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Humoral Hypercalcemia of Malignancy: Some Enigmas on the Clinical Features

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Abstract Humoral hypercalcemia of malignancy (HHM) is a common paraneoplastic syndrome mediated by tumor-derived parathyroid hormone-related peptide (PTHRP), which bears structural and functional similarities to PTH. Thus the clinical features of HHM are very similar to those of primary hyperparathyroidism (1° HPT), a prototype of humoral hypercalcemia caused by PTH. On the other hand, HHM syndrome differs from 1° HPT in several aspects, including serum $1,25(OH)_2D$ levels, acid-base balance, and bone remodeling process, the reason of which remains largely unknown. We approached these questions using a unique animal model of HHM, nude rats implanted with PTHRP-overproducing human carcinomas. In this review we will summarize the results and discuss the implications in understanding the disease mechanism. © 1995 Wiley-Liss, Inc.

Key words: cancer-associated hypercalcemia, parathyroid hormone-related peptide (PTHRP), nude rat model, bone remodeling, gene regulation

Primary hyperparathyroidism (1° HPT) and cancer-associated hypercalcemia represent the most common causes of hypercalcemia. Cancer-associated hypercalcemia is one of the most frequent paraneoplastic syndromes and believed to occur through two general mechanisms, one humoral and the other local, of which the former mechanism, termed humoral hypercalcemia of malignancy (HHM), is responsible for more than 80% of cases [1]. One of the major breakthroughs in this field is the identification of parathyroid hormone-related peptide (PTHRP) in various carcinomas as a major "humor" responsible for HHM syndrome [2–4]. Specific and sensitive immunoassays for human PTHRP have been developed and provided evidence that circulating PTHRP concentrations are elevated in most cancer patients with hypercalcemia [5]. Another outstanding achievement is the molecular cloning of a receptor for PTH, which turned out to bind PTHRP as well with an almost identical affinity [6]. These findings led to the current concept that PTHRP overproduced and secreted by certain cancers enters the circulation, interacts with a common receptor for PTH/PTHRP in bone and kidney, and causes hypercalcemia through stimulation of calcium mobilization from the bone (bone resorption) and calcium reabsorption from the kidney. Although the major humor responsible for HHM as well as the receptor molecule that is essential for its biological actions has been identified, there are still some enigmas regarding the clinical features of HHM syndrome, which will be the main focus of this review.

HHM VS. 1° HPT: SIMILARITIES AND DIFFERENCES

Although hypercalcemia had been a well-known complication of cancer patients since the 1920s, its pathogenesis remained unclear until the clinical features of HHM syndrome were biochemically defined by Stewart et al. in early 1980s [1]. Their study demonstrated that a majority of patients with cancer-associated hypercalcemia showed biochemical characteristics, such as hypercalcemia, hypophosphatemia, and increased urinary cAMP excretion [1], which are the same as those with 1° HPT (Fig. 1). These observations led to the hypothesis that cancer-

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associated hypercalcemia was mediated in most cases by a tumor-derived factor with PTH-like properties, and thus the term *HHM syndrome* was introduced. This hypothesis was further substantiated by the findings that human tumors associated with HHM syndrome indeed contained a PTH-like (adenylate cyclase-stimulating and bone-resorbing) activity in vitro [7].

On the other hand, the putative PTH-like factor appeared clearly distinct from PTH itself in terms of molecular weight and immunogenicity. Consistent with this notion are the clinical observations that patients with HHM display decreased immunoreactive PTH levels, suppressed $1,25(OH)_2D$ concentrations, and metabolic alkalosis [1], whereas those with 1° HPT are associated with elevated PTH levels, increased $1,25(OH)_2D$ concentrations, and hyperchloremic metabolic acidosis (Fig. 1). Subsequent bone histomorphometric analysis in HHM patients revealed another distinct feature, a phenomenon called uncoupling, that is, excessive bone resorption and suppressed bone formation vis-à-vis "coupling" of bone remodeling process in 1° HPT (increased bone formation coupled to increased bone resorption) [8]. To explain the differences in their clinical features and fully understand the pathogenesis of HHM syndrome, the identification of the causative PTH-like substance was eagerly awaited.

