

Mechanisms of Humoral Hypercalcemia of Malignancy in Leukemia/Lymphoma

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1. Introduction

Hypercalcemia is one of the most common paraneoplastic syndromes. The incidence of hypercalcemia is 50-90% in adult T-cell leukemia/lymphoma (ATLL), 27-35% in lung cancer, 25-30% in breast cancer, 7-30% in multiple myeloma, and less than 10% in other types of cancer patients (Mundy & Martin, 1982; Roodman, 1997). Patients with severe hypercalcemia (>12 mg/dL; > 6.0 mM) usually develop neuromuscular, gastrointestinal and renal symptoms including lethargy, depression, anorexia, nausea, vomiting, polyuria and polydipsia. Patients with serum calcium concentrations >15 mg/dL (7.6 mM) can develop renal failure or cardiovascular abnormalities with arrhythmias and coma (Mundy & Martin, 1982). Depending on the sources of the stimulating factors, hypercalcemia in cancer can be divided into 3 types: (1) humoral hypercalcemia of malignancy (HHM) in which humoral factors secreted by tumor cells directly or indirectly affect cells in the target organs including bone, kidney and intestine that regulate calcium homeostasis; (2) local osteolytic hypercalcemia in which factors secreted by either primary or metastatic tumor cells locally in the bone microenvironment stimulate osteoclastic bone resorption; and (3) primary hyperparathyroidism that coexists with the malignancy (Stewart, 2005). This review will focus on HHM, although some types of cancers may induce both HHM and local osteolytic hypercalcemia, since several factors can function both systemically and locally.

2. Overview of humoral hypercalcemia of malignancy

Humoral hypercalcemia of malignancy (HHM) is characterized by (1) circulating humoral factors derived from cancer cells; (2) uncoupling of bone formation and bone resorption; (3) increased renal calcium reabsorption even though there is hypercalciuria caused by increased Ca^{2+} in the glomerular filtrate. In contrast to HHM in primary hyperparathyroidism, bone formation and resorption are both increased resulting in fibrous osteodystrophy in patients with longstanding disease.

HHM is a common complication of certain lymphoma/leukemias; squamous cell carcinomas (e.g., of the lung or other organs); renal and breast carcinomas, and occasionally other tumors (Stewart, 2002). Factors secreted by the cancer cells (see Table 1 below) stimulate osteoclastic bone resorption (directly or indirectly through osteoblasts) by increasing the activity and/or survival of osteoclast precursors or mature osteoclasts. Most

cancer-derived hypercalcemic factors stimulate osteoclastic bone resorption indirectly by inducing osteoclast-stimulating factors from osteoblasts or bone stromal cells. Under normal physiological conditions, increased serum calcium concentration can be compensated for by decreasing the intestinal calcium absorption, increasing renal calcium excretion, decreasing PTH secretion from the parathyroid glands, and decreasing bone resorption. However, secretion of the cancer-related factor, parathyroid hormone-related protein (PTHrP), also increases renal calcium reabsorption in the kidney through the activation of parathyroid hormone receptor 1 (PTH1R), which facilitates the development of HHM.

Factors	Origin	Target cells/molecule	Function in bone
PTHrP	Cancer cells	Osteoblast	Catabolic and anabolic
RANKL	Osteoblast, cancer cells	Osteoclast	Catabolic
OPG	Osteoblast	RANKL	Inhibit RANKL
MIP-1 α	Cancer cells	Osteoblast	Catabolic
Calcitriol	Kidney, cancer cells, tumor-associated macrophages	Intestines, kidney, osteoclast, osteoblast, parathyroid chief cells	Increases calcium in blood
TNF- α , IL-1, IL-6, IL-17	T-cells	Osteoclast	Catabolic
OPG, IL-3, IL-4, IL-10, IL-13, IFN- β , IFN- γ , GM-CSF, and sFRPs	T-cells	Osteoclast	Inhibit osteoclast formation and/or function
Calcium (Ca ²⁺)	Bone Kidney Intestinal tract	Calcium-sensing receptor (CaR) on bone and cancer cells and parathyroid chief cells	Anabolic (bone cells) Catabolic (cancer cells) Decreases PTH secretion

Table 1. Factors associated with HHM that cause bone formation (anabolic action) or resorption (catabolic action) in bone.

3. Factors involved in the pathogenesis of HHM

3.1 Parathyroid hormone-related protein (PTHrP)

PTHrP was first cloned by Suva et al. (Suva et al., 1987) and purified by Broadus et al. (Broadus et al., 1988). PTHrP is widely expressed in normal tissues and functions as an endocrine, autocrine, paracrine and intracrine hormone. PTHrP is a polyhormone that results from alternative mRNA splicing and post-translational proteolytic processing. During development, PTHrP is essential for the growth and regulation of endochondral bone (Karaplis et al., 1994; Wysolmerski et al., 1998), epithelial-mesenchymal interactions in mammary gland (Wysolmerski et al., 1998), and has important functions in many other tissues. PTHrP knockout mice die soon after birth due to asphyxia caused by developmental abnormalities of bones in the thorax (Karaplis et al., 1994). PTHrP has been shown to be the principal factor in most cases of cancer-induced HHM. The expression of PTHrP is up-

regulated by NF- κ B, TGF- β , and/or Ras-MAPK signaling (Nadella et al., 2007; Richard et al., 2005). In adult T-cell leukemia/lymphoma, the promoter of PTHrP can be activated by the binding of HTLV-1 oncoprotein, Tax (Ejima et al., 1993; Watanabe et al., 1990).

The functions of PTHrP depend on the activation of different signal transduction pathways, including cyclic adenosine monophosphate/protein kinase A (cAMP/PKA) and protein lipase C/protein kinase C (PLC/PKC) cascades. In addition, PTHrP fragments have different functions that depend on the tissue-specific expression of distinct receptors. Activation of the cAMP-PKA pathway is required for PTHrP to induce bone resorption through the PTH1R, which recognizes both PTH and PTHrP (Greenfield et al., 1995).

