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Twenty-five Years of PTHrP Progress from Cancer Hormone to Multifunctional Cytokine

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Abstract

Twenty-five years ago a 'new' protein was identified from cancers that caused hypercalcemia. It was credited for its ability to mimic parathyroid hormone, and hence was termed parathyroid hormone-related protein (PTHrP). Today it is recognized for its widespread distribution, its endocrine, paracrine, and intracrine modes of action driving numerous physiologic and pathologic conditions with a central role in organogenesis. The multiple biological activities within a complex molecule with paracrine modulation of adjacent target cells present boundless possibilities. The protein structure of PTHrP has been traced, dissected and deleted comprehensively and conditionally, yet numerous questions lurk in its past that will carry into the future. Issues of the variable segments of the protein including the enigmatic nuclear localization sequence are only recently being clarified. Aspects of PTHrP production and action in the menacing condition of cancer are emerging as dichotomies that may represent intended temporal actions of PTHrP. Relative to PTH, the hormone regulating calcium homeostasis, PTHrP 'controls the show' locally at the PTH/PTHrP receptor throughout the body. Great strides have been made in our understanding of PTHrP actions, yet years of exciting investigation and discovery are imminent.

Keywords

PTHrP; PTH; paracrine; hypercalcemia; bone; cartilage

Historical Perspective

Parathyroid hormone-related protein (PTHrP) seemed almost to come out of nowhere, produced by certain cancers, mimicking PTH action and causing the complication of hypercalcemia. Fuller Albright in 1941 (1), when discussing a patient with renal carcinoma,

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a solitary metastasis and hypercalcemia, suggested that some tumors might cause hypercalcemia by secreting PTH or something very like it. As with many of his predictions he was ultimately proved correct, but not before the passing of a few decades during which the concept of “ectopic PTH production” by cancers was promulgated as the cause of non-metastatic hypercalcemia (2;3). Doubts began to appear in the 1970’s, when improved radioimmunoassays for PTH indicated that the immunoreactivity in tumor or plasma of patients with cancer differed from authentic PTH (4–7), and with some assays PTH immunoreactivity could not be detected at all (8). From this background three excellent clinical studies put beyond reasonable doubt the biochemical similarity between primary hyperparathyroidism and this syndrome of humoral hypercalcemia of malignancy (9–11). By that time, rapid, sensitive, robust biological assays of PTH had developed and extracts and culture supernatants of hypercalcemic animal and human tumors were found to contain PTH-like adenylate cyclase responses in osteoblast and kidney targets (12–14). This paved the way for purification of PTHrP from a human lung cancer cell line (15), a breast cancer (16) and a renal cancer cell line (17). The cloning of its cDNA (18;19) showed 8 of the first 13 residues of PTHrP identical to those in PTH, any remaining identities no more than expected by chance, and the structural requirements for full biological activity of PTHrP contained within the first 34 amino acids, as was known to be the case with PTH (20). These findings explained the biochemical similarities between syndromes of PTH excess and non-metastatic hypercalcemia in cancer, signaling the discovery of an evolutionary relationship between these two molecules, most likely derived from a common ancestor and evolving from a gene duplication event. The *PTHrP* gene had a more complex structure than that of *PTH*, but with similar intron-exon boundaries, and the marked conservation of the PTHrP amino acid sequence in human, rat, mouse, chicken and canine up to position 111 indicated that important functions are likely to reside in this region. This was the beginning of the rise of PTHrP to a position of great interest; yet in many circles it still plays a secondary role in the family of parathyroid hormones.

Soon after its discovery, it became apparent, that far from being simply an evolutionary relic that mimics PTH action in non – metastatic cancer, PTHrP has major roles in other aspects of cancer, in development, and in normal physiological functions in post-natal life. At the time the receptor was discovered, it was evident that this receptor functioned to relay both PTH and PTHrP biological activity (21), yet knock out of this PTH/PTHrP receptor (PPR) highlighted the importance of PTHrP (22;23). Indeed, PTHrP is the master regulator with widespread paracrine actions (Figure 1), and as illustrated for example by the pharmacological use of PTH as an anabolic agent in the treatment of osteoporosis where PTH administration actually mimics PTHrP actions locally in the bone microenvironment (24). Still, many questions remain unanswered. This perspective piece will briefly consider aspects of PTHrP function in development and disease and pose outstanding questions that despite 25 years of inquiry still linger.