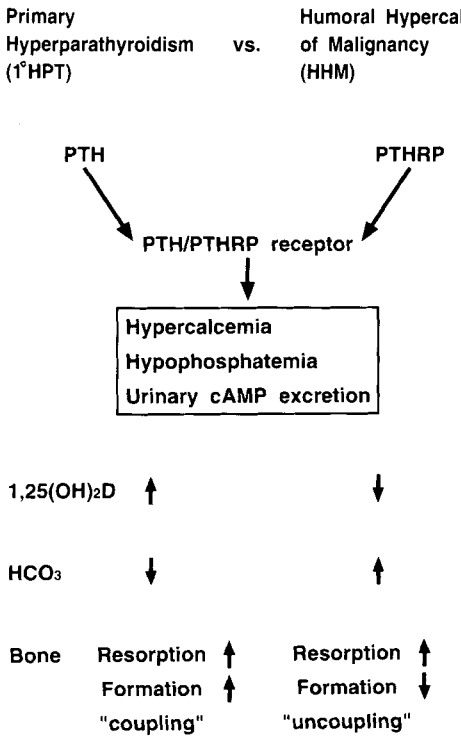

Fig. 1. Humoral hypercalcemia of malignancy (HHM) vs. primary hyperparathyroidism (1° HPT): similarities and differences in their clinical features.

PTHRP: MOLECULAR STRUCTURE AND FUNCTIONAL DOMAINS

After intensive work for several years, the PTH-like factor, now called PTHRP, was purified from human tumors, and its amino-terminal sequence was determined in 1987 by three groups almost at the same time [9–11]. Soon after the purification, a cDNA encoding PTHRP was cloned by the same groups [12–14]. The original cDNA encodes a 141 amino-acid protein with 8 out of 13 amino acids at the amino terminus being identical with those of PTH but complete sequence divergence thereafter [12,13]. The sequences up to the residue 111 are remarkedly conserved among species, suggesting the existence of functionally important domains peculiar to PTHRP.

The human PTHRP gene has also been isolated and its complex structure with multiple promoters clarified [15–17]. Alternative splicing at the 3′ ends has been postulated to give rise to three primary translation products of 139, 173, and 141 amino acids (in mature protein) with distict carboxyl terminal sequences (Fig. 2). In addition, PTHRP appears to be subject to a variety of posttranslational processing and modifications [18,19]. Although the secretory and/or circulating forms of PTHRP remain to be clarified, the data thus far available suggest the existence of at least three forms: amino-terminal, mid-region, and carboxyl-terminal fragment [20] (Fig. 2). The amino-terminal fragment, PTHRP(1-36), corresponds to the PTH-like domain (Fig. 2) and has been shown to reproduce the cardinal features of HHM syndrome, such as hypercalcemia, hypophosphatemia, and increased cAMP production in bone and kidney [21–25]. However, like PTH, PTHRP(1-36) excess results in increased $1,25(OH)_2D$ production as well as stimulation of both bone formation and resorption, suggesting that the full spectrum of HHM syndrome cannot be explained by the PTH-like actions of PTHRP(1-36).

A question arises as to whether or not some of the distinct features of HHM syndrome are mediated by a PTHRP molecule other than PTHRP(1-36). The mid-region fragment appears to start with Ala^{38} and consist of 60–70