Besides the catabolic effect of PTHrP on bone, intermittent administration or pulsatile secretion of the N-terminal fragment of PTH (1-34) and PTHrP (1-36) by a neuroendocrine tumor, such as islet cell carcinoma, can induce bone anabolic effects (Takeuchi et al., 2002). When osteoblastic cells were transiently exposed to the C-terminus of PTHrP (107-139), anabolic effects were induced by the upregulation of vascular endothelial growth factor receptor 2 (VEGFR2) through PKC/ERK activation (de Gortazar et al., 2006).

3.2 Receptor activator of nuclear kappa-B ligand (RANKL) and osteoprotegerin (OPG)

RANKL belongs to the tumor necrosis factor (TNF) family and represents one of the most common mediators for inducing osteoclastic bone resorption. Mature osteoblasts produce two forms of RANKL, a membrane-bound and secreted form. Both are important for osteoclast stimulation (Leibbrandt & Penninger, 2008). The expression of RANKL in osteoblasts and stromal cells is activated by the RUNX2 transcriptional factor under the regulation of PTHrP, 1,25-dihydroxyvitamin D₃, and prostaglandins (Lipton et al., 2009). RANKL binds and activates its receptor, RANK, which is expressed on the surface of osteoclasts. The binding of RANKL to RANK activates at least three major signaling pathways, NFAT, p38 and JNK, resulting in up-regulation of genes that are required for induction of osteoclast fusion, differentiation, activation and survival (Leibbrandt & Penninger, 2008). The secreted form of RANKL is important for the recruitment of osteoclast precursors and osteoclastogenesis. RANKL is an essential activator for normal bone remodeling. RANKL knockout mice have severe osteopetrosis (Lomaga et al., 1999).

OPG, or osteoclastogenesis inhibitory factor (OCIF), is a secreted member of the tumor necrosis receptor superfamily that is expressed by osteoblasts and functions as a RANKL 'decoy receptor' (Simonet et al., 1997). OPG binds to RANKL and blocks its interaction with RANK on osteoclast precursors (Lacey et al., 1998); therefore, it is a potent inhibitor of osteoclast formation and bone resorption. OPG knockout mice have early-onset osteopenia (Bucay et al., 1998; Mizuno et al., 1998), whereas OPG overexpressing mice develop osteopetrosis that results from the failure to form osteoclasts (Simonet et al., 1997). PTH and PTHrP suppress OPG expression by downregulating the promoter of OPG through activation of the cAMP/PKA pathway (Yang et al., 2002). PTHrP, on the other hand, stimulates RANKL expression to induce bone resorption. The ratio between RANKL and OPG levels in osteoblasts is a key factor in the regulation of osteoclast activity (Horwood et al., 1998).

In ATLL, the expression of RANKL in tumor cells correlated with hypercalcemia in patients. RANKL on the surface of leukemia cells induced osteoclastogenesis through direct contact with precursor cells (Nosaka et al., 2002). The direct activation of osteoclasts by tumor cells may play a key role in the decoupling of bone formation and bone resorption in HMM.

Therefore, RANKL from either osteoblasts or tumor cells is an important mediator of bone resorption in many forms of HHM.

3.3 Cytokines

The increased secretion of inflammatory cytokines from cancer cells, such as tumor necrosis factor- α (TNF- α), IL-1, IL-6, IL-8, M-CSF, CCL2 and CXCL12, is one of the key mechanisms by which NF- κ B is constitutively activated in tumor cells (Lu & Stark, 2004). The proinflammatory cytokine, TNF- α , is a critical factor in the pathogenesis of many inflammatory and non-inflammatory diseases which are characterized by increased osteoclastic bone resorption including rheumatoid arthritis (Chu et al., 1991), osteoporosis (Horowitz, 1993), osteomyelitis (Meghji et al., 1998) and aseptic loosening (an osteolysis syndrome caused by macrophages in joint replacements) (Merkel et al., 1999). TNF- α mediates lipopolysaccharide-stimulated osteoclastogenesis through the p55 receptor expressed on bone marrow macrophages and activation of NF- κ B (Abu-Amer et al., 1997; Abu-Amer et al., 1998). Whether osteoclastogenesis induced by TNF- α depends on RANKL or not remains controversial. Some studies have shown that without exogenous RANKL, TNF- α is sufficient to induce osteoclast differentiation (Kudo et al., 2002), and the increase of osteoclastogenesis was inhibited by OPG (Hounoki et al., 2008). However, Boyle et al. have shown that administration of TNF- α to RANK-deficient mice failed to induce osteoclastogenesis and restore hypocalcemia, suggesting that TNF- α cannot substitute for RANKL (Li et al., 2000). Teitelbaum et al. have reported that TNF- α alone was not able to induce osteoclastogenesis in murine osteoclast precursors; rather, RANKL was required for TNF- α to stimulate osteoclast precursors to form osteoclasts (Lam et al., 2000). Regardless, TNF- α has shown its potential to be a therapeutic target for bone resorption. Increased TNF- α levels have been found in patients with advanced chronic lymphocytic leukemia (CLL) and acute nonlymphocytic leukemia with HHM (Ferrajoli et al., 2002).

Increased plasma IL-6 has been found in cancer patients with HHM, including patients with multiple myeloma, squamous cell carcinoma of the liver, acute nonlymphocytic leukemia, and adult T-cell leukemia/lymphoma (Asanuma et al., 2002; Kounami et al., 2004; Roodman, 1997). IL-6 increases osteoclast recruitment by binding to its receptor on osteoblasts and inducing the signal of transducer and activator of transcription (STAT)-1/3 and mitogen-activated protein kinase (MAPK) signaling pathways (Sims et al., 2004). It also enhances the effects of PTH and PTHrP and mediates the effects of inflammatory cytokines, such as IL-1 and TNF- α , on osteoclast formation (Roodman, 2001). IL-6 does not directly increase RANKL expression; therefore, its osteoclastogenic effect is RANKL-independent (Hofbauer et al., 2000). However, there is no correlation between serum IL-6 and HHM in cancer patients, suggesting that IL-6 functions additively or synergistically with other factors and may be a redundant factor for development of HHM (Vanderschueren et al., 1994).

