EXPRESSION AND REGULATION OF PARATHYROID HORMONE-RELATED PROTEIN DURING LYMPHOCYTE TRANSFORMATION AND DEVELOPMENT OF HUMORAL HYPERCALCEMIA OF MALIGNANCY IN LYMPHOMA

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
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* * * * *

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ABSTRACT

Adult T-cell leukemia/lymphoma (ATLL) is caused by infection with the human T-lymphotropic virus type-1 (HTLV-1). HTLV-1 Tax plays an important role in the transformation of lymphocytes; however, the exact mechanisms remain unclear. Parathyroid hormone-related protein plays an important role in the pathogenesis of humoral hypercalcemia of malignancy (HHM) observed in the majority of ATLL patients. However, PTHrP is up-regulated in HTLV-1-carriers and ATLL patients with normocalcemia, indicating that PTHrP is expressed before malignant transformation of lymphocytes.

Using long-term co-culture assays, herein we demonstrate very high levels of PTHrP and PTHrP receptor (PTH1R) expression during HTLV-1-mediated immortalization of human T-lymphocytes. PTHrP expression did not correlate temporally with expression of HTLV-1 tax and other accessory proteins known to regulate Tax. Co-transfection of HTLV-1 expression plasmids and PTHrP P2/P3-promoter-driven luciferase reporter plasmids demonstrated that HTLV-1 mildly up-regulated PTHrP expression. This indicated that other cellular factors or events are required for increased expression of PTHrP in ATLL cells.

PTHrP is regulated by three distinct promoters P1, P2 and P3. HTLV-1-infected T-cells and ATLL cells predominantly utilize the P2 and P3 promoters. We characterized an NF-κB binding site in the P2 promoter of human PTHrP. Using electrophoretic mobility shift assays, we detected a specific complex in Tax-
expressing human T-cells composed of p50/c-Rel, and two distinct complexes in
ATLL cells consisting of p50/p50 homodimers and a second unidentified protein(s).
Chromatin immunoprecipitation assays confirmed *in vivo* binding of p50 and c-Rel on
the PTHrP P2 promoter. Using transient co-transfection with NF-κB expression
plasmids and PTHrP P2 luciferase reporter-plasmid, we showed that NF-κB p50/p50
alone and p50/c-Rel or p50/Bcl-3 cooperatively up-regulated the PTHrP P2 promoter.
These data demonstrate that transcriptional regulation of PTHrP in ATLL cells can be
controlled by NF-κB activation.

Finally we developed a xenograft model of canine T-cell lymphoma with HHM by
injecting lymphoma cells from a dog with spontaneous lymphoma intraperitoneally
into NOD/SCID mice. The mice developed multicentric lymphoma along with HHM
and increased PTHrP as occurs in dogs with T-cell lymphoma. Quantitative RT-PCR
of T-cell lymphoma samples from hypercalcemic canine patients showed that PTHrP
likely plays a central role in the pathogenesis of HHM and that hypercalcemia is the
result of a combinatorial effect of different hypercalcemic factors. Finally, we
monitored *in vivo* tumor progression and metastases in the mouse model by
transducing the lymphoma cells with a lentiviral vector that encodes a luciferase-
yellow fluorescent protein reporter and showed that *in vivo* trafficking patterns in this
model were similar to that seen in dogs. This unique mouse model will be useful for
comparative and/or translational research and for investigating the pathogenesis of
HHM in T-cell lymphomas in dogs and humans.
In conclusion, these data demonstrate that 1) PTHrP and its receptor are markedly up-regulated during immortalization of lymphocytes due to HTLV-1 infection, 2) The PTHrP P2 promoter is regulated by NF-κB pathway in HTLV-1-infected and ATLL cells, 3) Hypercalcemia in canine T-cell lymphoma patients is multifactorial and PTHrP plays an important role in the pathogenesis of HHM.
Dedicated to my loving and supporting parents Tenugutalli and Subba Rao Nadella
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**FIELDS OF STUDY**

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CHAPTER 1

INTRODUCTION

PATHOGENESIS OF BONE LESIONS AND HYPERCALCEMIA IN ADULT T-CELL LEUKEMIA/LYMPHOMA

Pathogenesis of Adult T-cell Leukemia/Lymphoma

Adult T-cell leukemia/lymphoma (ATLL) is a highly aggressive malignancy of CD4+ T lymphocytes caused by infection with human T-lymphotropic virus type-1 (HTLV-1)(1). HTLV-1 infection is also associated with inflammatory diseases including HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP), uveitis, arthropathy and infective dermatitis(2). About 10 to 20 million people worldwide are infected with HTLV-1, and it is endemic in southwestern Japan, the Caribbean basin, and Central Africa(3). HTLV-1 exists predominantly as a cell-associated provirus and cell-free infection is inefficient(4). Transmission occurs via cell-cell contact through exposure to contaminated blood, sexual contact, or vertically from mother to child either transplacentally or through infected lymphocytes in breast milk(5). About 2-5% of HTLV-1-infected people develop ATLL after a long latency period of 20-40 years post-infection indicating that additional cellular events besides HTLV-1 infection are required for development of the disease(6).
The HTLV-1 genome encodes typical retroviral structural, enzymatic, and envelope proteins, HTLV-1-specific regulatory proteins (Tax and Rex), several accessory proteins (p12, p13, p21, p30) and a minus-strand protein HTLV-1 bZIP-factor, HBZ\(^{(7)}\). The molecular mechanisms by which HTLV-1 induces transformation are not clearly understood, but the 40-kDa nuclear protein Tax is thought to be the viral oncogene and plays important role in the pathogenesis of ATLL\(^{(8)}\). Tax not only increases the expression of viral genes through viral LTRs but also stimulates the transcription of cellular genes through the signaling pathways such as nuclear factor kappa B (NF-κB), serum responsive factor (SRF), cyclic AMP response element-binding protein (CREB), and activated protein 1 (AP-1)\(^{(9,10)}\). Furthermore, Tax alters the activity of several cell cycle regulators, tumor suppressor genes, and genes involved in DNA repair and apoptosis resulting in deregulation of cellular growth mechanisms\(^{(11)}\). Its ability to transcriptionally activate various cellular genes, including proto-oncogenes, is a key mechanism in the transformation of the T-cells. Tax expression induces an immune response and is the major target of cytotoxic T lymphocytes; however, the majority of ATLL cells do not express significant levels of Tax\(^{(12)}\). Currently, it is believed that Tax plays an important role in the persistent proliferation of HTLV-1-infected cells during the carrier state, and the mutator phenotype of Tax accumulates genetic and epigenetic changes in the host genome that lead to Tax-independent proliferation and escape from the host immune system by inactivation of Tax\(^{(13)}\).
The regulator protein, Rex regulates the expression of incompletely spliced viral RNAs through its interaction with the Rex response element in the viral RNA and cellular proteins involved in chromosomal region maintenance-dependent nuclear export\(^{(14)}\). Although Rex is not required for immortalization of lymphocytes in vitro\(^{(15)}\), it is required for infectivity and persistence in vivo\(^{(15)}\). The functions of HBZ are still uncertain, but it has been shown to interact with CREB-2 and suppress Tax-mediated viral transcription\(^{(16)}\). Interestingly, HBZ is thought to have bimodal functions in its RNA and protein molecular forms and the RNA form of HBZ is speculated to be important in the transforming activity of HTLV-1\(^{(17)}\). The accessory proteins, p12, p27, p13, and p30 are important for in vivo viral infectivity, host cell activation, and regulation of gene transcription. Their role in HTLV-1-mediated transformation is not clear\(^{(18)}\).

Clinical Features of ATLL and Hypercalcemia

ATLL is characterized by four clinical subtypes: acute, lymphoma, chronic and smoldering. About 55% are the acute type, 20% are the lymphoma type, 20% are the chronic type and 5% are the smoldering type. Acute and lymphoma-type show aggressive clinical courses, whereas the second two types progress more indolently\(^{(19)}\). ATLL is characterized by generalized lymph node, peripheral blood and/or skin involvement by pleomorphic tumor cells with hyperlobated nuclei (flower cells), lytic bone lesions, hypercalcemia, a rapidly progressive course, and a relatively
short survival\textsuperscript{[20,21]}. Lytic bone lesions are observed in about 5.5% of ATL patients, especially in the tibia, ulna, scapula, femur, and clavicle\textsuperscript{[22]}. Hypercalcemia occurs with a high frequency in acute ATLL patients.

Hypercalcemia is a potentially life-threatening complication that usually affects several organ systems, but most commonly the gastrointestinal, nervous and urinary systems. The severity of symptoms usually depends on the degree of hypercalcemia, the rapidity of the rise in serum calcium, and on the general medical condition of the patient. Gastrointestinal symptoms are the earliest and most frequent features that occur in the syndrome, including anorexia, nausea and vomiting. Neurological manifestations occur in more than 50% of the patients and consist of loss of concentration, fatigue, and depression, and are followed by psychiatric symptoms and neuromuscular disturbances if the condition is untreated. In severe cases, drowsiness and coma may occur. Renal manifestations include polyuria and polydypsia which occur because hypercalcemia interferes with antidiuretic hormone action at the distal nephron resulting in a syndrome-like diabetes insipidus\textsuperscript{[23]}.