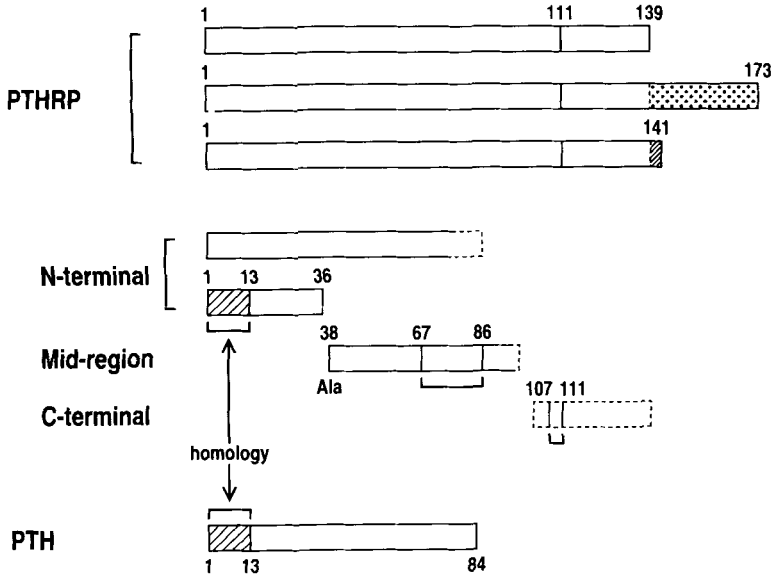

Fig. 2. PTHRP: molecular structure and functional domains.

amino acids [20] (Fig. 2). PTHRP(67-86) contained in this domain exerts PTHRP-specific actions, such as calcium transport across the placenta [26]. In addition, it has been shown in isolated perfused rat kidney that a mid-region PTHRP, contained in PTHRP(1-108) but not in PTHRP(1-34), does not cause a sustained stimulation of HCO_3 excretion, as compared with PTH(1-34), PTH(1-84), or PTHRP(1-34) [27]. These results raise the possibility that metabolic alkalosis in HHM patients, in contrast to metabolic acidosis in 1° HPT, is due to the PTH-unlike action of a mid-region PTHRP.

It has been shown that a carboxyl-terminal fragment is excreted from the kidney and thus accumulates to very high levels in renal insufficiency [5]. Interestingly, a pentapeptide corresponding to PTHRP(107-111) has been shown to inhibit osteoclastic bone resorption [28], which is the opposite action to that of PTHRP(1-36) [2–4]. Although the biological significance of the carboxyl-terminal PTHRP fragment in renal failure and HHM remains to be clarified, it would be intriguing to surmise that the full spectrum of HHM syndrome is explained by the diverse actions of multiple species of PTHRP molecule.

NUDE RAT MODEL OF HHM

Experimental models of HHM produced by implanting human tumors into immunodeficient animals have contributed much to our understanding of the disease mechanisms. We developed a nude rat model of HHM, a unique experimental system which enabled us to analyze the blood and urinary biochemical parameters as well as bone histology with more ease and in much more detail than a nude mouse model. After surveying dozens of models implanted with a variety of human cancers, we identified two models, OCC and UCC, which turned out to be particularly interesting and valuable for further studies [29]. OCC, derived from a hypercalcemic patient with squamous carcinoma of oral cavity, induced very aggressively growing tumors with marked hypercalcemia, and OCC-bearing nude rats usually died in 3 weeks after transplantation [29,30]. UCC, derived from a hypercalcemic patient with squamous carcinoma of uterine cervix, caused less aggressive tumors and hypercalcemia, and UCC-bearing nude rats survived as long as 3 months [29]. Both tumors were confirmed to contain a PTH-like bioactivity in vitro [29], express the typical multiple transcripts of PTHRP mRNA [K Ikeda, unpublished observations], and display elevated circulating PTHRP concentrations, as determined by our two-site immunoradiometric assay (IRMA) for human PTHRP(1-87) [31].

OCC-bearing animals displayed increased serum $1,25(OH)_2D$ concentrations, like most other animal models of HHM, despite the fact that $1,25(OH)_2D$ are usually low normal or markedly suppressed in HHM patients. On the other hand,

UCC-bearing animals showed decreased $1,25(OH)_2D$ concentrations, making it a valuable model that completely mimics human syndrome of HHM and thus is particularly useful to shed new light on the characteristic vitamin D metabolism in the disease [29]. Dr. Fukumoto, Dr. Matsumoto and colleagues found as they attempted to purify the PTH-like activity from these tumors that UCC tumors contained two peaks (a major peak A and a minor peak B) corresponding to PTH-like activity, whereas OCC tumor contained only the major peak A [32]. The presence of two peaks of PTH-like activity was also documented by Stewart et al. in various human tumors derived from HHM patients [33].