Granulocyte macrophage-colony stimulating factor (GM-CSF) also plays a role in osteoclastogenesis and it has two distinct effects on osteoclast activity depending upon the presence of RANKL. GM-CSF increases the proliferation of osteoclast progenitors when RANKL is absent. On the other hand, it induces osteoclast progenitors to differentiate into dendritic cells when RANKL is present (Gillespie, 2007).

Macrophage colony-stimulating factor (M-CSF) is essential for osteoclast differentiation and proliferation of osteoclast progenitor cells (Tanaka et al., 1993). Increased serum M-CSF

concentration has been reported in acute nonlymphocytic leukemia patients with HHM (Kounami et al., 2004).

Interferon (IFN)- γ disrupts JAK/STAT signaling in osteoblasts to induce the expression of OPG and Wnt, causing decreased cathepsin K and tartrate-resistant acid phosphatase (TRAP) in mature osteoclasts (Gallo et al., 2008; Gillespie, 2007). Transgenic mice with the HTLV-1 viral oncoprotein, Tax, and knockout of IFN- γ developed more severe osteolytic bone lesions and increased osteoclast activity. Administration of IFN- γ to mice transplanted with Tax-positive tumors inhibited tumor growth and decreased hypercalcemia, suggesting a protective role for IFN- γ in HHM development (Xu and Hurchla et al., 2009).

Cytokines have been shown to play a more important role in the pathogenesis of HHM in patients with lymphoma and leukemia compared to patients with carcinoma. However, further investigation is needed to clarify the additive and synergistic roles of cytokines in causing hypercalcemia because multiple cytokines are often produced by the tumor cells.

3.4 Macrophage inflammatory protein-1 alpha (MIP-1 α)

MIP-1 α is a pro-inflammatory chemokine expressed by many different cell types including macrophages, dendritic cells and lymphocytes. It normally functions as a chemoattractant for T-cells, macrophages, and other proinflammatory cells at the site of inflammation. It also regulates the transendothelial migration of NK cells, monocytes and dendritic cells (Maurer & von, 2004). Therefore, it plays an important role in autoimmune and inflammatory diseases, such as multiple sclerosis, rheumatoid arthritis, asthma, and organ transplant rejection (Maurer & von, 2004). MIP-1 α binds to several chemokine (c-c motif) G-protein-coupled receptors including CCR1, CCR5 and CCR9, which are expressed in lymphocytes and monocytes/macrophages. It activates several signaling pathways, including PI3K, PLC, PKC, MAP kinase and JAK/STAT pathways (Tsubaki et al., 2007). MIP-1 α can potently inhibit the binding of HIV to CCR5 on macrophages (Baba et al., 1999; Maurer & von, 2004).

Both CCR1 and CCR5 are expressed in bone marrow stromal cells. MIP-1 α is chemotactic for osteoclasts and osteoclast precursors (Fuller et al., 1995). MIP-1 α was first identified as a putative osteoclastogenic factor in a human myeloma cDNA expression library derived from marrow samples of myeloma patients (Zlotnik & Yoshie, 2000). Subsequently, CCR1 and CCR5 expression was demonstrated in human and murine multiple myeloma cell lines (Menu et al., 2006). Furthermore, MIP-1 α enhances osteoclast formation induced by IL-6, PTHrP and RANKL in multiple myeloma (Han et al., 2001) and increases adhesion of myeloma cells to bone marrow cells through its binding to both CCR1 and CCR5 receptors (Oba et al., 2005). *In vivo*, mice bearing Chinese hamster ovary cells that overexpress MIP-1 α develop more severe osteolytic lesions after intramuscular inoculation (Oyajobi et al., 2003).

The mechanisms by which MIP-1 α induces osteoclastic bone resorption are controversial. One study demonstrated that a RANKL-dependent pathway was responsible for activation of osteoclasts (Tsubaki et al., 2007). MIP-1 α enhances RANKL expression in mouse bone marrow stromal cells and osteoblasts through the MAPK and PI3K/Akt signaling pathways. On the other hand, RANK-Fc did not block the activation of osteoclasts by MIP-1 α , indicating that MIP-1 α used a RANKL-independent pathway to increase bone resorption (Han et al., 2001). Therefore, it has been suggested that MIP-1 α is a RANKL-independent osteoclastogenic factor that acts directly on osteoclast precursors (Choi et al., 2000). In any case, it is apparent that MIP-1 α is a significant osteoclast activator. The role of MIP-1 α in

HHM is highlighted by the fact that HTLV-1 infected T-cells express and secrete MIP-1 α (Shu et al., 2007; Shu et al., 2010), and the increased serum levels of MIP-1 α correlated well with the development of HHM in HTLV-1-infected patients (Okada et al., 2004).

3.5 1 α ,25-Dihydroxyvitamin D (Calcitriol)

Vitamin D is an essential hormone for calcium homeostasis. Its active form, 1 α ,25-dihydroxyvitamin D₃ or calcitriol, is synthesized by hydroxylation of vitamin D in the kidney. The renal 25-hydroxyvitamin D 1- α hydroxylase is the rate limiting enzyme for production of calcitriol. Calcitriol increases calcium absorption from the intestinal tract, increases osteoclastic bone resorption, and decreases PTH gene expression in the parathyroid glands (Guise et al., 2005). Calcitriol is an uncommon primary cause of HHM in patients with leukemia or lymphoma even though serum calcitriol concentrations may be increased in up to 50% of patients (Seymour & Gagel, 1993). Some lymphomas or tumor-associated macrophages express 25-hydroxyvitamin D 1- α hydroxylase, which is responsible for the increased production of calcitriol (Hewison et al., 2003).