**Calcium Homeostasis and Normal Bone Remodeling**

Knowledge of calcium homeostasis and normal bone remodeling is required to understand the mechanisms involved in the pathogenesis of hypercalcemia and bone lesions in ATLL patients. Normally, calcium homeostasis is a tightly regulated process involving the coordinated efforts of calcitropic hormones (parathyroid
hormone, calcitonin and calcitriol) controlling fluxes of calcium between the extracellular fluid and the skeleton, kidney, and gastrointestinal tract\textsuperscript{(24)}. Calcium in the diet that is absorbed by the intestines enters the bloodstream and is filtered by the kidney, where the majority of it is reabsorbed in the proximal renal tubules. Parathyroid hormone (PTH) is synthesized by the chief cells of the parathyroid gland and regulated by serum ionized calcium concentrations, which are sensed by the cell membrane Ca\textsuperscript{2+} receptor\textsuperscript{(25)}. Serum PTH increases as the serum calcium concentration decreases creating a negative feedback loop\textsuperscript{(26)}. PTH functions by binding to the PTH receptor type-1 (PTH1R) on osteoblasts and renal epithelial cells to stimulate osteoclastic bone resorption and release calcium from the bone, increase renal calcium reabsorption, and induce production of calcitriol that increases the intestinal absorption of calcium\textsuperscript{(27)}. Calcitonin is secreted by the parafollicular cells of the thyroid gland and directly inhibits osteoclastic bone resorption and decreases renal tubular reabsorption of calcium; however, the effects of calcitonin are transient and it likely does not have an important role in chronic calcium homeostasis\textsuperscript{(28,29)}. Calcitriol or 1,25-dihydroxyvitamin D\textsubscript{3} (1,25-(OH)\textsubscript{2}D\textsubscript{3}), the biologically active metabolite of vitamin D\textsubscript{3}, is produced in the kidney through the action of 1\textalpha-hydroxylase on the precursor, 25-hydroxyvitamin D\textsubscript{3}. Calcitriol increases plasma calcium concentrations by increasing the absorption of calcium from the intestines\textsuperscript{(30)}.

The majority of calcium in the body is stored in the skeleton (99%), and the remaining 1% is present in the extracellular fluid and soft tissues. Bone undergoes constant remodeling with osteoclasts removing bone and osteoblasts forming new
bone at sites of previous bone resorption in a closely coupled fashion\(^{31}\). Hypercalcemia and bone resorption associated with cancer is typically due to humoral factors secreted by tumors that act systemically on the target organs of bone, kidney and intestines, to disrupt normal calcium homeostasis, or from the local production of factors in the bone microenvironment at sites of bone tumors that stimulate osteoclastic bone resorption.

**Osteoclastogenesis:** Osteoclasts are highly specialized multinucleated cells formed by the fusion of osteoclast precursors of the monocyte-macrophage lineage circulating in the peripheral blood\(^{32}\). Osteoclasts are activated to resorb bone\(^{33}\). Both systemic and local factors in the bone microenvironment play important roles in the regulation of osteoclast differentiation and activity. It has been shown that osteoclastogenesis is regulated by a common molecular signaling pathway that involves members of the tumor necrosis family; including the receptor activator of nuclear factor-\(\kappa\)B (RANK), osteoprotegerin (OPG), and receptor activator of nuclear factor-\(\kappa\)B ligand (RANKL)\(^{34}\). RANKL is expressed as a membrane-bound protein (cleaved into a soluble form by metalloproteinases) on several cell types, including osteoblasts and bone marrow stromal cells, and is also secreted by activated T-cells\(^{35;36}\). RANKL plays a critical role in the differentiation and fusion of osteoclast precursors by binding to the specific receptor RANK expressed on these cells. RANKL not only promotes osteoclastogenesis but also activates mature osteoclasts to resorb bone\(^{37}\). In contrast, OPG, which is also produced by osteoblasts, acts as a decoy receptor for RANKL and blocks osteoclast differentiation and stimulation of bone-resorbing
activity \textsuperscript{(38)}. Humoral hypercalcemia of malignancy and tumor-associated bone lesions primarily result from increased bone resorption and uncoupling of the normal remodeling due to increased osteoclast numbers and activity \textsuperscript{(39)}.

**Cancer-Associated Hypercalcemia**

Hypercalcemia occurs when the serum ionized calcium concentrations are greater than the normal range. Typically total serum calcium concentrations are greater than 12 mg/dL, after adjusting for protein concentration \textsuperscript{(40)}. Hypercalcemia occurs in a wide variety of cancers in addition to ATLL\textsuperscript{(41)}. Hypercalcemia occurs with a high frequency (70-80\%) in ATLL patients compared to other hematologic malignancies and is one of the distinguishing features of ATLL\textsuperscript{(42)}. Although clinical presentation and the underlying pathogenesis can sometimes overlap, cancer-associated hypercalcemia can be divided into two syndromes, humoral hypercalcemia of malignancy (HHM) and local osteolytic hypercalcemia (LOH), depending on whether a circulating hormone or local paracrine factors mediate the increased bone resorption \textsuperscript{(43)}.

1. **Local Osteolytic Hypercalcemia** is caused by increased osteoclastic bone resorption due to skeletal metastases of tumor cells that secrete paracrine factors to stimulate osteoclasts to resorb bone while tumor-free sites do not have excessive bone resorption. Although the majority of ATLL patients present with HHM, a small percentage of them also may have local osteolytic lesions. Some patients have been
reported to have lytic bone lesions in the absence of a detectable primary tumor\(^{(44-47)}\). Local osteolytic hypercalcemia also occurs in patients with extensive osteolytic metastases such as those with myeloma, breast cancer and lymphoma. Several cytokines including interleukin-1\(\alpha\), interleukin-1\(\beta\), interleukin-6, tumor necrosis factor-\(\alpha\), tumor necrosis factor-\(\beta\), transforming growth factor-\(\alpha\), and parathyroid hormone-related protein have been implicated in the pathogenesis of osteoclastic bone resorption in LOH\(^{(48)}\).

2. **Humoral Hypercalcemia of Malignancy** is a syndrome associated with widespread osteoclastic bone resorption caused by tumors distant from bone and results from factors secreted into the systemic circulation affecting the entire skeleton\(^{(48)}\). Biochemical characteristics that define HHM include increased serum calcium, low serum phosphorus, low PTH concentrations and increased elevated nephrogenous cyclic adenosine mono-phosphate (NcAMP) and phosphate excretion rates\(^{(49)}\). Several factors have been implicated in the pathogenesis of HHM, particularly in lymphoma patients, but it is believed that parathyroid hormone-related protein (PTHrP) plays a central role in the development of HHM in most patients\(^{(48)}\).

**Factors implicated in the bone lesions associated with ATLL:** ATLL cells express a variety of factors that directly and/or indirectly stimulate osteoclast differentiation and activity and are detailed below:

**Interleukin-1 (IL-1):** IL-1\(\alpha\) and -1\(\beta\) are potent stimulators of bone resorption\(^{(50)}\). Dewhirst et al. purified the osteoclast activating factor from the culture supernatant of human peripheral blood lymphocytes and reported that it corresponded to IL-1\(\beta\)\(^{(51)}\). In
1986, Gowen et al. reported that recombinant IL-1β had bone resorbing activity in vitro\(^{(52)}\). Shirakawa et al. reported that there was a significant correlation between the level of IL-1α and bone resorbing activities produced by ATLL cells and serum calcium concentrations of ATLL patients\(^{(53)}\). Wano et al. showed that IL-1 was increased in T lymphocytes infected with HTLV-1, and further studies showed that Tax can transactivate IL-1α gene expression\(^{(54,55)}\). Interestingly, IL-1 can act synergistically with tumor necrosis factor and PTHrP to increase osteoclast formation and bone resorption\(^{(56)}\). However, a direct role for IL-1 in HHM in ATLL patients has not been established and further studies are required to understand its role in HHM in these patients.

**Interleukin 6 (IL-6):** IL-6 is a potent osteoclastogenic factor produced by many cell types, including lymphocytes and PTH-stimulated osteoblasts \(^{(57)}\). Ishimi et al. have shown that IL-6 can enhance osteoclastic bone resorption in a mouse calvarial resorption assay\(^{(58)}\). High levels of IL-6 have been reported in patients with solid tumors and HHM\(^{(59,60)}\) and IL-6 is one of the factors produced by multiple myeloma that causes osteolysis\(^{(61)}\). Consistent with these observations, Matshushima et al. described multiple osteolytic lesions in a patient with ATLL caused by elevated IL-6 levels in the absence of hypercalcemia and elevated PTHrP expression\(^{(62)}\). Increased expression of IL-6 has also been described in a pathologic fracture in an ATLL patient\(^{(63)}\). IL-6 has been shown to be transactivated by Tax\(^{(64)}\). Some reports showed that IL-6 stimulated osteoclast-like cell formation by inducing IL-1\(^{(65)}\). Furthermore, IL-6 can enhance the effect of other osteoclastogenic factors such as RANKL\(^{(66)}\) or
PTHrP\(^{(67)}\), thus it is possible that IL-6 can function synergistically with other cytokines in the induction of bone lesions in ATLL patients. In conclusion, IL-6 likely plays a role in the osteolytic lesions in some ATLL patients but its role in HHM has not been established.

**Tumor necrosis factor (TNF):** TNF-\(\alpha\) and -\(\beta\) (lymphotoxin) have been shown to enhance osteoclastic bone resorption and induce hypercalcemia *in vivo*\(^{(68)}\). Ishibashi et al. analyzed the serum levels of TNF-\(\beta\) in twenty-eight ATLL patients without hypercalcemia and eight ATLL patients with hypercalcemia and found that TNF-\(\beta\) was increased in seven of eight ATLL patients with hypercalcemia indicating that TNF-\(\beta\) might be one of the factors responsible for bone resorption in these patients\(^{(69)}\). Stashenko et al. reported synergistic interactions between IL-1 and TNF-\(\beta\) in bone resorption of normal rats. Both TNF-\(\alpha\) or -\(\beta\) can have synergistic effects with IL-1\(\alpha\) or -1\(\beta\)\(^{(70)}\). Therefore, it is possible that TNF and IL-1 could act synergistically to induce HHM in ATLL patients. More detailed studies are required to determine their roles in HHM.