According to Fukumoto et al., the common peak A stimulated $1,25(OH)_2D$ production, whereas the UCC-specific peak B suppressed PTH-stimulated production of $1,25(OH)_2D$ in the primary culture system of rat kidney cells [32]. These results may suggest that a human tumor associated with HHM contains a PTHRP molecule which suppresses the production of $1,25(OH)_2D$ as well as another molecule that stimulates $1,25(OH)_2D$ production and that suppressed $1,25(OH)_2D$ concentrations in HHM patients can be explained by the interplay of multiple PTHRP species. However, the identity of peak A and peak B remains to be determined, and it is not clear why some tumors lose peak B after transferred to nude rat or nude mouse models leading to increased $1,25(OH)_2D$ levels.

BONE REMODELING IN HHM

Bone histomorphometric analysis has demonstrated that bone in HHM patients is characterized by a marked uncoupling process—that is, excessive bone resorption with suppressed bone formation [8]—whereas an increase in both bone formation and resorption (coupling) is a common feature of 1° HPT. Characteristic bone involvement in HHM patients cannot be explained by the PTH-like actions of PTHRP(1-36) since infusion of amino-terminal PTHRP fragments, like PTH, causes an increase in bone formation as well as resorption [34,35]. In this respect, it is puzzling that stimulation of bone formation has not been documented in HHM patients. It is to be noted that previous bone histological studies were performed on patients with advanced disease [8], and no study has been focused on the bone involvement at its very early stage. Our nude rat model may provide a suitable in vivo system to clarify a sequence of events in the bone remodeling process at the tissue and cell levels from a very early phase of HHM through its terminal stage.

As mentioned earlier, OCC-bearing animals developed progressive hypercalcemia as a function of tumor growth and died in 3 weeks [29,30] (Fig. 3). Based on the results of detailed biochemical analysis, we divided the disease process into three phases (Fig. 3): a very early stage with marginal hypercalcemia and modestly elevated circulating PTHRP levels on day 6, an intermediate stage with a clear-cut elevation in serum calcium and plasma PTHRP on day 12, and a terminal stage with a marked increase in both serum calcium and plasma PTHRP on day 18. In order to clarify the temporal profile of the bone remodeling process as a function of tumor growth, bone histomorphometric analysis was

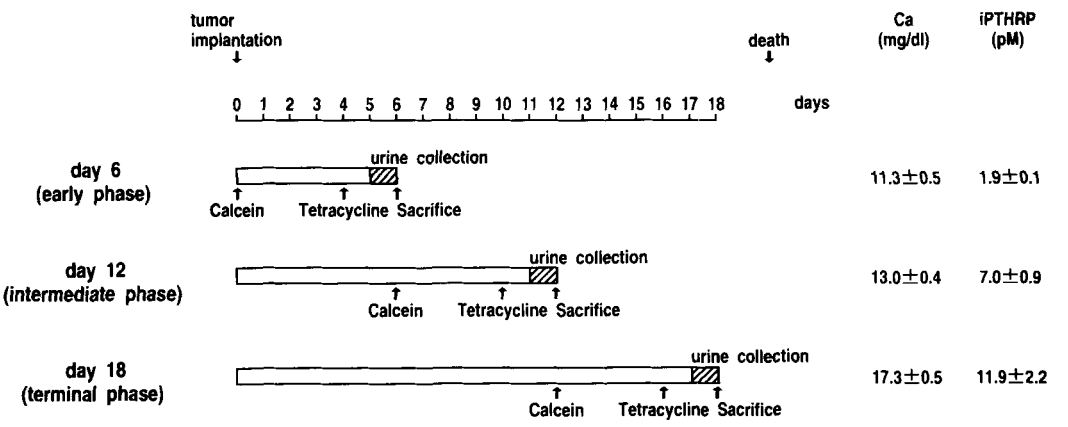

Fig. 3. Experimental schedule for bone histomorphometric studies in a nude rat model of HHM.

performed on day 6, 12, and 18, according to the experimental schedule shown in Figure 3.