3.6 Cell membrane calcium-sensing receptor (CaR)

CaR is expressed on multiple cell types including the chief cells of the parathyroid gland where it regulates PTH expression and secretion. Under normal conditions, CaR, a G protein-coupled receptor, senses extracellular calcium concentrations and activates downstream signaling pathways, such as the PLC/inositol trisphosphate (IP₃) and ERK1/2 pathways, in a tissue-specific manner (Saidak et al., 2009). In bone, CaR is expressed in osteoblasts, osteoclasts, stromal cells, monocytes-macrophages, and chondrocytes. CaR promotes osteoblast proliferation, differentiation and mineralization (Sharan et al., 2008). It also mediates osteoclast differentiation and apoptosis. Therefore, CaR in bone cells promotes the bone formation phase of bone remodeling (Yamaguchi, 2008). CaR is also expressed in some cancer cells, such as breast and prostate cancers (Liao et al., 2006). However, in these cells, increased extracellular calcium leads to increased PTHrP expression (Chattopadhyay, 2006). Increased PTHrP induces osteoclastic bone resorption and renal calcium reabsorption, resulting in HHM. The positive feedback loop formed by PTHrP, calcium and CaR is a unique phenomena in HHM. In addition to the specific activation of PTHrP expression, gain-of-function mutations in CaR have been demonstrated in breast cancer. Lorch *et al.* have found single nucleotide polymorphisms in CaR in human lung squamous cell carcinoma (Lorch et al., 2011). Functional evaluation of a nonconservative amino acid substitution (R990G) in CaR induced HHM in patients with lung squamous cell carcinoma. Dysregulation of PTHrP expression and HHM caused by CaR signaling has been demonstrated in breast and prostate cancer (Saidak et al., 2009). However, the role of CaR in HHM induced by lymphoma/leukemia remains to be determined.

3.7 Role of T cells in calcium homeostasis

The skeletal and immune systems share many regulatory molecules and systems. An interdisciplinary research area called "Osteoimmunology" has been developed recently to understand the interplay between these two systems. Cytokines, receptors, signaling molecules and transcriptional factors, and their signaling pathways are comprehensively reviewed by Takayanagi (Takayanagi, 2007). T-cells have both pro- and antiresorptive effects on osteoclasts. The proresorptive effect is present in osteoclast-stimulating T-cells,

which secrete a soluble form of RANKL. TNF- α is also expressed by activated T-cells to act in concert with RANKL. IL-1, IL-6 and IL-17 secreted from T-cells increase RANKL expression in osteoblasts. This mechanism is important for rheumatoid arthritis where TH17 cells have been shown to be an immunomodulator of osteoclastic bone resorption (Sato et al., 2006). In addition, T-cells produce IL-7 which increase bone resorption in a RANKL-independent mechanism (Weitzmann & Pacifici, 2005). T-cells also play a major role in postmenopausal osteoporosis induced by decreased estrogen levels and decreased transforming growth factor (TGF)- β expression in bone cells (Gillespie, 2007).

In contrast, T-cells can also exert an antiresorptive effect directly by secreting OPG, IL-3, IL-4, IL-10, IL-13, IFN- β , IFN- γ , GM-CSF, and secreted frizzled-related proteins (sFRPs) to inhibit osteoclastogenesis (Quinn & Gillespie, 2005). In addition, T-cells can inhibit osteoclast formation and activity indirectly by expressing GM-CSF (induced by the upregulation of IL-18 in bone microenvironment), IFN- γ (by IL-12) and OPG (by leptin) (Horwood et al., 2001). It will be important to understand the effects of T-cells on osteoblast and stromal cell function, as well as signaling in the immune response to further understand the interactions between the immune system and bone biology.

4. HHM in leukemia/lymphoma in humans and animals

4.1 Adult T-cell leukemia lymphoma (ATLL)

50-90% of ATLL patients develop HHM and osteolytic lesions in the long bones and calvaria (Olivo et al., 2008). Bone resorption may act as a 'vicious cycle' for ATLL growth in bone, since factors released by resorbing bone increased the growth of ATLL and HTLV-1-infected T-cells *in vitro* (Shu et al., 2010). The mechanisms by which HTLV-1 induces HHM are not completely known. We and others have demonstrated that ATLL primary cell lines (T-cell lines derived from leukemic ATLL patients) and *in vitro* HTLV-1 transformed T-cell lines express and secrete PTHrP, particularly transcripts from the P3 promoter (Nadella et al., 2007; Richard et al., 2005; Shu et al., 2010) (Figure 1). The expression of PTHrP was upregulated by both oncoviral protein Tax-dependent and -independent pathways. Tax cooperates with Ets to activate the P3 promoter of PTHrP, while constitutive activation of NF- κ B in ATLL cells contributes to expression of the PTHrP P2 promoter (Nadella et al., 2007; Richard et al., 2005). Despite the essential role of PTHrP in HHM in carcinomas (such as lung, breast and prostate cancers), the correlation between the plasma PTHrP and HHM in ATLL patients has been controversial. It has been concluded that PTHrP is not the sole factor that induces HHM in ATLL, but it likely plays an important cooperative or synergistic role with other humoral factors (Figure 1).

In ATLL, there were increased plasma MIP-1 α concentrations in mice with human ATLL cells and HHM (Shu et al., 2007). In a human clinical study, plasma MIP-1 α concentrations had a strong correlation with HHM in ATLL patients (Okada et al., 2004). MIP-1 α expression in ATLL cells is induced by Tax and increased plasma calcium concentrations may further up-regulate MIP-1 α expression through the calmodulin-dependent protein kinase kinase (CaM-KK) cascade (Matsumoto et al., 2008; Sharma & May, 1999). Treatment with a neutralizing MIP-1 α antibody decreased osteoclast formation induced by ATLL cells *in vitro* (Okada et al., 2004).