**Macrophage colony stimulating factor:** M-CSF is a critical factor for differentiation of osteoclast precursors\(^{(71)}\) and has been implicated in bone lesions associated with some cancers. Yamada et al. demonstrated high expression of plasma M-CSF in acute ATLL patients\(^{(72)}\). Nosaka et al. reported expression of M-CSF in eight hypercalcemic ATLL patients and showed that high levels of MCSF may play an important role in the pathogenesis of HHM in association with RANKL\(^{(73)}\). However, M-CSF was increased in patients without hypercalcemia, suggesting that M-CSF alone was not
responsible for hypercalcemia. Therefore, a definitive role for M-CSF in the pathogenesis of HHM in these patients has not been established.

**Calcitriol:** Increased extra-renal production of calcitriol (1,25-dihydroxyvitamin D, the active form of vitamin D) is believed to be one of the factors responsible for the pathogenesis of HHM in patients with non-Hodgkin’s lymphoma. Hypercalcemia in these patients results from increased intestinal absorption of calcium and osteoclastic bone resorption\(^{[74]}\). Because of the association of HHM with calcitriol in lymphoma patients, expression of calcitriol in ATLL patients with HHM has been measured. Increased circulating concentrations of calcitriol have been reported in ATLL patients indicating that calcitriol may play a role in the pathogenesis of HHM in these patients\(^{[75]}\). Furthermore, Fetchik et al. have shown that infection of lymphocytes with HTLV-1 results in the production of 1-\(\alpha\) hydroxylase activity that converts 25-hydroxy D3 to the active metabolite, calcitriol\(^{[76]}\). However, a study on five hypercalcemic patients with ATLL showed that the serum levels of calcitriol were within or below the normal range in these patients. Moreover, NcAMP levels were increased in these patients indicating that PTH-like activity was produced by the lymphoma cells and was responsible for the HHM\(^{[77]}\).

**Parathyroid hormone-related protein (PTHrP):** In 1941 Fuller Albright first proposed the syndrome of ectopic PTH production by a tumor in a patient with renal carcinoma and hypercalcemia with normal parathyroid glands. However, PTH was not detected in the tumor and subsequent studies showed that the factor was not PTH but instead an immunologically distinct factor with ‘PTH-like’ biological activity\(^{[78]}\).
Moreover, several of the factors described previously did not satisfactorily explain the other biochemical features of HHM, including elevated NcAMP. In pursuit of the factor responsible for this syndrome, several groups simultaneously purified PTHrP from human lung cancer, breast cancer and renal carcinoma, and PTHrP was shown to be the major mediator of humoral hypercalcemia of malignancy\textsuperscript{(79-81)}. The first 13 amino acids of PTHrP are homologous (70\%) to the N-terminal portion of PTH. The hypercalcemic effects of PTHrP are attributed to its N-terminal portion (1-36) which binds to the common PTH/PTHrP receptor (PTH\textsubscript{1}R) and increases osteoclastic bone resorption and renal reabsorption of calcium\textsuperscript{(82)}, which mimics the effects of PTH. PTHrP has been found in multiple tumor types associated with HHM including squamous, breast and renal carcinomas, and lymphomas. Circulating levels of PTHrP are increased in approximately 80\% of the patients with HHM\textsuperscript{(83)}. A direct causal role for PTHrP in HHM was established when antibodies to PTHrP lowered serum calcium in xenograft mouse models of HHM\textsuperscript{(84)}.

Motokura et al. reported the expression of PTHrP mRNA by northern blot analysis and demonstrated PTH-like bioactivity in a HTLV-1-infected cell line, MT-2. The authors concluded that PTHrP likely is responsible for the hypercalcemia seen in ATLL patients\textsuperscript{(85)}. This report was followed by studies showing that PTHrP was secreted not only by MT-2 cells, but also by cultured peripheral lymphocytes from ATLL patients\textsuperscript{(86)}. Later, Watanabe et al. analyzed PTHrP gene expression in thirteen ATLL patients, four HAM/TSP patients and four healthy HTLV-1-carriers by RT-PCR and showed that PTHrP mRNA was expressed in all the samples. Furthermore,
the authors reported for the first time transactivation of PTHrP by Tax, providing a mechanistic basis for the up-regulation of PTHrP in these patients\(^{(87)}\). However, it was not until the advent of the immunoradiometric assay (IRMA) to measure the circulating protein that PTHrP was measured in ATLL patients with hypercalcemia. Matsumoto’s group first identified increased circulating PTHrP protein in nine out of ten hypercalcemic ATLL patients by IRMA\(^{(88)}\). Subsequent studies also have circulating PTHrP in ATLL patients with hypercalcemia\(^{(89,90)}\). Mori et al. measured the urinary levels of PTHrP in ATLL patients and reported that the urinary level of PTHrP were useful for evaluation of ATLL\(^{(91)}\). Another study demonstrated that urinary excretion of C-terminal PTHrP could be a valuable predictor for the development of HHM in ATLL patients\(^{(92)}\).

Several mechanistic studies using molecular biology approaches have shown that Tax transactivated the PTHrP P3 promoter via the ETS-binding site and SP-1 site present within the promoter\(^{(93)}\). However, constitutively high PTHrP expression in ATLL cells in the absence of Tax expression has led researchers to postulate that PTHrP can be transactivated in a Tax-independent manner. Consistent with this hypothesis we have shown that activation of T-cell receptor signaling in Jurkat T-cells by phytohemagglutinin and ionomycin up-regulated PTHrP expression in the absence of Tax and the protein complexes formed in these cells were similar to those seen in ATLL cells\(^{(94)}\). Some reports have shown that IL-2 and prostaglandin E\(_1\) can up-regulate PTHrP expression in HTLV-1-infected T-cells providing additional mechanisms for the up-regulation of PTHrP observed in ATLL cells\(^{(95,96)}\).
Although many studies identified a central role for PTHrP in the pathogenesis of HHM, other studies have shown that there was no strong correlation between PTHrP expression and hypercalcemia, and that PTHrP was up-regulated in ATLL patients without hypercalcemia\(^{(97)}\). Some studies have implicated PTHrP in combination with other cytokines in the pathogenesis of HHM, suggesting that HHM in ATLL patients is likely multifactorial. IL-6 has been shown to act synergistically with PTHrP in the induction of HHM in mice bearing renal carcinoma\(^{(98)}\) and factors such as IL-1 and TNF can enhance the hypercalcemic effects of PTHrP\(^{(99,100)}\). Other cytokines, such as TGF-\(\beta\), TNF-\(\alpha\), and IL-1 up-regulate PTHrP gene expression in a variety of nonlymphoid cell lines and tissues\(^{(101)}\). Taken together, these studies suggest that HHM in ATLL patients is caused by the synergistic effects of PTHrP in conjunction with various other cytokines.

PTHrP has been shown to be up-regulated during homotypic adhesion of ATLL cells via the LFA1/ICAM-1 pathway indicating diverse roles for PTHrP in ATLL\(^{(102)}\). In addition to its well-documented role in HHM, PTHrP is now known to have pleiotrophic effects including regulation of cell differentiation, proliferation and apoptosis in various cell types\(^{(103)}\). McCauley et al. demonstrated that PTHrP bound to the HTLV-1-infected cell line, MT-2, indicating a potential autocrine role for PTHrP in these cells\(^{(104)}\). More recently, we have shown that PTHrP is up-regulated during immortalization of human peripheral blood mononuclear cells by HTLV-1 (Nadella and Rosol; unpublished data), indicating that PTHrP is expressed not only during HHM but also during early HTLV-1 infection. In conclusion, due to the
pleotrophic properties of PTHrP it is likely that PTHrP has several functions in ATLL besides its central role in HHM.

**Receptor activator of nuclear factor \( \kappa \)B ligand (RANKL):** RANKL produced by osteoblastic lineage cells and activated T-cells is an essential factor for osteoclast formation, fusion and activation. RANKL binds to and activates its receptor RANK present on osteoclasts that in turn results in bone resorption\(^{(105)}\). Recently Nosaka et al. measured the expression of M-CSF, PTHrP, TNF-\( \alpha \) and RANKL in eight hypercalcemic and seven normocalcemic ATLL samples. They showed that M-CSF and PTHrP were present in the serum from both groups, whereas the expression of RANKL mRNA was very high in the ATLL samples from hypercalcemic patients compared to the normocalcemic ATLL samples. The authors concluded that expression of RANKL is critical for induction of hypercalcemia\(^{(106)}\).

**Macrophage inflammatory protein-1\( \alpha \) (MIP-1\( \alpha \)):** MIP-1\( \alpha \) expression can be induced in most mature hematopoietic cells including monocytes, T and B lymphocytes, macrophages, neutrophils and dendritic cells by a variety of stimuli ranging from bacterial LPS, IL-2, and phytohemagglutinin (PHA) to HIV and HTLV-1 infection\(^{(107)}\). MIP-1\( \alpha \) has been shown to stimulate RANKL, osteoclast formation, and bone resorption\(^{(108};109)}\). In the most recent and largest study, Okada et al. analyzed twenty-four ATLL patients with hypercalcemia and thirty-seven patients without hypercalcemia and reported that MIP-1\( \alpha \) was increased in 100% of the ATLL patients with hypercalcemia and only 8% of the ATLL patients with normal calcium levels. They also showed that PTHrP was increased in only 25% of the ATLL patients with
hypercalcemia. IL-1β was increased in 17%, IL-6 was increased in 55%, TNFα was elevated in 12.5% of the ATLL patients with hypercalcemia suggesting that MIP-1α is a specific factor responsible for hypercalcemia in ATL patients\(^{(110)}\). Studies such as this one that analyzed more than one factor will be required in the future to better understand the pathogenesis of HHM in ATLL patients. It is likely that the ATLL cells secrete several factors that act in concert with PTHP to induce HHM.