The tumor-bearing animals showed progressive and marked bone loss, as determined by bone mineral density (BMD) at the proximal tibia. The results of histomorphometric analysis confirmed the previous observations that bone in overt HHM syndrome (day 12 and day 18) is characterized by a striking uncoupling mechanism. In addition, we have demonstrated that a substantial increase in bone formation as well as bone resorption does occur at a very early stage of HHM (day 6). The increase in bone formation rate on day 6 was associated with an increase in both mineral apposition rate and osteoblast surface, suggesting that at the cell level the osteoblastic activation as well as the recruitment of new osteoblasts contributes to the stimulation of osteoblastic bone formation at this stage [30]. These findings were in marked contrast with those at later stages (day 12 and 18), when both mineral apposition rate (as a parameter of osteoblastic activity) and osteoblast surface (as a parameter for recruitment of new osteoblasts) were markedly suppressed [30].

It is generally believed that communication between osteoblasts and osteoclasts is a critical event in the stimulation of osteoclastic bone resorption in response to systemic calciotropic hormones, such as PTH, PTHRP, and $1,25(OH)_2D_3$ [36]. It has been shown, for example, that PTHRP acts on osteoblasts to stimulate the release of a soluble factor(s), which in turn stimulates osteoclasts to resorb bone [37]. These findings, taken together with our results of histomorphometric analysis, raise an intriguing possibility that the activation of osteoblasts by tumor-derived PTHRP at a very early phase of HHM plays an important role in the acceleration of osteoclastic bone resorption at later stages.

It is well known that multiple myeloma (MM) often causes hypercalcemia through a local mechanism and is characterized by marked uncoupling of bone. It has recently been reported that recruitment of new osteoblasts is observed in patients with early MM in contrast to the marked suppression of osteoblastic function in patients with overt MM [38]. Taken together with our observations in a nude rat model of HHM, it is tempting to speculate that the transient increase in osteoblastic activity at an early stage may be a critical event leading to bone diseases characterized by excessive bone resorption, whether the mediator by systemic or local.

What causes a marked suppression of bone formation in overt HHM syndrome? One possible explanation is that a PTHRP molecule other than PTHRP(1-36) exerts a distinct biological action from that of PTH and suppresses bone formation, based on the emerging evidence that PTHRP is subject to a variety of posttranslational processing and that the mid-region or carboxyl-terminal fragment of PTHRP exerts various PTH-unlike actions [20]. The second possibility is that other cytokines derived either from tumors or from the host immune system in response to the presence of tumors, such as interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) [39], modulate the bone remodeling process during the development of HHM syndrome. In this respect, we have recently found that the OCC tumor overproduced IL-6 in addition to PTHRP and that a neutralizing antibody against human IL-6 not only inhibited the osteoclastic bone resorption but partially reversed the marked suppression of mineral apposition rate in the tumor-bearing animals, raising the possibility that tumor-derived IL-6 is involved, at least in part, in the uncoupling process of bone [Y. Nagai, H. Yamato, et al., manuscript in preparation].

ACTIVATION OF PTHRP GENE TRANSCRIPTION WITH TUMOR GROWTH

Although PTHRP was initially identified in a variety of carcinomas as a humoral mediator of cancer-associated hypercalcemia [2–4], it is now evident that PTHRP is a product of almost all normal tissues and plays diverse physiological roles in fetal as well as adult life mainly through a local mechanism [40]. However, the molecular and cellular mechanism(s) by which PTHRP is overproduced in certain malignancies remains largely unknown. It is not clear, for example, how and when PTHRP gene is activated during the multistep process of carcinogenesis.

We have found using the transfection-focus formation assay that malignant transformation of normal mammalian cells by introduction of oncogenes induces the expression of PTHRP gene in vitro and causes HHM syndrome in vivo (T. Motokura et al., manuscript submitted). Similarly, Li and Drucker have recently demonstrated that a single oncogene, either src or ras, is capable of activating PTHRP gene transcription in established cell lines [41]. These results