The increase in RANKL expression observed in the ATL leukemic cells has been shown to correlate with the occurrence of HHM in ATLL patients (Nosaka et al., 2002). Although the levels of RANKL expression were not high in HTLV-1-infected cell lines *in vitro* (Shu et al.,

2010), leukemic cells isolated from ATLL patients did have up-regulation of RANKL expression (Nosaka et al., 2002). In addition, HTLV-1 infected leukocytes *in vitro* were able to convert 25-dihydroxyvitamin D₃ to its active form, 1 α ,25-dihydroxyvitamin D₃ or calcitriol (Fetrick et al., 1986). High levels of calcitriol have been found in ATLL patients with hypercalcemia (Johnston & Hammond, 1992; Seymour & Gagel, 1993)

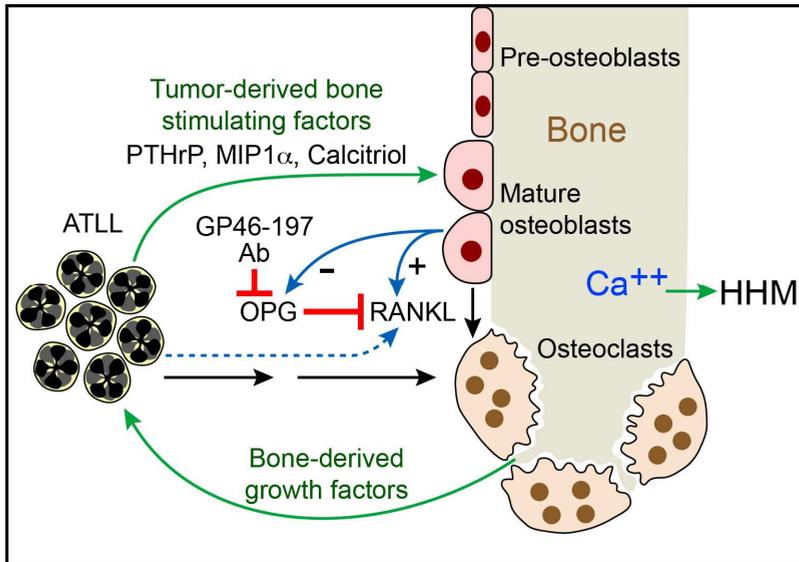


Fig. 1. HHM in ATLL is caused by increased osteoclastic bone resorption. Cancer cells secrete bone regulatory factors, including PTHrP, MIP-1 α , calcitriol, and RANKL to increase osteoclast activity. The resorbing bone secretes bone-derived growth factors that increase ATLL cell growth. In osteoblasts, the RANKL/OPG expression ratio increases when cells are co-cultured with T-cell leukemia lines. Antibodies against HTLV-1 Gp46 can inhibit OPG and contributes to the pathogenesis of hypercalcemia.

In addition to the direct effects of factors secreted from ATLL cells on osteoclasts, we recently found decreased OPG expression and secretion in osteoblasts that were co-cultured with leukemic T-cell lines (Shu et al., 2010). This suggests that the regulation of gene expression in osteoblasts by leukemic T-cells plays an indirect role in HHM in ATLL.

Finally, an endogenous antibody recognizing the HTLV-1 viral envelope protein Gp46-197 can occur in ATLL patients, which correlated with disease progression. The amino acid sequence of Gp46-197 is homologous with the C-terminus of OPG. Rabbits immunized with the Gp46-197 peptide developed hypercalcemia and died. Sprague-Dawley rats injected with Gp46-197 peptide developed decreased bone mineral density and hypercalcemia. Administration of recombinant human OPG restored femoral bone growth. These data suggest that HTLV-1 Gp46 contributes to the pathogenesis of hypercalcemia due to cross-reactive antibodies in the patients that antagonize the action of OPG (Sagara et al., 2009).

Other factors proposed to be humoral factors for HHM in ATLL include TNF- β (lymphotoxin) (Ishibashi et al., 1992), IL-6 (Chiba et al., 2009), and IL-1 (Wano et al., 1987).

4.2 Other leukemias/lymphomas that develop HHM (Table 2)

4.2.1 De novo acute nonlymphocytic leukemia (ANLL)

Hypercalcemia in de novo ANLL patients can be caused by either local osteolytic hypercalcemia or HHM. Elevated circulating concentrations of several humoral factors, including PTHrP, TNF- α , IL-6, and M-CSF, in de novo ANLL patients with HHM have been reported supporting a role for HHM in ANLL (Kounami et al., 2004). Generalized osteoporosis with normal renal function was observed in these patients, indicating that the increased calcium was mainly from bone.

Cancer Type	Incidence of HHM	Humoral factor(s)
ATLL	50-90%	PTHrP, MIP-1 α , RANKL, IL-1, TNF- β , IL-6
Diffuse large B cell lymphoma	Rare	PTHrP, IL-6, 1,25-dihydroxyvitamin D
Acute nonlymphocytic leukemia	Rare	PTHrP, IL-6, TNF- α , M-CSF
Primary cutaneous B-cell lymphoma	Rare	1,25-dihydroxyvitamin D
Canine T-cell lymphoma	40% in mediastinal lymphoma; 10-20% in multicentric lymphoma	PTHrP, 1,25-dihydroxyvitamin D
Feline lymphoma	Has been reported	PTHrP
Feline leukemia virus associated leukemia/lymphoma	Has been reported	Unknown
Avian malignant lymphoma	Has been reported	Unknown

Table 2. Incidence and humoral factors of HHM in leukemia/lymphoma in humans and animals. Other undefined factors or cytokines may also be involved.

4.2.2 Diffuse large B-cell lymphoma

Increased PTHrP, IL-6, and 1,25-dihydroxyvitamin D concentrations have been reported in the serum from patients with diffuse large B cell lymphoma that developed hypercalcemia (Amezyane et al., 2008; Chang et al., 2008). Diffuse osteolytic lesions and nephrocalcinosis also occurred in these patients.