### Animal Models of HTLV-1-Associated Diseases and Bone Lesions

Because HHM requires a complex interactions of tumor-secreted factors with bone and kidney, animal models are necessary to understand the pathogenesis of HHM in ATLL patients. There is no animal model that can recapitulate the complete disease progression from infection to manifestation of disease. Therefore, the animal models of HTLV-1 can be divided into two categories: infection models and pathology models. The infection models provide information focusing on viral infection, transmission, replication, immune response, and genetics. The pathology models provide information on viral oncogenesis, disease manifestations and pre-clinical therapeutic studies\(^{(111)}\).

To investigate the pathogenesis of HTLV-1, several lines of transgenic mice (primarily targeting the Tax viral oncoprotein) have been developed using different viral gene segments and promoters. The following animal models developed bone lesions:
1. **HTLV-1 LTR-Tax model:** Transgenic mice with the HTLV-1 LTR-Tax developed neurofibromas and adrenal medullary tumors. Tax mRNA was expressed at very high levels in osteoclasts and endosteal spindle-shaped cells within the myelofibrotic lesions of the bone. Generalized skeletal alterations in the femurs, tibias, humeri and tail bones included thicker but fragile bones and alteration in the external shape with a thick diaphysis and an irregular external surface were observed. The transgenic mice had marked bone remodeling with 2-3-fold thicker cortices and trabeculae compared to the control mice. The authors also reported increases in the number, size and degree of multinucleation of the osteoclasts, increases in osteoclast and osteoblast numbers and high bone formation rates in the transgenic mice. Myelofibrosis also occurred in the mice. Finally, bone turnover did not correlate with tumor burden in these mice (112).

2. **Tax\(^+\) C57B6/SJL:** In this model, Tax was targeted to mature T-lymphocyte compartment by utilizing the human granzyme B promoter. The mice developed tumors on the ears, legs, and tails. Tumors with large granular lymphocytes occurred in the cervical, axillary, popliteal, and mesenteric lymph nodes and the spleen. In addition, there were increased WBC counts in the mice. The mice spontaneously developed mild hypercalcemia, enhanced osteoclast activity and a high-frequency of osteolytic bone metastases. The authors showed that Tax\(^+\) tumor cells expressed mRNA for IL-6, M-CSF, IL-1\(\alpha\), IL-1\(\beta\), TGF-\(\beta\), and TNF-\(\alpha\), all of which have been shown to activate osteoclasts and to enhance tumor-associated bone loss. It was shown that intervention with bisphosphonates before the development of lymphoma
prevented tumor-associated osteolysis, dissemination of soft-tissue tumors, and prolonged overall survival, suggesting a role for early bisphosphonate therapy in HTLV-1-infected people\(^{(113;114)}\).

The Tax transgenic models revealed the importance of the Tax viral oncoprotein, but tumor development usually takes longer than 6 months. To circumvent this problem, immunodeficient mice engrafted with primary cancer cells from ATLL patients have been used to test novel therapies for ATLL. The following xenograft models exhibited bone lesions and hypercalcemia as seen in the patients:

1. **ATLL xenograft model:** Uchiyama’s group described a severe combined immunodeficient mouse (SCID) xenograft model developed by intraperitoneal injection of lymph node cells from a lymphoma-type ATLL patient. These mice developed tumors along with hypercalcemia within three weeks after inoculation of tumor cells. There was a marked increase in serum C-terminal PTHrP concentrations along with decreased bone formation rates reported in these mice\(^{(115)}\).

2. **RV-ATL xenograft model:** Richard et al developed this model by intraperitoneal injection of ATLL cells from a patient into SCID/beige mice. The mice developed lymphoma in the mesentery, liver, thymus, lungs, and spleen approximately 1 month after the inoculation of tumor cells. The mice developed severe hypercalcemia with marked increases in serum PTHrP concentrations and increased osteoclastic bone resorption\(^{(116)}\). It was also shown that PTHrP expression in these cells was independent of HTLV-1 Tax expression as occurs in many ATLL patients\(^{(117)}\). Recently the model was refined using luciferase-expressing RV-ATL cells and
subsequent bioluminescent imaging to monitor tumor burden non-invasively. Novel combined therapy with PS-341, a proteasome inhibitor and zoledronic acid, a potent inhibitor of osteoclastic bone resorption showed that the combination was very effective in reducing tumor burden and HHM. (Shu and Rosol; unpublished data).

**Treatment of Hypercalcemia and Bone Lesions**

Prognosis of patients with HHM is generally poor because it generally occurs in patients with advanced malignancy. Treatment of hypercalcemia is usually palliative without prolonging survival. Definitive treatment of HHM is eradication of the underlying tumor. Most experience in the treatment of HHM comes from treating patients with solid cancers and has been adapted to patients with ATLL because the underlying mechanisms of HHM are similar. Palliative treatment for hypercalcemic patients include rehydration with intravenous administration of isotonic saline, which increases glomerular filtration rate and reduces the fractional reabsorption of calcium. Loop diuretics have been used to enhance calcium excretion. Glucocorticoids have been particularly useful for treating lymphoma patients with HHM\(^\text{118}\).

ATLL is a highly aggressive malignancy. Several therapies including doxorubicin-based combination protocols, nucleoside analogues, topoisomerase inhibitors, interferon, zidovudine, arsenic trioxide and monoclonal antibodies have been used with limited success, but the prognosis is still poor due to non-responsiveness, drug resistance, and marrow or hematological toxicity\(^\text{119}\). In patients with symptomatic or
life-threatening hypercalcemia, therapy must be targeted at the inhibition of bone resorption and promoting renal calcium excretion. Bisphosphonates are important principal treatments for long-term treatment of hypercalcemia and the bone disease associated with various cancers. Bisphosphonates have a strong affinity to bone mineral and are released into the bone microenvironment during bone resorption. The nitrogen-substituted bisphosphonates, such as alendronate, risedronate and zoledronic acid are potent inhibitors of the enzyme farnesylidiphosphate synthase, which blocks protein isoprenylation and induces apoptosis of osteoclasts. The non-nitrogen-containing bisphosphonates, clodronate and etidronate, are less potent and also induce osteoclastic apoptosis by inhibition of ATP-dependent intracellular enzymes\(^{120;121}\). The majority of bisphosphonates have limited antitumor activity, a recent report from Mori’s group reported that incandronate inhibited growth of HTLV-1-infected cells and ATLL cells and was effective in reducing tumor burden in a sub-cutaneous mouse xenograft model\(^{122}\). Because the majority of ATLL patients have either hypercalcemia or bone lesions, combination therapy with a bisphosphonate and various anti-tumor drugs should be tested. We recently found that a combination treatment with zoledronic acid and PS-341, a potent proteasome inhibitor of a xenograft mouse model with ATLL cells was very effective in decreasing tumor burden and reducing hypercalcemia (Shu and Rosol; unpublished data). Other novel therapies including RANKL inhibitor (OPG-Fc) and anti-PTHrP antibodies have been promising in decreasing hypercalcemia in mouse models of cancer and HHM\(^{123;124}\). Similar treatments need to be tested in mouse models of ATLL or in ATLL patients.
**Conclusion:** Although the exact mechanisms involved in the pathogenesis of HHM and bone lesions associated with ATLL patients are not clearly understood, research over the past several years has shown that HHM in these patients is likely multifactorial and that PTHrP, MIP-1α and RANKL play important roles in HHM. Studies involving large groups of ATLL patients with and without hypercalcemia need to be analyzed for several hypercalcemic factors simultaneously so that any synergistic or cooperative roles played by the different cytokines in this syndrome can be better understood. There is also a great need for the development of new animal models to study both the molecular pathogenesis of HHM associated with ATLL and to test novel therapies for treating this life-threatening complication.
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CHAPTER 2

EXPRESSION OF PARATHYROID HORMONE-RELATED PROTEIN DURING IMMORTALIZATION OF HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS BY HTLV-1: IMPLICATIONS IN TRANSFORMATION

2.1 ABSTRACT

Although adult T-cell leukemia/lymphoma (ATLL) is initiated by infection with human T-lymphotropic virus type-1 (HTLV-1), additional host factors are required for T-cell transformation and development of ATLL. HTLV-1 Tax plays an important role in the transformation of lymphocytes; however, the exact mechanisms remain unclear. Parathyroid hormone-related protein (PTHrP) plays an important role in the pathogenesis of humoral hypercalcemia of malignancy (HHM) that occurs in the majority of ATLL patients. However, PTHrP is also up-regulated in HTLV-1-carriers and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) patients with normocalcemia, indicating that PTHrP is expressed before malignant transformation of lymphocytes. The role of PTHrP in the transformation of lymphocytes by HTLV-1 infection has not been investigated. In this study, we report high levels of PTHrP expression during immortalization of lymphocytes due to
HTLV-1 infection using long-term co-culture assays. PTHrP expression did not correlate temporally with expression of HTLV-1 Tax and other accessory proteins known to regulate Tax. HTLV-1 infection also up-regulated the PTHrP receptor (PTH1R) in peripheral blood mononuclear cells (PBMCs) indicating a potential autocrine role for PTHrP. Furthermore, co-transfection of HTLV-1 expression plasmids and PTHrP P2/P3-promoter-driven luciferase reporter plasmids demonstrated that HTLV-1 mildly up-regulated PTHrP expression. This indicated that other cellular factors and/or events are required for increased expression of PTHrP in ATLL cells. We also report that macrophage inflammatory protein-1α (MIP-1α), a cellular gene known to play an important role in the pathogenesis of HHM in ATLL patients, was highly expressed during early HTLV-1 infection indicating that its expression was enhanced due to activation of lymphocytes by HTLV-1 infection. These data demonstrate that PTHrP and its receptor are up-regulated during immortalization of lymphocytes by HTLV-1 infection and may facilitate the transformation process.