PTHrP – evolutionary insights

It has long been accepted that PTH and PTHrP arose from gene duplication, PTH located on human chromosome 11 and PTHrP on chromosome 12. If conservation across species is evidence of gene importance, PTHrP prevails – fugu fish PTH has 44% identity with human

PTH whereas fugu fish PTHrP bears 53% similarity with human PTHrP (25). At the amino acid level, chicken PTH shares 88% similarity with human, and chicken PTHrP shares 91% similarity with human (26). The first 111 amino acids of PTHrP are extraordinarily conserved among many species. Interestingly, PTH appears to play a paracrine role in lower vertebrates but evolved in higher vertebrates with a more restricted yet vital endocrine role. In fish, PTH produced in the gills is responsible for local calcium regulation; whereas via evolution to tetrapods the parathyroid gland assumed an endocrine role as calcium requirements shifted from an aquatic to a terrestrial environment (27). PTHrP, having a fairly simple gene structure in lower vertebrates acquired a more complex structure with added exons and alternative promoters with progression to humans, and in parallel picked up a stronger paracrine emphasis (26;28).

PTHrP – more than an endocrine factor

A great surge of interest and research activity came with the finding that PTHrP is normally produced in many tissues and acts in those sites in a paracrine manner. There are only three identified circumstances in which PTHrP species are present in the circulation and act in an endocrine manner: 1) the humoral hypercalcemic syndrome, in which PTHrP is produced by tumors and circulates to the bone to stimulate bone resorption (29;30), 2) lactation, in which PTHrP is made in the breast and reaches the circulation (31), and 3) fetal life, where PTHrP regulates maternal-to-fetal placental calcium transport (32;33). There remains to the present time no convincing evidence of biologically relevant circulating PTHrP levels otherwise in normal humans. Hence, the vast majority of PTHrP actions, unlike PTH are paracrine in nature.

There are three splice variant isoforms of PTHrP rendering PTHrP 1-139, 1-173, or 1-141 with transcriptional regulation from three distinct promoters (34). The multiple products of post-translational processing including glycosylation, the short half-life of PTHrP mRNA, and the multiple biological activities contained within PTHrP, equip it ideally to function as a paracrine effector with a developmental focus (35–38). Together with the obvious susceptibility of PTHrP to post-translational modification through proteolysis (39), and the generation of several constituent peptides, this increased complexity highlights the leading role of PTHrP, yet leaves questions that remain unanswered to this day. ***Why are there 3 PTHrP splice variants in humans but not in other mammals? What is the extent of biologically relevant PTHrP peptide fragments, and how do they function? Biologically active PTHrP peptide fragments functioning independent of the N-terminus raise the question – are there yet unidentified receptors ?***

Skeletal actions

The physiological importance of PTHrP in the skeleton was evident with deletion of PTHrP resulting in death in mice immediately after birth from respiratory failure, attributed to defective rib cage formation (40). Here PTHrP stands out from PTH, whose later gene deletion resulted in a comparatively mild phenotype (41). Multiple defects in skeletal development confirmed the importance of PTHrP in fetal bone development (42). Whereas haploinsufficient PTHrP (+/-) mice are phenotypically normal at birth, by three months of