4.2.3 Primary cutaneous B-cell lymphoma

Hypercalcemia has been reported in patients with primary cutaneous B-cell lymphoma (Habra et al., 2007; Narimatsu et al., 2003). Increased serum 1,25-dihydroxyvitamin D and undetectable PTH and PTHrP levels were found in one patient. The pathogenesis of hypercalcemia in these patients has not been determined.

4.2.4 HHM in animals with leukemia/lymphoma

HHM occurs in 10-40% of dogs with T-cell lymphoma (Fournel-Fleury et al., 2002). Increased circulating PTHrP and 1,25-dihydroxyvitamin D were found in dogs with lymphoma and HHM, but the serum concentrations did not always correlate with

hypercalcemia. Therefore, it was speculated that additional cytokines are involved in the pathogenesis of HHM in dogs with lymphoma (Mellanby et al., 2006; Rosol et al., 1992). In a xenograft mouse model of canine lymphoma, there was increased expression of TNF- α in the tumor *in vivo* (Nadella et al., 2008). Bone histomorphometry indicated that increased osteoclastic bone resorption was the major cause of HHM in these mice. Increased circulating PTHrP has also been reported in cats with lymphoma and HHM (Bolliger et al., 2002). Cats may also develop HHM due to unknown humoral factors induced by feline leukemia virus infection (Engelman et al., 1985). Hypercalcemia has also been reported in an Amazon parrot with lymphoma (de Wit et al., 2003).

5. Therapy of HHM in leukemia/lymphoma

For urgent care, saline hydration is the first step to correct hypercalcemia by diluting the serum calcium concentration and increasing the clearance of calcium by the kidneys. Treatment of the underlying leukemia/lymphoma, including chemotherapy and radiation therapy, is necessary. Several drugs have been used for long-term management of hypercalcemia associated with HHM.

5.1 Bisphosphonates

Bisphosphonates are structural analogs of pyrophosphoric acid and have been widely used to treat cancer patients with hypercalcemia, heritable skeletal disorders in children, and postmenopausal and glucocorticoid-induced osteoporosis patients with significant bone loss (Fleisch, 1997). Intravenous aminobisphosphonates are a standard of care for the treatment of HHM. The newest generation of bisphosphonates, nitrogen-containing aminobisphosphonates, inhibits farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) synthetases in the mevalonic acid metabolic pathway resulting in decreased prenylation of low-molecular-weight G proteins including Ras. Functional Ras signaling is necessary for osteoclast activity and survival (Fleisch, 1998). It also has been suggested that bisphosphonates exert a cytotoxic effect on HTLV-1-infected T-cells. However, high doses of bisphosphonates were needed for cytotoxic effects *in vitro* and *in vivo* (Gao et al, 2005; Ishikawa et al., 2007; Shu et al., 2007; Hirbe et al., 2009). The clinical relevance of a direct effect of bisphosphonates on ATLL cells needs to be further evaluated. Bisphosphonates have a high affinity for bone mineral and bind to hydroxyapatite crystals *in vivo*. Therefore, bisphosphonates are rapidly depleted from the circulation and extracellular fluid and become highly concentrated in bone. Bisphosphonate therapy may have complications including hypocalcemia, osteonecrosis of the jaw, low bone turnover with pathologic fractures, and increased incidence of atrial fibrillation (Drake et al., 2008). In addition to the effects on osteoclasts, a new bisphosphonate, YM527/ONO-5920, decreased MIP-1 α expression and secretion through inhibition of the transient increase of phosphorylated ERK1/2 and Akt in mouse myeloma cells after lipopolysaccharide (LPS) stimulation (Drake & Rajkumar, 2009). Since MIP-1 α has been shown to play an important role in cancer cell growth and osteolysis in multiple myeloma, bisphosphonates may be useful for inhibiting the growth of myeloma cells and to prevent osteolysis by decreasing MIP-1 α expression. This may also apply to ATLL. Commonly used bisphosphonates include pamidronate and zoledronic acid.

5.2 Calcitonin

Calcitonin is a 32-amino acid peptide secreted by thyroid C-cells. It inhibits calcium absorption in the intestine and reabsorption of calcium and phosphate in renal tubules. It is

a potent (but transient) inhibitor of osteoclastic bone resorption. The mechanisms of action have been shown to involve several signaling pathways including cAMP/PKA, PKC, and pyk2/src activity. It has also been shown that calcitonin up-regulates renal 1α -hydroxylase, an important enzyme for the synthesis of calcitriol, through the binding of C/EBP β and brahma-related gene 1 (BRG1), an ATPase in the SWItch/Sucrose NonFermentable (SWI/SNF) chromatin remodeling complex, on the 1α -hydroxylase promoter (Zhong et al., 2009). Because of its potent inhibitory effect on bone resorption, calcitonin has clinical applications in the treatment of Paget's disease, osteoporosis, and hypercalcemia. However, due to its short duration of action, calcitonin may not be suitable for treatment of chronic hypercalcemia. Salmon calcitonin is used clinically due to its greater potency compared to human calcitonin (Zaidi et al., 2002).

5.3 Corticosteroids

Corticosteroids have been used for the treatment of hypercalcemia induced by multiple myeloma and certain types of lymphoma. Corticosteroids act by decreasing calcium absorption in gastrointestinal tract, decreasing bone resorption, and increasing renal calcium excretion (Unal et al., 2008; Yarbro et al., 2003). Corticosteroids generally are not effective in patients with HHM induced by solid tumors.