2.2 INTRODUCTION

Human T-lymphotropic virus type I (HTLV-I) is the etiological agent of adult T-cell leukemia/lymphoma (ATLL), HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) and a variety of other disorders\(^{(1,2)}\). Approximately 10-20 million people worldwide are infected with HTLV-1\(^{(3)}\). HTLV-1 predominantly exists
as a cell-associated provirus and cell-free infection is inefficient\(^4\). Transmission occurs via cell-cell contact through exposure to contaminated blood, sexual contact, or from mother to child either transplacentally or by infected lymphocytes in breast milk\(^5\).

ATLL is an aggressive malignancy of CD\(^+\) T cells that occurs in approximately 5% of infected individuals after a long latency period of 20–40 years; however the majority of HTLV-1-infected individuals are lifelong asymptomatic carriers of the virus\(^6\). The long latent period and the relatively low proportion of HTLV-1-infected people developing ATLL reflects the inefficiency of the virus to transform cells and the need for multiple cooperative changes in growth control mechanisms to induce leukemogenesis. ATLL cells are typically clonal and contain a single or multiple copies of integrated provirus. HTLV-1 is a complex deltaretrovirus and its genome not only encodes for the essential viral proteins Gag, Pol, and Env, but also additional HTLV-1-specific regulatory proteins Tax and Rex, several accessory proteins p12, p13, p30 and a minus-strand encoded protein, HTLV-1 bZIP-factor (HBZ)\(^7\). Although the precise mechanisms underlying transformation are not completely understood, the 40-kDa transcriptional trans-activator Tax is considered the viral oncogene and is thought to be mainly responsible for tumorigenesis\(^8\). In addition to activating transcription from the viral long terminal repeat, Tax activates the expression of multiple cellular genes, which either encode proteins involved in the regulation of cellular proliferation such as interleukin 2 (IL-2)\(^9\), IL-2 receptor \(\alpha\) chain\(^10\), and proliferating cell nuclear antigen (PCNA)\(^11\) or are proto-oncogenes
such as c-fos\textsuperscript{(12)} and c-sis\textsuperscript{(13)}. Furthermore, Tax interacts with numerous proteins to deregulate cellular processes including transcription, cell cycle regulation, DNA repair, and apoptosis\textsuperscript{(14)}.

The ability to activate cellular genes, including proto-oncogenes, is a key mechanism leading to immortalization and transformation of HTLV-1-infected cells. Rex regulates the expression of incompletely spliced viral RNAs by the interaction of the Rex response element in the viral RNA and cellular proteins used by CRM-dependent nuclear export\textsuperscript{(15)}. Although Rex is not required for immortalization of lymphocytes \textit{in vitro}, it is required for infectivity and persistence \textit{in vivo}\textsuperscript{(16)}. The accessory genes \textit{p12}, \textit{p30}, \textit{p13} and \textit{HBZ} contribute to establishing persistent viral infection \textit{in vivo} but are not required for transformation of cells \textit{in vitro}\textsuperscript{(17,18)}.

About 80\% of ATLL patients develop humoral hypercalcemia of malignancy (HHM), a life-threatening paraneoplastic syndrome, that occurs in a wide variety of cancers in addition to ATLL\textsuperscript{(19)}. ATLL cells express factors that directly and/or indirectly stimulate osteoclast differentiation and activity, such as interleukin-1, tumor necrosis factor \(\beta\), parathyroid hormone-related protein (PTHrP), macrophage inflammatory protein-1\(\alpha\) and receptor activator of nuclear factor-\(\kappa\)B ligand (RANKL), that have been associated with induction of hypercalcaemia\textsuperscript{(20-24)}. PTHrP has been shown to play a central role in the pathogenesis of HHM in ATLL patients\textsuperscript{(25)}. However, there is only a weak correlation between hypercalcemia and PTHrP expression. PTHrP expression was detected in asymptomatic HTLV-1-carriers and HAM/TSP patients with no evidence of hypercalcemia, which suggests that PTHrP is
expressed before malignant transformation of lymphocytes\(^{(26)}\). Moreover, ATLL cell adhesion up-regulated PTHrP expression\(^{(27)}\) indicating additional roles for PTHrP besides its central role in the pathogenesis of HHM. PTHrP, although initially discovered for its role in HHM, is known to regulate a wide variety of cellular functions including apoptosis and proliferation\(^{(28-30)}\).

The goal of this study was to investigate the expression of PTHrP, its receptor, and other factors during the early stages of immortalization of human lymphocytes by HTLV-1. Using long-term immortalization and transformation assays we showed that PTHrP and PTH1R were expressed early in immortalization of lymphocytes. PTHrP expression was independent of HTLV-1 viral gene expression and IL-2 stimulation. Co-transfection of HTLV-1 with a PTHrP P2/P3 luciferase reporter showed that PTHrP was up-regulated by HTLV-1 infection. In addition, Rex alone or in the context of the provirus, increased PTHrP expression indicating that Rex might play an important role in the up-regulation of PTHrP during HTLV-1 infection.

2.3 MATERIALS AND METHODS

Cells

293T cells were maintained in Dulbecco’s modified Eagle medium (DMEM) medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, penicillin (100 U/mL), and streptomycin (100 µg/mL). PBMCs were isolated from blood of healthy
donors by centrifugation over Ficoll-paque (Pharmacia, Piscataway, NJ). PBMCs were cultured in RPMI 1640 medium supplemented with 20% FBS, 2 mM glutamine, and antibiotics in the presence or absence of 10 U/mL IL-2 (Boehringer Mannheim, Mannheim, Germany).

**Long-term immortalization and transformation assays**

Immortalization and transformation assays were performed as described previously\(^{(31)}\). Briefly, 2 x 10\(^6\) PBMCs were cultured alone or co-cultured with 10\(^6\) SLB-1 producer cells irradiated with 10,000 rad in 24-well culture plates in the absence or presence of 10 U/mL human IL-2 (hIL-2) for transformation or immortalization assay, respectively. Viable cells were counted weekly by trypan blue exclusion. Cells that continued to produce p19 Gag antigen and proliferate 12 weeks after co-culture in the presence of exogenous IL-2 were identified as HTLV-1-immortalized (IMM-1, IMM-2, IMM-3) and in the absence of exogenous IL-2 as HTLV-1-transformed cells (TRAN-1, TRAN-2). PBMCs cultured alone (PBMC-1, PBMC-2) or the in the presence of IL-2 (PBMC-1 + IL-2, PBMC-2 + IL-2) or phytohemagglutinin (PHA) (PBMC-1+PHA, PBMC-2 + PHA) without HTLV-1 infection were used as controls.

**Real time RT-PCR**

Total RNA was extracted using TRIZOL\textsuperscript{®} Reagent (Invitrogen, CA). To measure the total PTHrP, 1 µg RNA was reverse-transcribed and submitted for real-time RT-PCR.
analysis using TaqMan® Gene Expression assays (4331182, Applied Biosystems, CA). β2M (4333766, Applied Biosystems) was used as a reference gene. PTHrP P2 and P3 promoter-initiated transcripts and PTHR1 were measured as described previously(32;33). HTLV-1 viral transcripts p13, p30 and HBZ were measured as previously described(34) except that SYBR Green technique was used instead of the TaqMan assays.

**PTHRP Immunoradiometric Assay**

PTHRP concentrations were measured in the conditioned medium using a two-site immunoradiometric assay (DSL, Webster, TX) specific for the PTHrP N-terminal region (amino acids 1 to 40) and mid-region (amino acids 57 to 80).

**Enzyme Linked Immunosorbant Assays**

p19 Gag protein in the culture supernatant was measured using a commercially available ELISA kit (Zeptometrix, Buffalo, NY). MIP-1α protein in the conditioned medium was measured using Quantikine Human CCL3/MIP-1α Immunoassay (R&D systems, Minneapolis, MN).

**Plasmids and transfections**

The PTHrP P2/P3 luciferase construct was made by cloning the PTHrP P2/P3 promoter fragment (-1120 Bam H1 to +1 Hind III) into pGL2 basic vector. ACH, PcTax, BCRex, HBZ plasmids were obtained from the laboratory of Dr. Patrick
Green (The Ohio State University). P12, p13 and p30 expression plasmids were obtained from the laboratory of Dr. Michael Lairmore (The Ohio State University). 293T cells were transfected with either PTHrP P2/P3 PGL2 Luc plasmid alone or with ACH, PcTax, BCRex, HBZ, p12, p13, p30. pcDNA-3.1 was used as a “filler” plasmid so that the total amount of DNA would be the same in all transfection groups. The plasmid pβgal-Control Vector (250 ng) was included in each transfection and served as an internal control to correct for transfection efficiency. Luciferase activity was measured with the Luciferase Assay System (Promega, Madison, WI) using 40 µl of lysate. Simultaneously, β-galactosidase activity was measured with the Luminescent β-Galactosidase Detection Kit II (BD Biosciences).