age they have low bone mass, with a marked decrease in trabecular thickness and connectivity and an abnormally high number of adipocytes in the bone marrow (43). PTHrP +/- mice have compromised recruitment of bone marrow precursors and increased osteoblast apoptosis compared to wild-type mice. Importantly, this phenotype was recapitulated in transgenic mice with osteoblast-specific knockout of PTHrP thereby confirming the role of osteoblast-derived PTHrP in the process of bone formation (24). These mice also demonstrated reduced osteoclast formation likely due to impaired ability of PTHrP null osteoblasts to support osteoclast formation. Confounding work in this area, is the nature of PTHrP, its low-abundance mRNA and protein products that have been difficult to identify by conventional immunohistochemical approaches. Coincident expression of PTHrP mRNA and protein was noted in both chondrocytes and osteoblasts in endochondral bone formation in the mouse, and both also in preosteoblasts and actively synthesizing osteoblasts in a regenerating bone model in the rabbit (44). On the other hand, using a PTHrP-lacZ knock-in mouse, Chen and colleagues were not able to demonstrate osteoblast derived PTHrP production suggesting osteoblast-derived PTHrP would not drive local bone formation (45).

In the growth plate, chondrocyte maturation is tightly regulated by a paracrine PTHrP/Indian hedgehog (Ihh) signaling loop. PTHrP produced by the distal perichondrium interacts with the PPR expressed in the proliferative and perhypertrophic zones of the growth plate. These findings support a key role of PTHrP in controlling the pace of growth plate development via preventing premature differentiation of chondrocytes into prehypertrophic and hypertrophic chondrocytes (46). More recently, findings have extended to articular joints where evidence suggests PTHrP is produced in response to loading and functions in a similar Ihh signaling loop to support articular cartilage maintenance (47).

The favored current concept is that PTH the hormone, regulates calcium homeostasis in development and maturity. PTHrP the local factor, on the other hand, directs growth plate development by controlling chondrocyte proliferation and differentiation, while of these two proteins postnatally, PTHrP is the main factor generated locally in bone and acting through the PPR in bone remodeling, without normally contributing to the maintenance of serum calcium levels. These studies also highlight emerging evidence and pose questions – *Does anabolic PTH essentially co-opt PTHrP physiologic actions? Might we regard the use of PTH in skeletal anabolic therapy as an attempt to reproduce the local action of PTHrP?*

Placental calcium transport

A role for PTHrP action in the placenta is highlighted by its ability to promote trans-placental calcium transport in sheep (32), with PTHrP (67–86) and (38–94) the most active peptides (48;49), and no action of amino-terminal PTHrP. Studies in genetically manipulated mice confirm that PTHrP controls placental calcium transport to bring about mineralization of the fetal skeleton (50), with the main PTHrP source being the placenta.

Smooth muscle relaxation

A particularly instructive example of the paracrine actions of PTHrP is found in the smooth muscle beds of the vasculature. It had been known since the 1920's that injection of parathyroid extract in animals results in dose-dependent increases in blood flow through a range of vascular beds, and decreases in blood pressure (51–54). When PTHrP was discovered it became clear that this was not the normal function of PTH but instead, a local physiological role of PTHrP, produced in smooth muscle beds of the stomach and intestine, uterus, urinary bladder, and arterial vessels, acting in all those tissues as a muscle relaxant (36;55;56). PTHrP expressed in smooth muscle acts rapidly to relax the vasculature (57) through an endothelium – independent mechanism, and vasoconstrictors such as angiotensin II induced a rapid rise in PTHrP production (58). Thus increased PTHrP production following vasoconstriction could provide a mechanism to limit or reverse this effect through the relaxing action of PTHrP on smooth muscle. The paracrine production and action of PTHrP in local vascular beds comes into action as required physiologically. On the other hand, when PTH is administered systemically, with simultaneous activation in many sites, the response of general vasodilatation and decline in blood pressure is not surprising. *Yet, where does PTHrP stand in the hierarchy of paracrine vasoactive peptides?*

Mammary gland development

Although the neonatal lethality of *PTHrP*^{-/-} mice initially presented difficulty in identifying tissue specificity, rescue of these mice was achieved by directing PTHrP production to cartilage with use of the collagen II promoter, allowing study of the effect of the PTHrP null phenotype on several other organs (59). In the case of the breast, “rescued” PTHrP null mice show failure of early breast ductal development, providing strong evidence of a further paracrine role for PTHrP in promoting branching morphogenesis (59). With such dramatic expressions of PTHrP involvement in early breast development, it is perhaps not surprising that PTHrP emerges as a factor important in breast cancer biology yet a recent study provides evidence against a PTHrP role in post-natal breast development (60). The discovery of RANKL production by primitive ductal cells, acting on RANK in mammary stem cells to promote their expansion (61) highlights the need for further investigation of the role of PTHrP and RANKL in the breast.