5.4 Bortezomib (PS-341)

Bortezomib is a selective proteasome inhibitor that decreases the ubiquitination of I κ B, the inhibitor for NF- κ B, thereby stabilizing I κ B and inhibiting NF- κ B. NF- κ B is necessary for osteoclast function and constitutively activated NF- κ B plays an important role in ATLL cells. Bortezomib has been shown to inhibit tumor growth in models of ATLL (Mitra-Kaushik et al., 2004b; Nasr et al., 2005; Satou et al., 2004; Shu et al., 2007; Tan & Waldmann, 2002). Shu et al. reported that bortezomib not only decreased tumor burden in mice bearing ATLL cells, but also decreased the severity of HHM (Shu et al., 2007). Although the decrease in serum calcium concentrations was mainly due to the decreased tumor burden, several studies have shown that bortezomib inhibited osteoclastogenesis by decreasing p38, AP-1 and NF- κ B activity in osteoclasts (von Metzler et al., 2007) and increased bone formation by decreasing the expression of Dkk-1, a potent osteoblast inhibitor (Drake & Rajkumar, 2009; Heider et al., 2009; Pennisi et al., 2009; Qiang et al., 2009; Terpos et al., 2006). Bortezomib is now a standard of care for treatment of multiple myeloma patients. Several clinical trials involving bortezomib are ongoing for the treatment of prostate cancer, nonsmall cell lung cancer, acute myelogenous leukemia, and other cancers.

5.5 Humanized anti-parathyroid hormone-related protein antibody and small molecule antagonists of the PTH/PTHrP receptor (PTH1R)

Although bisphosphonates have been widely used for treatment of HHM to inhibit osteoclastic bone resorption, they do not function on the kidney to decrease renal calcium reabsorption. To eliminate the actions of PTHrP in bone and kidney, an anti-PTHrP antibody has been developed and tested (Sato et al., 2003). Anti-PTHrP antibody prevented hypercalcemia and skeletal metastasis, but not visceral metastasis, induced by PTHrP-producing cancer cells in mice (Guise & Mundy, 1996; Sato et al., 2003). Although it has been difficult to identify small molecule antagonists for type B G-protein-coupled receptors, including the PTH1R, two compounds have been recently developed that antagonize PTH1R. SW106 was discovered by Bristol-Myers-Squibb Company by screening compounds

that inhibited targets downstream of the PTHR1 (Carter et al., 2007). The benzoxazepinone non-peptide inhibits the cAMP response induced by a PTH1R agonist. The pharmacological behavior of SW106 is not yet completely understood. A similar compound, 1,3,4-benzotriazepine was identified and serves as a base molecule to develop non-peptide PTH1R antagonist derivatives (McDonald et al., 2007). Using a radioligand binding assay that measures cAMP production, N-1 anilino-substituted compounds were identified that have up to a 1000-fold more potency at inhibiting PTH1R compared to the original compound. Efforts are ongoing to determine the effects of these compounds on bone metastasis, hypercalcemia and hyperparathyroidism.

5.6 RANKL inhibitors

Ever since RANKL and OPG were discovered to be major regulators of osteoclastic bone resorption, RANK-Fc, Fc-OPG, antibodies targeting RANKL or inhibitors imitating OPG function have been developed for the treatment of osteolytic bone diseases (Schwarz & Ritchlin, 2007). RANK-Fc was generated by combining the carboxyl-terminus of RANK with the Fc portion of human IgG1. It has been shown to decrease tumor burden in two multiple myeloma mouse models, inhibit prostate cancer bone metastasis, and decrease the incidence of lung metastasis and bone lysis in an osteosarcoma mouse model (Lamoureaux et al., 2008; Sordillo & Pearse, 2003; Zhang et al., 2003). Fc-OPG was generated by combining the Fc portion of the immunoglobulin heavy chain to the amino-terminus of OPG. The inhibitory effect of Fc-OPG on bone resorption is similar to pamidronate (Bekker et al., 2001; Body et al., 2003). However, Fc-OPG and RANK-Fc have been replaced by denosumab (Amgen), which is a fully human monoclonal antibody developed using the xenomouse technology that specifically inhibits primate RANKL (Green, 1999). Denosumab does not bind to murine RANKL, human TRAIL, or other TNF family proteins and has a longer half-life than Fc-OPG (Kostenuik et al., 2009). Denosumab has been tested in patients with varying diseases/conditions, including osteoporosis, treatment-induced bone loss, bone metastases, multiple myeloma and rheumatoid arthritis, and is now a standard of care for patients with solid tumor bone metastases (Schwarz & Ritchlin, 2007). Denosumab can significantly delay or prevent skeleton-related events (SREs), including hypercalcemia, in patients with bone metastasis (Castellano et al., 2011). Due to its longer half life, higher specificity and lower toxicity, the therapeutic potential of denosumab is superior to that of Fc-OPG and RANK-Fc (Schwarz & Ritchlin, 2007).

6. Development of *in vivo* models of HHM in leukemia/lymphoma

6.1 Mouse models of HHM and ATLL

Animal models of ATLL are divided into infectious models, which are useful to study viral infection, viral transmission and the immune response, and pathogenesis models, which are useful for preclinical therapy studies. Pathogenesis models, including tumor xenografts and transgenic mice that develop tumors, are useful to study the development and treatment of HHM in ATLL. Unfortunately, there are few animal models of ATLL that develop HHM. The following animal models are currently available for studying HHM in ATLL.

6.1.1 Human RV-ATL xenograft mouse model of ATLL

The RV-ATL model was first developed by the laboratory of Dr. Irvin Chen by injecting RV-ATL cells, derived from an ATLL patient, into severe combined immunodeficient (SCID)

mice (Feuer et al., 1993). Richard et al. characterized the tumorigenesis and HHM of this cell line in SCID/beige mice (Richard et al., 2001). SCID/beige mice developed severe hypercalcemia one month after intraperitoneal injection of RV-ATL cells. The mice had bone loss due to increased osteoclastic bone resorption. Shu et al. introduced the luciferase gene into the RV-ATL cells by lentiviral infection and developed a bioluminescent model of HHM in ATLL for preclinical studies (Shu et al., 2007). It was found that both zoledronic acid, a nitrogen-containing bisphosphonate, and PS-341, a selective proteasome inhibitor, decreased tumor burden and HHM in mice. No complication of using the combination of PS-341 and zoledronic acid was observed in this preclinical study (Shu et al., 2007).