Statistical analyses

For the co-culture and transfection experiments, several independent measurements were taken across time and thus ANOVA models were used to study the effects of time, treatment and the interaction between time and treatment. The square-root transformation was used for cell number and MIP-1α data to achieve normality and homogeneous variances. Dunnett’s method was used to adjust for multiple comparisons versus control group. For PTHrP mRNA fold changes, some of the treatments were zero after the 3rd week (PBMC, PBMC+IL2, PBMC+PHA). Non-parametric method (Wilcoxon sum rank) was used for the comparison among non-zero groups to the zero groups. A p value less than 0.05 was considered significant.
2.4 RESULTS

HTLV-1-infected PBMCs proliferate beyond six weeks

To investigate the expression of PTHrP early in HTLV-1 infection, we used long-term co-culture assays of PBMCs from healthy human donors in the presence or absence of irradiated HTLV-1 producer cells (SLB-1). Viable cells were counted by typan blue exclusion and the results are shown in Figure 2.1A. Irradiated SLB-1 cells lived up to 1 week in culture. PBMCs, in the absence of stimulation with either IL-2 or PHA, progressively decreased in numbers and failed to grow in vitro as expected\(^{(35)}\). PBMCs supplemented with IL-2 or PHA lived and proliferated up to 4 weeks in culture, at which time they entered a “growth crisis” phase and decreased in numbers and did not live beyond 6 weeks in culture. In contrast, HTLV-1-infected PBMCs continued to proliferate beyond 6 weeks for up to at least 13 weeks in culture. Cells that continued to proliferate beyond 8-9 weeks in culture in the absence of exogenous IL-2 were referred to as transformed cells (TRAN-1 and -2) and in the presence of IL-2 were referred to as immortalized cells (IMM-1, -2 and -3). High levels of p19 Gag protein were detected at very high levels throughout the co-culture demonstrating viral production (Figure 2.1B).

PTHrP was up-regulated during immortalization of PBMCs with HTLV-1

To determine the temporal expression of PTHrP during immortalization of PBMCs with HTLV-1, PTHrP mRNA and protein expression were analyzed at various time
points from the long-term co-culture assays (Figure 2.2). Freshly isolated PBMCs expressed barely detectable PTHrP. There was no increase in PTHrP mRNA or protein expression in unstimulated PBMCs in culture. IL-2 stimulation up-regulated PTHrP mRNA expression in the first week (3.8- and 12-fold) compared to unstimulated PBMCs. Beyond 1 week there was no up-regulation of PTHrP mRNA in the IL-2-stimulated PBMCs. Although there was an increase in the PTHrP mRNA expression due to IL-2 stimulation, PTHrP protein (2.6 pM) was detected only in one of the samples (PBMC-1 + IL-2). No increase in PTHrP mRNA and protein occurred with PHA stimulation of PBMCs. In contrast, HTLV-1 infection markedly up-regulated PTHrP mRNA expression compared to the uninfected PBMCs. In the immortalization assays, PTHrP mRNA was up-regulated 300- to 500-fold compared to uninfected PBMCs at day 0. In the transformation assays, PTHrP mRNA was up-regulated 1300- to 3800-fold compared to uninfected PBMCs at day 0. PTHrP protein was detectable in the conditioned medium 1 week following co-culture with HTLV-1 producer cells and peak PTHrP protein expression was detected between weeks 10 and 13 post-infection. For the immortalization assays, peak PTHrP protein expression ranged from 153 to 212 pM and in the transformation assays it ranged from 130 to 160 pM.

**Up-regulation of PTHrP was mediated by the PTHrP P2 and P3 promoters**

PTHrP is regulated by three distinct promoters that are transactivated by divergent cellular signal transduction pathways\(^{(36)}\). To understand the molecular mechanisms
involved in the transcriptional up-regulation of PTHrP following HTLV-1 infection, we investigated the preferential promoter usage using real time RT-PCR to detect specific promoter-initiated transcripts. As shown in Figure 2.3, there was preferential usage of the PTHrP P2 and P3 promoters during immortalization and transformation. However the ratio of P2 to P3 promoter-initiated transcripts was at least 2-fold higher during immortalization and transformation of PBMCs with HTLV-1 (1:2) (Figure 2.3A-E) compared to transformed MT-2 cells (1:4) (Figure 2.3E).

**HTLV-1 infection up-regulated PTH1R expression**

Since many of the biological properties of PTHrP are the result of its interaction with the PTH1R, which is coupled to adenylyl cyclase (AC) and/or phospholipase C (PLC) and subsequent signaling pathways\(^{(37,38)}\), we analyzed the expression of PTH1R during immortalization and transformation of PBMCs with HTLV-1. As shown in Figure 2.4A, there was very low PTH1R expression in freshly isolated PBMCs. Stimulation of PBMCs with IL-2 or PHA did not up-regulate PTH1R. However, following infection with HTLV-1 there was marked induction of PTH1R in PMBCs. We also analyzed the expression of PTH1R in various HTLV-1-infected and ATLL cells. As shown in Figure 2.4B, HTLV-1-negative Jurkat cells did not express PTH1R. High Tax-expressing HTLV-1-positive cells (MT-2, SLB-1, HT-1RV) expressed moderate levels of PTH1R. ATLL cells (Met-1 and RV-ATL) did not express PTH1R.
**PTHRP expression did not correlate with HTLV-1 viral transcripts**

HTLV-1 Tax has been shown to transactivate PTHrP; however, ATLL cells that lack significant Tax expression have very high levels of PTHrP indicating that PTHrP can be expressed in a Tax-independent manner\(^{(39)}\). To investigate the basis for up-regulation of PTHrP due to HTLV-1 infection we analyzed by quantitative real-time RT-PCR the temporal expression of HTLV-1 viral transcript Tax and other HTLV-1 viral accessory proteins known to regulate Tax expression (Figure 2.5). The high HTLV-1 viral transcript expression during the first week in the co-cultures (data not shown) was contributed by the residual live irradiated SLB-1 cells. After the first week, the decline in the viral gene expression correlated with the death of the irradiated SLB-1 cells and the subsequent viral gene expression was from the HTLV-1-infected PBMCs. Among the viral transcripts analyzed, Tax expression was the greatest. Tax mRNA expression increased from 3 to 7 weeks and then decreased between 9-11 weeks post-infection (Figure 5A). HTLV-1 accessory genes, p30 and HBZ have been shown to regulate Tax expression\(^{(40-42)}\). In order to determine if the expression of any of these genes that regulate Tax expression correlated with PTHrP expression, we analyzed the expression pattern of p13, p30 and HBZ mRNAs using quantitative real-time RT-PCR. As shown in figure 2.5B-D, the expression of the accessory genes was lower than that of Tax expression. Among the accessory genes analyzed, the expression patterns of these genes did not correlate with the expression of PTHrP.
HTLV-1 infection and HTLV-1 Rex up-regulated PTHrP expression

In order to investigate the direct effect of each of the HTLV-1 viral proteins on PTHrP expression, we co-transfected a PTHrP P2/P3 promoter-driven luciferase plasmid with an HTLV-1 expression plasmid (ACH) and/or expression plasmids for p12, p13, p30, Tax, Rex and HBZ (Figure 2.6). Infection with HTLV-1 up-regulated PTHrP expression only 1.6-fold 48 h after transfection. The expression of HTLV-1-p12, p13, p30, HBZ or Tax did not alter PTHrP expression (Figure 2.6A). Co-expression of Rex and ACH up-regulated PTHrP expression, and expression of Rex alone up-regulated PTHrP. Over-expression of p12, p13, p30, HBZ or Tax along with the ACH plasmid did not up-regulate PTHrP expression (Figure 2.6B).

MIP-1α expression correlated with activation of PBMCs following HTLV-1 infection

Since PTHrP was specifically up-regulated during the immortalization of PBMCs with HTLV-1, we also measured the expression of MIP-1α, another chemokine known to be involved in the pathogenesis of HHM in ATLL patients. As shown in Figure 2.7, MIP-1α expression was induced by IL-2 (4- to 14-fold) or PHA (3- to 9-fold) stimulation of PBMCs as expected\(^{(43,44)}\). However, there was marked up-regulation of MIP-1α in the first week post co-culture in PBMCs infected with HTLV-1.
The expression of MIP-1α in the immortalization assays ranged from 10,000 to 46,000 pg/mL and in the transformation assays the expression was about 45,000 pg/mL. After the peak induction of MIP-1α at week 1, there was consistent, although lower, MIP-1α expression at all later time points.

2.5 DISCUSSION

Although HTLV-1 Tax is known to have pleiotropic effects that either directly or indirectly contribute to immortalization and transformation of infected T-cells, the exact mechanisms of transformation are unclear. In this study we analyzed temporal PTHrP gene expression during virus-mediated immortalization of lymphocytes to characterize its role in the transformation process. We present data to show that PTHrP is markedly up-regulated during the immortalization process.

An important step in HTLV-1-induced leukemogenesis is the induction of abnormal T-cell growth. Long-term immortalization assays have been used to study the kinetics of HTLV-1 infection and abnormal T-cell growth that leads to transformation. The growth curves in our study are similar to previous reports that PBMCs cultured in the presence of IL-2, but not exposed to the virus, survived *in vitro* only for a few weeks. Following exposure to HTLV-1, PBMCs initially underwent a proliferative response due to HTLV-1 infection after which the cells entered a “growth crisis” between weeks 5-7 followed by expansion resulting in the immortalized cells. The high levels of HTLV-1 p19 antigen expression in the first few weeks of co-culture was likely
contributed by the irradiated SLB-1 cells, however the p19 expression following three weeks in culture was from the newly infected PBMCs and was indicative of active HTLV-1 viral infection.