Teeth and skin

Further evidence supporting a prominent role for PTHrP in development came with the PTHrP type II collagen promoter rescue. These mice lack tooth eruption, a cardinal sign of defective osteoclastogenesis (62). PTHrP is produced by cells of the enamel organ during development (63) and receptors for PTHrP exist in the bone surrounding the developing tooth, and also in the dental follicle and in cementoblasts lining the tooth root surface (64–66). Philbrick and colleagues used a cytokeratin, K14-PTHrP transgene, to show that replacement of PTHrP in the enamel epithelium restores tooth eruption (62). Since osteoclasts do not express PPR receptors, this supported the developmental role of PTHrP to drive osteoclasts necessary for clearing the path for the erupting tooth through a paracrine/

juxtacrine interaction. Murine studies were validated with human analyses of loss of function of the PPR revealing ankylosed and distorted tooth development (67).

Yet another tissue/organ site of PTHrP paracrine actions was identified in the hair follicle with reciprocal expression of the PPR in the dermal components. Overexpression of PTHrP led to premature termination of anagen and entrance to catagen in the hair cycle (68). PTHrP expression was identified to be high in late anagen and thought to participate in the hair cycle but not necessarily be essential for hair cycle progression. Similarly, studies of PTHrP expression in keratinocytes found temporal dependent production of PTHrP with a reduction as keratinocytes differentiate (69;70) suggesting a regulatory loop similar to that found in cartilage development.

Pancreas

In the pancreas, islet cells were found to produce PTHrP as well as bear the PPR, and PTHrP provides a robust increase in intracellular calcium in beta cells (71). All four endocrine cell types, alpha, beta, delta, and pancreatic polypeptide cells produce PTHrP (72). Overexpression of PTHrP as well as PTHrP 1-36 administration increases beta cell proliferation via cell-cycle specific activation (73). PTHrP overexpression in beta cells results in islet hyperplasia and insulin-mediated hypoglycemia associated with reduced apoptosis (74). This effect was also shown via exogenous administration of PTHrP 1-36 supporting a local mediated action at the PPR. PTHrP also increases beta cell production of insulin suggesting its consideration in therapeutic strategies to improve islet growth and function.

PTHrP in other locations

Beyond the organ - focused investigations discussed above, PTHrP has been detected in nearly every tissue/organ in the body. Early reports of PTHrP and PPR expression in the heart, brain, skeletal muscle, bladder, lungs, bile ducts, immune system, liver, uterus, testes, as well as most endocrine organs including the pituitary and thyroid gland C-cells (36;75) leave unanswered questions years later as to the tissue-specific significance of PTHrP in health and disease. However, many of the early studies in this area did not have PTHrP knockout mice available as negative controls, and did not use *in situ* hybridization to detect PTHrP mRNA. To this day, challenges surrounding the specificity of PTHrP immunohistochemistry still need to be overcome.

PTHrP – the intracrine factor

A most intriguing early finding was the discovery that PTHrP attains a nuclear/nucleolar location through a specific transport process, and is likely to exert some of its functions from that site. Nucleolar localization of PTHrP through a defined sequence in the mid-region is associated with enhanced chondrocyte survival following prolonged periods of serum starvation (76;77). Expression of PTHrP is cell cycle-specific in smooth muscle cells (78) and keratinocytes (78;79), and its mRNA highest at the G1 phase, when localized to the

nucleolus (80), with cyclin-dependent kinase phosphorylation of T⁸⁵ resulting in exclusion of PTHrP from the nucleus (81).