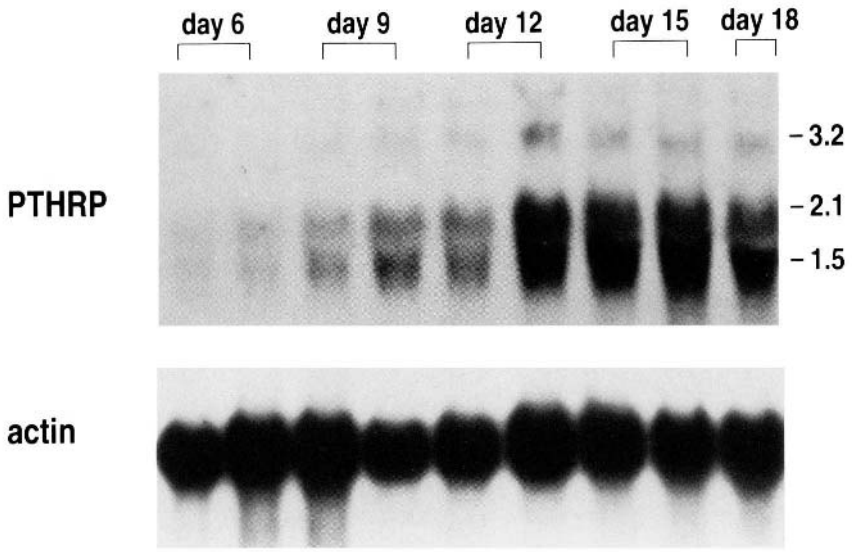

Fig. 4. Temporal profile of PTHRP mRNA expression with tumor growth. Steady-state levels of PTHRP mRNA in the tumors were determined by Northern blot analysis on the indicated days after tumor implantation. (Reproduced from Yamato et al. [30], with permission of Blackwell Science, Inc.)

suggest that genetic alterations in cancer cells play a pivotal role in the induction of PTHRP gene expression and the development of the paraneoplastic syndrome. However, the mechanism by which PTHRP gene is activated and HHM syndrome develops in vivo with tumor growth appears even more complex. If the genetic alterations in cancer cells were the only determinant of PTHRP gene transcription, it would follow that the level of PTHRP gene expression is fixed during the process of tumor growth. Using the nude rat model, we obtained in vivo evidence that this is not the case.

As described earlier, OCC tumors express high levels of PTHRP mRNA, and tumor-bearing animals display a progressive increase in circulating PTHRP concentrations with marked hypercalcemia especially 12–15 days after tumor implantation [30] (Fig. 3). When a piece of the tumor was implanted back into nude rats, the level of PTHRP mRNA in the tumor was rather low at an early phase (day 6) but markedly elevated after days 12–15 [30] (Fig. 4). Nuclear run-off assays using nuclei prepared from the tumors demonstrated that the increase in the steady-state levels of PTHRP mRNA was due to an increase in the transcription rate of the gene [30]. Histological examination revealed that although the tumor tissue contained a variety of cell types, including white blood cells, macrophages, connective tissues, blood vessels, and necrotic tissues as well as squamous carcinoma cells, the relative percentage of the cancer cells in the tumor did not differ between day 6 and day 12, making it unlikely that the increased transcription is due to the increase in the percentage of squamous carcinoma cells in the tumor tissue. These results suggest that a marked increase in PTHRP gene transcription is not simply due to an increase in tumor burden but due to progressive stimulation of PTHRP gene transcription per se along with tumor growth. Furthermore, it is tempting to speculate that the transcription rate of PTHRP gene within individual cancer cells is increased through some host- or tumor-derived stimulatory factor(s). The characterization and identification of such a stimulatory factor may be important to clarify the in vivo activation mechanism of PTHRP gene in tumors and also to design therapeutic strategies for cancer-associated hypercalcemia.

CONCLUSION

In order to obtain some insight into the pathogenesis of HHM syndrome, we have developed nude rat models implanted with PTHRP-overproducing human carcinoma. Bone histomorphometric analysis demonstrated that the bone remodeling process changes dynamically during the course of HHM syndrome: the advanced stage is characterized by a marked uncoupling process, as documented in HHM patients, whereas a transient increase in bone formation occurs at an early stage, raising the possibility

that the transient activation of osteoblastic functions at an early phase may be important for the acceleration of osteoclastic bone resorption at later stages.

There is emerging evidence that the induction of PTHRP gene expression is closely related to cellular transformation. In addition, our in vivo studies in a nude rat model suggest that some tumor- or host-derived stimulatory factor(s) is also important for progressive activation of PTHRP gene transcription with cancer growth. These findings may have important implications to better understand the pathogenesis of cancer-associated hypercalcemic syndrome and to design therapeutic approaches.

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