Although PTHrP was discovered for its role in the pathogenesis of HHM, PTHrP is now known to be a complex factor with various properties and a broad range of physiologic and/or pathophysiologic actions in different cells and tissues. Watanabe et al. have shown that PTHrP was constitutively expressed in HTLV-1-carriers and ATLL patients with or without hypercalcemia. Moreover, homotypic ATLL cell adhesion up-regulated PTHrP expression, which supported a unique role for PTHrP in the pathogenesis of ATLL. In this study, we investigated the expression of PTHrP during transformation of lymphocytes following early HTLV-1 infection using long-term immortalization assays. Our data showed that PTHrP mRNA expression was gradually up-regulated in PBMCs following HTLV-1 infection; however, marked expression of PTHrP protein occurred at the time when the PBMCs were undergoing immortalization. This supports an important role for PTHrP during immortalization and the subsequent transformation process. There was some discrepancy between PTHrP mRNA and protein expression. This is likely due to differences in the translation, processing of the mature protein, and/or its secretion from the cells. Secretion of PTHrP is a complex process and it has been shown that some PTHrP is not secreted, but can be targeted to the nucleus and function in an intracrine fashion\(^{(45)}\).
Motokura et al. have shown that PTHrP gene expression was induced in close association with transformation of normal rat embryo fibroblasts by co-transfection with an activated \( \text{ras} \) gene and a mutated p53 gene\(^{46}\). Insogna et al. have shown that PTHrP induced transformation of rat fibroblasts in conjunction with epidermal growth factor\(^{47}\). Moreover, co-transfection of rat embryonic fibroblasts with tax and ras transformed the fibroblasts and they were highly tumorigenic \textit{in vivo}\(^{48}\). Based on these reports, it is possible that PTHrP can function as a transforming factor in conjunction with other oncogenes.

PTHRP has been shown to be an auto/paracrine cell growth regulator that increases proliferation of several cell types including chondrocytes and renal epithelial cells\(^{49}\). PTHrP is known to stimulate proliferation through the PTH1R via mechanisms involving both PKA and PKC signaling pathways. It has been shown that anti-PTHrP antibodies or PTH1R antagonists inhibited the growth of cells\(^{50}\). In our investigation, the induction of both PTH1R and PTHrP by HTLV-1 suggests that PTHrP may have functioned as an autocrine growth regulator in the transformation process.

PTHRP is a complex gene that is regulated by three distinct promoters P1, P2 and P3 and transactivated by diverse cellular signal transduction pathways. We and others have shown that the P3 promoter in ATLL cells is regulated by the ETS signaling pathway\(^{51}\) and recently have shown that the P2 promoter is regulated by the NF-κB
pathway\textsuperscript{(52)}. Our data, in this investigation demonstrated that PTHrP was up-regulated during immortalization by the P2 and P3 promoters. The ratio of the P2/P3 promoter-initiated transcripts during the immortalization phase was higher (1:2) than in the transformed cells (MT-2; 1:4) or ATLL cells (data not presented)\textsuperscript{(53)}. NF-κB is known to play an important role during the immortalization process and our data showed that the P2 promoter was highly expressed during immortalization. This suggests that NF-κB activity known to play an important role in the transformation process transactivated the PTHrP P2 promoter.

HTLV-1 tax has been shown to transactivate PTHrP; however, ATLL cells with no significant Tax expression have very high levels of PTHrP. Recently, we have shown that Tax mRNA expression was inversely proportional to PTHrP mRNA expression and PTHrP can be regulated in a Tax-independent manner in ATLL cells\textsuperscript{(54)}. To investigate possible mechanisms for up-regulation of PTHrP in our co-culture assays, we measured the expression of Tax/Rex mRNA. Our data showed that there was no correlation between PTHrP and Tax/Rex mRNA expression. Thus, induction of PTHrP could either be an indirect effect of Tax or possibly due to a Tax-independent mechanism. Tax expression has been shown to be regulated by HTLV-1 p13, p30 and HBZ\textsuperscript{(55)}. To investigate the possible effect of any of these viral genes on PTHrP expression, we analyzed their expression in the co-cultures and our data showed that the expression pattern of these genes did not correlate with PTHrP.
Data from the transfection experiments showed that HTLV-1 infection up-regulated PTHrP expression mildly and suggests that additional cellular events are required for the dramatic PTHrP expression seen in ATLL cells. Alternatively, PTHrP expression might be dependent on cell-type and require lymphocyte-specific factors for marked up-regulation. Interestingly, over-expression of either p12, p13, p30 or Tax did not up-regulate PTHrP while over-expression of Rex alone resulted in the up-regulation of PTHrP. Over-expression of the viral genes in the context of the provirus showed that Tax decreased PTHrP expression whereas over-expression of Rex in the context of the provirus up-regulated PTHrP. Interestingly, Rex and PTHrP have a similar nuclear localization signal and can bind to importin β \(^{(56,57)}\). Therefore, the increased expression of PTHrP in the presence of Rex may have been due to increased nuclear export of PTHrP or alternatively could be due to the increased PTHrP mRNA stability since Rex has been shown to increase mRNA stability of genes such as IL-2Rα \(^{(58)}\). Finally, the data from the transfection experiments correlated with the PTHrP expression data from co-culture experiments. The data demonstrated that HTLV-1 infection increased PTHrP expression and cellular transformation was associated with marked up-regulation of PTHrP.

We analyzed the expression of MIP-1α, another cellular gene that is known to play an important role in the pathogenesis of HHM, in the co-cultures. The data showed that MIP-1α was markedly up-regulated as early as 1 week following HTLV-1 infection of PBMCs. These data are in agreement with reports \(^{(59)}\) that showed MIP-1α
was up-regulated during activation of lymphocytes. Our data demonstrated that MIP-1α was up-regulated early in co-cultures with HTLV-1 infection due to activation of lymphocytes. In contrast, the up-regulation of PTHrP occurred later during immortalization, which supports a role for PTHrP in the transformation process.

In conclusion, our data demonstrated that PTHrP was dramatically up-regulated during the immortalization of PBMCs with HTLV-1 in a Tax-independent manner. PTHrP likely functions in an autocrine mechanism with thePTH1R facilitating the transformation process. Although further investigations are required to understand the role of PTHrP in the transformation process, it is apparent that PTHrP is up-regulated not only during HHM but also during early HTLV-1 infection implicating an important role for PTHrP in the pathogenesis of ATLL. Novel therapies directed against PTHrP might be an important strategy to prevent ATLL in HTLV-1-infected people.
2.6 REFERENCE LIST