Within the PTHrP sequence there are nucleus (CcN) and nucleolus localization motifs, with the former being similar to that described for the archetypal CcN-containing protein, SV40 T-antigen (82). The mechanism of nuclear import requires PTHrP interaction with importin β and GTP-Ran expression (83–85), and cyclin-dependent kinase phosphorylation of T⁸⁵ results in exclusion of PTHrP from the nucleus (81). A nuclear targeting sequence that inhibits apoptosis exists at PTHrP (87–107) (76;86–88), and PTHrP (109–139) is involved in its nuclear export (89). Evidence supports direct binding of PTHrP to RNA through a distinct motif in the NTS (90) and further points to PTHrP as likely to exert important functions from its nuclear site. PTHrP appears so far to be the only protein classed at least in some circumstances as a hormone, which possesses a CcN motif and displays differential cellular localization (nuclear/nucleolar versus cytoplasmic). There must be some important purpose behind the evolutionary conservation of this property, and prompts the question, *what is the significance of nuclear entry of PTHrP in the many tissues in which PTHrP is considered to play a local role?*

The impact of other biological activities exerted by domains within PTHrP was exemplified in two studies in mice, in one of which knock-in of PTHrP (1–84), lacking both the nuclear localization sequence (NLS) and C-terminal region while retaining the bioactive amino-terminal, resulted in multiple abnormalities and early lethality in mice (91). Homozygous mice exhibited skeletal growth retardation and osteopenia associated with reduced proliferation and increased apoptosis of osteoblasts as well as early senescence with altered expression patterns and subcellular distribution of proliferative- and senescence-related genes in multiple tissues. A further knock-in of PTHrP (1–66) excluding a significant part of the mid-region resulted in an even more severe phenotype and highlighted the role of PTHrP in stem cells as well as later lineage cell commitment (92). These genetic studies in mice show that many of the actions of PTHrP are not mediated by the amino-terminal region, and among the generalized abnormalities, absence of the mid-region, NLS and C-terminal region result in greatly impaired commitment and survival of osteogenic and hematopoietic precursors.

PTHrP and Cancer

The significance of PTHrP in cancer was not confined to the humoral hypercalcemic syndrome. Breast cancer was one of the original sources of PTHrP (16). Hypercalcemic breast cancer patients with metastatic bone disease have elevated plasma PTHrP levels (30), and 60% of primary breast cancers and 90% of bone metastases are positive for PTHrP by immunohistochemistry (93;94). From this arose the concept that PTHrP production in the bone marrow by breast cancer cells promotes bone resorption, thus favoring tumor establishment and expansion. Extensive experimental evidence was produced in support of this (95;96), including prevention and treatment of tumor growth by inhibiting bone resorption, using bisphosphonates or neutralizing monoclonal antibodies against PTHrP (95;97). All of this accorded with the “seed and soil” hypothesis developed by Stephen Paget (98), which depicted bone as the favorable soil for the “seed” of breast cancer. A major

contributor to this co-operation is tumor production of PTHrP, together with the several other cancer-derived factors that influence bone metastasis establishment, including prostaglandins and cytokines (96) and of factors favoring the homing and/or adherence of cancer cells to bone (99). Although this emphasizes PTHrP involvement in breast cancer, increasing evidence implicates it in prostate, lung, renal, colon, lymphoid and other cancers (100–104).

The history and findings need to be considered when evaluating the place of PTHrP in the pathogenesis of bone metastasis formation and progression. ***What is the relative importance of PTHrP compared to other tumor derived factors and the type of cancer (e.g. breast vs. prostate and their different bone phenotypes)? When a PTHrP-producing tumor metastasizes to bone, how does this locally produced factor compare in its biologic impact with other tumor-derived factors? Is there a very early role for PTHrP as an endocrine/tumor-derived circulating factor in conditioning the bone microenvironment, i.e. a pre-metastatic niche? Does PTHrP influence tumor cell dormancy?***

Does PTHrP indeed have an entirely independent function, perhaps early in cancer development, of contributing to a less invasive phenotype of the cancer? That suggestion comes from a long-term, prospective study of consecutive patients at a single center, that tumors positive for PTHrP at surgery were independently predictive of improved patient survival, with reduced metastases at all sites, including bone (105;106). Such a mechanism is distinct from the bone resorbing action later in disease, that can explain the association of PTHrP production with bone metastases (107–110). Highlighting the controversy, of two independent studies of genetically induced breast cancer in mice, one concluded that loss of PTHrP expression resulted in poorer outcomes in breast cancer (111), the other concluded the opposite, that PTHrP promotes the initiation and progression of primary tumors (112).

