized recently that such effects could be mediated through activation of opioid receptors located peripherally. In fact, although more anecdotal than scientific in nature, already in the previous century [Wood, 1885] it was reported that morphine elicited analgesic effects when applied topically in the periphery. In the last few years several studies employing laboratory animals as well as human subjects point to the intriguing possibility that opioids produced in the periphery react with peripheral opioid specific receptor to produce anti-nociceptive effects. Several studies have demonstrated that opiate antagonists can block several forms of "stress induced analgesia." This phenomenon develops cross-tolerance with morphine administration, is attenuated by adrenalectomy and adrenal demedullation, suggesting that it depends on enkephalins released from a peripheral source of opioids, the adrenal gland. Another line of research revealed that lymphoid cells express opioid-encoding genes and release products which have previously been considered to be exclusively of neuroendocrine origin. PENK mRNA and PENK-derived peptides in the immune system were demonstrated in murine T

helper cell lines, in leukocytes of leukemia patients, in normal rat lymphocytes, and in rodent and human mononuclear phagocytes [Zurawski et al., 1986; Monstein et al., 1986; Padros et al., 1989; Rosen et al., 1989; Linner et al., 1991; Kuis et al., 1991]. A recent report demonstrated that PENK expression by monocytes is induced by endotoxin [Behar et al., in press], and that the monocyte-derived enkephalins interact with receptors on sensory nerves to inhibit nociception of inflamed tissues [Stein et al., 1990; Przewlocki et al., 1992]. Finally, and of special interest, is a recent report [Stein, 1991] which demonstrated that low doses of intraarticular morphine can significantly reduce pain after knee surgery through an action specific to local opioid receptors. This analgesic activity was inhibited by the opioid receptor antagonist, naloxone. Taken together, these studies indicate that opioid system in peripheral tissues (outside the CNS) is capable of modulating nociception.

CONCLUDING REMARKS

In our survey we have concentrated on two novel aspects of the opioid system: The first is the transient nature of PENK expression during embryonic development in skeletal tissues and the modulated expression of this gene in bone cells in culture which support the notion that PENK-derived peptides play a role in the maturation/differentiation of skeletal tissues. The other aspect is the capability of the endogenous opioid system in the periphery, outside of the CNS, to participate in the control of pain in the adult.

On the basis of these two phenomena, we would like to propose the following hypothesis: The potential of embryonic skeletal tissue to synthesize PENK-derived peptides is retained in the adult in small defined undifferentiated cell populations. This potential is realized in certain situations requiring rapid growth, such as remodeling or fracture repair. We suggest that in these processes, similarly to the situation in the embryo, the dividing cells which are the undifferentiated cells, produce the endogenous opioids. In the adult these peptides may have a dual function, namely the control of tissue regeneration and the control of pain of the injured tissue. A model describing our hypothesis is presented in Figure 1. Recently it was demonstrated that human articular chondrocytes also express proenkephalin mRNA and that it was controlled by factors modulating chondrocyte proliferation [Villiger and Lotz, 1992]. Transforming growth factor-β (TGF-β) and platelet-derived growth factor (PDGF) caused upregulation of PENK mRNA levels, while retinoic acid suppressed the opioid gene expression. The former factors enhance chondrocyte proliferation, while the latter is an inhibitor. Therefore, Villiger and Lotz concluded that

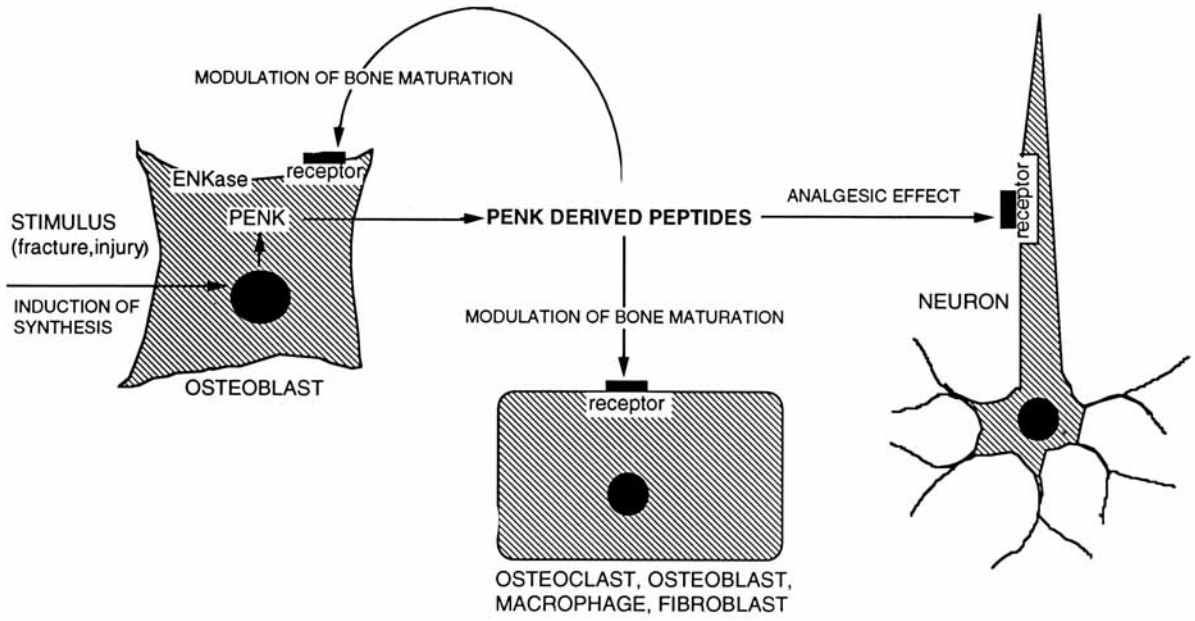

Fig. 1. A proposed model for the dual role of osteoblast-derived enkephalins.

proenkephalin mRNA is differentially controlled by cartilage regulatory factors and closely associated with cell proliferation. This study proves the opioid potential of adult skeletal-derived cells in vitro and is an important step in establishing our hypothesis. The next step in testing the hypothesis should be the examination in vivo of the opioid potential and the involvement of the endogenous peptides, produced locally in the control of pain in the same site. If our hypothesis is proven correct, it is expected to contribute to novel approaches towards pain control in fractures and wounds by activating the local production of endogenous opioids.

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