6.1.2 Uchiyama human xenograft mouse model of ATLL

The laboratory of Dr. Takashi Uchiyama developed a mouse xenograft model by injecting cells from the lymph node of a lymphoma-type ATLL patient intraperitoneally into SCID mice (Imada et al., 1996). The mice developed tumors and hypercalcemia within three weeks. There was a marked increase in serum C-terminal PTHrP concentrations with decreased bone formation rates reported in the mice (Takaori-Kondo et al., 1998). However, no significant increase in bone resorption measured by bone histomorphometry was observed in the mice, which is in contrast to ATLL patients with HHM.

6.1.3 Human MET-1 xenograft mouse model of ATLL

Recently, a NOD/SCID mouse model using human ATLL MET-1 cells has been developed (Phillips et al., 2000). The mice developed leukemia and HHM after intraperitoneal injection. Marked infiltration of tumor cells in multiple organs including spleen, lungs, liver, lymph nodes was observed. Increased plasma PTHrP concentrations in the mice and expression of PTHrP and RANKL in MET-1 cells were reported (Parrula et al., 2009). It is not known whether the HHM in this model was caused by an increase in bone resorption or other mechanisms.

6.1.4 HTLV-1 LTR-Tax transgenic model

Transgenic mice have been used to study the pathogenesis and role of the HTLV-1 viral oncoprotein, Tax. Several Tax transgenic mice have been developed using different promoters. A transgenic mouse model overexpressing Tax under the regulation of the HTLV-1 long terminal repeat (LTR) was generated (Ruddle et al., 1993). These mice developed neurofibromas and adrenal medullary tumors, but did not develop leukemia or lymphoma. Unexpectedly, a significant increase in bone remodeling with a net increase in bone volume was observed. This was surprising because the authors also demonstrated that osteoclasts were increased in number, size and degree of multinucleation, which would have been expected to lead to a net decrease in bone volume. It was not reported whether HHM developed in the mice.

6.1.5 Human granzyme B-Tax transgenic mice

The laboratory of Dr. Lee Ratner developed a tissue-specific Tax overexpressing mouse model (Grossman et al., 1995). Tax expression in the mice was under the regulation of the human granzyme B promoter, which limited Tax expression primarily to activated CD4⁺ and CD8⁺ T-cells and NK cells. The mice developed mild hypercalcemia and multifocal osteolytic bone lesions, especially in the tail, with increased osteoclastic bone resorption (Gao et al., 2005). The

mice have increased serum IL-6 concentrations, which is a potent osteoclast activator. When the mice were crossed to mice that overexpressed OPG, they were protected from the development of osteolytic lesions and soft tissue tumors, indicating that increased bone resorption in Tax transgenic mice was induced, at least in part, through a RANKL-dependent pathway. The mouse model also has been used for preclinical studies and zoledronic acid not only prevented the osteolytic bone lesions but also decreased tumor burden. After crossing the mice with IFN- γ knockout mice, the resulting Tax⁺IFN- γ ^{-/-} mice had accelerated tumor formation, dissemination, and death, when compared with Tax⁺IFN- γ ^{+/-} or Tax⁺IFN- γ ^{+/+} mice (Mitra-Kaushik et al., 2004a). The mice also develop increased osteolytic bone lesions, increased osteoclast formation and more severe hypercalcemia compared to Tax⁺IFN- γ ^{+/+} mice (Xu et al., 2009). These data indicate that IFN- γ may contribute to the host defense systems that prevent HTLV-1-induced malignancy, bone metastasis and HHM.

6.2 Mouse model of canine lymphoma and HHM

A bioluminescent NOD/SCID mouse model of canine T-cell lymphoma and HHM has been developed (Nadella et al., 2008). The mice developed multicentric lymphoma in the mesenteric lymph nodes after intraperitoneal injection of tumor cells. Moderate to marked splenomegaly and enlarged thymuses were observed. There was increased osteoclastic bone resorption in trabecular bone in mice with lymphoma. HHM developed 6-8 weeks after injection of tumor cells. The increase in plasma PTHrP concentrations likely played a central role in HHM in the mice. The cause of canine T-cell lymphoma is unknown and retroviruses have not been identified as a cause of lymphoma in dogs (in contrast to humans and cats).

7. Conclusion

HHM is a life-threatening complication in certain patients with lymphoma or leukemia. As outlined in this review, progress has been made in elucidating the mechanisms by which humoral factors from neoplastic lymphocytes induce HHM, including increased osteoclastic bone resorption and renal calcium reabsorption. However, further efforts are needed to fully understand the pathogenesis of HHM, including the endocrine or paracrine role of interactions between tumor-associated and host-produced cytokines. PTHrP plays a major endocrine and paracrine role in HHM, but the effects of other factors secreted from tumor cells or host cells cannot be neglected. For example, the expression of RANKL in ATLL cells suggests that ATLL cells may function directly as inducers of osteoclastic bone resorption. Additional effective treatments are needed for this paraneoplastic syndrome. Small molecules or humanized antibodies targeting essential factors or their receptors may be an attractive future therapeutic strategy for treatment of HHM.

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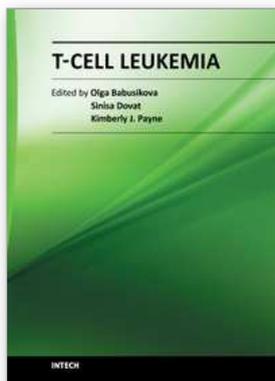
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The purpose of this book is to provide a comprehensive review of the scientific advances in T-cell malignancies and to highlight the most relevant findings that will help the reader understand both basic mechanisms of the disease and future directions that are likely to lead to novel therapies. In order to assure a thorough approach to these problems, contributors include basic scientists, translational researchers and clinicians who are experts in this field. Thus, the target audience for this book includes both basic scientists who will use this book as a review of the advances in our fundamental knowledge of the molecular mechanisms of T-cell malignancies, as well as clinicians who will use this book as a tool to understand rationales for the development of novel treatments for these diseases.

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