With PTHrP directing an important role in early mammary gland development (59;67;113), it might not be surprising if it were to play a part in early stages of cancer development – but is this concept of PTHrP a credible one - protective at one stage of cancer yet deleterious at another? Indeed PTHrP has been shown to have multiple and opposing roles in other circumstances such as in its ability to both protect and promote apoptosis in osteoblastic cells and pneumocytes (114–116). A similar example of a dual action is TGF β , which acts early as a tumor suppressor by inhibiting proliferation of epithelial, endothelial and hematopoietic cells. Refractoriness to these effects develops later, and over expression of TGF β leads to a microenvironment conducive to tumor growth (117–119). Confirmation of a dual role for PTHrP requires further clinical and basic study, with the critical question: ***What are the temporal implications of PTHrP in tumorigenesis; are there different and contrasting early and later actions?***

Summary

After 25 years, what does PTHrP really do? Can we regard PTH use as a pharmacological agent that is simply a surrogate for what PTHrP does physiologically? We can only speculate about the nature of the PTHrP molecule that gains access through a paracrine mechanism to its adjacent target cells, and the likelihood of multiple biological activities

within the molecule presents complex possibilities. Assuming that full-length PTHrP is secreted, is that the predominant form that interacts with target cells locally, or does its susceptibility to proteolytic breakdown yield shorter products, even in that local environment? PTHrP was designated as 'related' to PTH, but it is certainly not a distant cousin. Although knowledge of PTH actions far preceded our knowledge of PTHrP, over the past 25 years PTHrP has emerged as the key regulator of normal physiology as well as pathophysiologic events. Whereas PTH actions center on its role in calcium metabolism, the multifactorial nature of PTHrP will continue to give years of exciting investigation.

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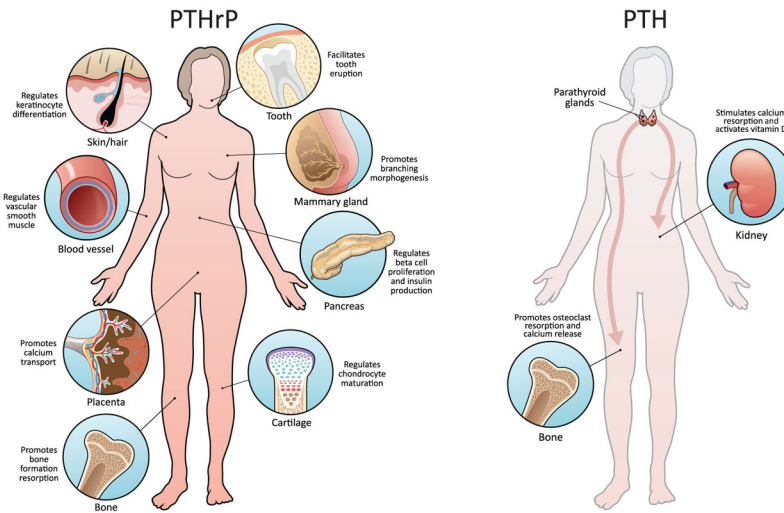


Figure one. PTHrP Paracrine actions

PTHrP has numerous paracrine actions in physiologic homeostasis including roles in keratinocytes/hair follicles, cartilage, vascular smooth muscle, bone, mammary gland development, tooth eruption, pancreas, and others not depicted. In comparison, PTH has relatively fewer direct physiologic targets via its endocrine mode of operation in bone and kidney.