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Figure 2.1 Growth curves and p19 Gag expression in HTLV-1 T-lymphocyte immortalization assays. Human PBMCs ($2 \times 10^6$) were cultured alone or with irradiated donor cells (SLB-1) in 24-well plates. Cell viability was measured weekly by trypan blue exclusion (0-13 weeks after co-cultivation) and growth curves are shown. (A) IMM-1, IMM-2 and IMM-3 represent immortalization assays in which IL-2 (10U/mL) was supplemented from day 1 following HTLV-1 infection; TRAN-1 and TRAN-2 represent transformation assays and did not receive exogenous IL-2. PBMCs-1&2 with no stimulation, PBMCs-1&2 stimulated with PHA and IL-2 served as controls. Only HTLV-1-infected cells continued to proliferate beyond 6 weeks in culture (B) p19 Gag expression in co-cultures. P19 production as a measure of HTLV-1 viral production was measured in the supernatant of the co-cultures by ELISA. The results showed that PBMCs infected with HTLV-1 produce p19 expression in the immortalization and transformation assays. (*) indicates significant differences in both immortalization (IMM-1,2,3) and transformation groups (TRAN-1, 2) compared to PBMC alone (PBMC-1,2) (p<0.05)
Figure 2.1 Growth curves and p19 Gag expression in HTLV-1 T-lymphocyte immortalization assays.
Figure 2.2 PTHrP was markedly up-regulated during immortalization of PBMCs with HTLV-1 infection. (A) PTHrP mRNA expression during immortalization of PBMCs with HTLV-1. Total RNA was extracted from the co-cultures at various time points and PTHrP mRNA expression was measured by real-time RT-PCR using the Taqman method. PTHrP expression was normalized to human β2M and the data was represented as fold change over uninfected PBMCs from day 0. The results showed marked up-regulation of PTHrP mRNA in PBMCs following HTLV-1 infection. (B) PTHrP protein expression during immortalization of PBMCs with HTLV-1. Secreted PTHrP was measured in the conditioned medium from the co-culture assays by IRMA. Results showed marked up-regulation of PTHrP secretion in PBMCs infected with HTLV-1 during the immortalization phase. (*) indicates significant differences in the immortalization (IMM-1,2,3) and transformation groups (TRAN-1,2) compared to PBMC alone (PBMC-1 and 2) (p<0.05).
Figure 2.2 PTHrP was markedly up-regulated during immortalization of PBMCs with HTLV-1 infection.
Figure 2.3 PTHrP was up-regulated by the P2 and P3 promoters. Specific PTHrP promoter-initiated transcripts were measured by real time quantitative RT-PCR using the SYBR green method. The data was normalized to human β2M gene expression. Specific PTHrP-promoter initiated transcripts at time points day 0, 3, 7 and 13 weeks post co-culture are shown for IMM-1 (A), IMM-2 (B), IMM-3 (C), TRAN-1 (D) TRAN-2; (E) MT-2 cells. The data showed that PTHrP was up-regulated in PBMCs following HTLV-1 infection by the activation of the P2 and P3 promoters.
**Figure 2.4 HTLV-1 infection up-regulated expression of the PTHrP receptor (PTH1R) in PBMCs.** PTHrP receptor expression and human β2M were measured by RT-PCR from total RNA at various time points in the co-culture assays. (A) Marked up-regulation of PTH1R in PBMCs at weeks 1, 3, 5, 7, 9, 11, and 13 following HTLV-1 infection in IMM-1, IMM-2, IMM-3, TRAN-1 and TRAN-2 assays compared to day 0; controls 1 and 4 are PBMC-1 and PBMC-2; controls 2 and 5 are PBMC-1 and PBMC-2 stimulated with PHA, controls 3, and 6 are PBMC-1 and PBMC-2 stimulated with IL-2 for one week. (B) PTH1R expression in HTLV-1-infected T-cells. Lane represents (1) Jurkat (2) MT-2 (3) SLB-1 (4) HUT102 (5) C8166 (6) MET-1 (7) RV-ATL (8) HT-1RV cells. The data showed that PTH1R expression was very low or absent in the ATLL cells (MET-1 and RV-ATL) compared to HTLV-1-infected T-cell lines (MT-2, SLB-1 and HT-1RV). Jurkat T-cells were used as a negative control. β2M was used a loading control.
Figure 2.4 HTLV-1 infection up-regulated expression of the PTHrP receptor (PTH1R) in PBMCs
Figure 2.5 PTHrP expression did not correlate with HTLV-1 Tax and HTLV-1 accessory genes known to regulate Tax expression. mRNA expression was measured by quantitative real time RT-PCR using the SYBR green method and the data was normalized to human β2M gene expression. (A) HTLV-1 Tax expression in co-cultures following HTLV-1 infection, (B) HBZ mRNA expression in co-cultures, (C) p30 mRNA expression in co-cultures.
Figure 2.6 HTLV-1 infection or over-expression of Rex alone up-regulated PTHrP expression. Relative luciferase activity in 293T cells transfected with either pGL2 or pGL2 PTHrP-P2/P3 Luc constructs alone or with expression plasmids for HTLV-1 (ACH), p12, p13, p30, HBZ, Rex and Tax. The quantity of the expression plasmid is indicated in µg. Bars represent the mean ± SD of three independent samples. The data showed that HTLV-1 infection or over-expression of Rex alone up-regulated PTHrP. (*) indicates significant differences between various groups compared to pGL2-PTHrP-P2/P3 Luc alone in figure A. In figure B, (*) indicates significant differences between various groups compared to pGL2-PTHrP-P2/P3 Luc + ACH (p<0.05).
MIP-1α induction due to activation of lymphocytes following HTLV-1 infection. MIP-1α was measured in the conditioned medium from the co-culture assays at various time points (day 0, 1, 3, 5, 7, 9, 11 and 13 weeks of co-culture) by ELISA. The results showed that MIP-1α expression was up-regulated in PBMCs following stimulation with PHA or IL-2. HTLV-1 infection markedly up-regulated MIP-1α expression in the first week after infection, which demonstrated that HTLV-1 infection activated the lymphocytes. (*) indicates significant differences between IMM-1, 2, 3 and TRAN-1 and 2 groups compared to PBMC-1 and 2 (p< 0.05).
CHAPTER 3

TRANSCRIPTIONAL REGULATION OF PARATHYROID HORMONE-RELATED PROTEIN PROMOTER P2 BY NF-κB IN ADULT T-CELL LEUKEMIA/LYMPHOMA

3.1 ABSTRACT

Parathyroid hormone-related protein (PTHrP) plays a primary role in the development of humoral hypercalcemia of malignancy (HHM) that occurs in the majority of patients with adult T-cell leukemia/lymphoma (ATLL) due to human T-cell lymphotropic virus type-1 (HTLV-1) infection. We previously showed that ATLL cells constitutively express high levels of PTHrP via activation of promoters P2 and P3, resulting in HHM. In this study, we characterized an NF-κB binding site in the P2 promoter of human PTHrP. Using electrophoretic mobility shift assays, we detected a specific complex in Tax-expressing human T-cells composed of p50/c-Rel, and two distinct complexes in ATLL cells consisting of p50/p50 homodimers and p50 with a second unidentified protein(s). Chromatin immunoprecipitation assays confirmed in vivo binding of p50 and c-Rel on the PTHrP P2 promoter. Using transient co-transfection with NF-κB expression plasmids and PTHrP P2 luciferase reporter-plasmid, we showed that NF-κB p50/p50 alone and p50/c-Rel or p50/Bcl-3
cooperatively up-regulated the PTHrP P2 promoter. Furthermore, inhibition of NF-κB activity by Bay 11-7082 reduced PTHrP P2 promoter-initiated transcripts in HTLV-1 infected T-cells. In summary, the data demonstrated that transcriptional regulation of PTHrP in ATLL cells can be controlled by NF-κB activation and also suggests a Tax-independent mechanism of activation of PTHrP in ATLL.

3.2 INTRODUCTION

Adult T-cell leukemia/lymphoma (ATLL) is a highly aggressive malignancy of peripheral helper T-cells associated with human T-cell lymphotropic virus type-1 (HTLV-1) infection\(^\text{(1)}\). About 80% of ATLL patients develop humoral hypercalcemia of malignancy (HHM), a life-threatening paraneoplastic syndrome, seen in a wide variety of cancers in addition to ATLL\(^\text{(2)}\). In HHM, increased circulating parathyroid hormone-related protein (PTHrP) stimulates the parathyroid hormone-1 receptor (PTH1R) to induce osteoclastic bone resorption and increase calcium reabsorption in kidneys, resulting in hypercalcemia\(^\text{(3)}\). In addition to its role in the induction of HHM, PTHrP has been shown to be involved in the regulation of cell proliferation and apoptosis in a wide variety of normal and neoplastic tissues\(^\text{(4)}\).

Transcriptional regulation of the human PTHrP gene is achieved by three distinct promoters identified as P1, P2 and P3. Promoters P1 and P3 (previously called the P2 promoter) contain a typical TATA box\(^\text{(5-7)}\), while P2 is a GC-rich promoter region located immediately upstream of exon 3 (also named exon 1c) with the transcription
initiation site located 11 nucleotides upstream of the exon 3 splice acceptor site. Several studies have shown that P2/P3 promoter usage is prevalent in many cancers such as breast cancer and bone cancer in addition to HTLV-1-positive and ATLL cells\(^{8-11}\). Furthermore, Brandt et al. reported a preferential usage of P2 over P1 and P3 promoters in different cancer cell lines examined\(^{12}\). Although this stimulation and activity of the PTHrP P2 promoter was identified ten years ago, little is known regarding the transcriptional mechanisms involving cis-acting regulatory sequences within this promoter.

HTLV-1-Tax is a potent transcriptional activator that not only drives the transcription of all HTLV-1 transcripts from the viral LTR but also activates numerous cellular genes through activation of nuclear factor-kappaB (NF-κB), cyclic AMP response element binding protein (CREB/ATF), and serum response factor (SRF)\(^{13-15}\). NF-κB, a cytokine-inducible family of transcription factors, controls expression of genetic networks important in cell survival, proliferation, inflammation, and T-cell transformation. Although tightly regulated in normal T-cells, NF-κB is constitutively activated in ATLL and Tax-expressing T-cells\(^{16}\). While Tax-mediated NF-κB activation serves as a critical step in the induction of T-cell transformation by HTLV-1, the presence of constitutively active NF-κB in freshly isolated ATLL cells that lack detectable Tax expression implicates a crucial role for NF-κB in the multistep process of leukemogenesis\(^{17}\).

The NF-κB family is composed of five structurally related protein subunits that can be divided into two groups: (1) p65/RelA, RelB, and c-Rel contain a well-defined
transactivation domain; and (2) p50 and p52, which are generated by proteolytic processing from their precursors, p105/NF-κB 1 and p100/NF-κB 2, respectively, and lack transactivation domains. Although the predominant complex in most cells is p50/p65, various dimeric transcription factor complexes can form between the family members\(^{(18)}\). Heterodimers containing p52 or p50 combined with p65, c-Rel, or RelB are capable of activating transcription. NF-κB dimers bind target gene regulatory regions through a wide variety of binding sites that generally match a 5’-GGGRNNYYCC-3’ consensus (R, purine; Y, pyrimidine; N, any base). Although their functions often overlap, NF-κB achieves target gene specificity in part through preferential binding of different subunit combinations to numerous similar DNA sequences\(^{(19)}\).

NF-κB is expressed in virtually all cell types, but in unstimulated cells the NF-κB homo- and heterodimers are sequestered in the cytoplasm in an inactive form complexed with one of the members of the family of regulatory IκBs, including IκB-α, IκB-β, IκB-γ and Bcl-3. IκB molecules are subject to phosphorylation, subsequent degradation, and release of active NF-κB upon reception of signals that lead to NF-κB activation\(^{(20)}\). In contrast to IκBα and IκBβ, which specifically interact with dimers containing p65 or c-Rel, Bcl-3 specifically interacts with p50 or p52 homodimers and contains N- and C-terminal regions that can act as transactivation domains\(^{(21,22)}\). Although the cellular function of Bcl-3 is still unclear, various studies have suggested that Bcl-3 acts to increase transcription from NF-κB responsive promoters by either 1) acting as an anti-repressor by removing homodimers from the κB sites so that
transactivating NF-κB dimers can bind, 2) forming a complex with homodimers at κB sites and acting as a transactivator, or 3) enhancing homodimer binding to κB sites. Interestingly, Bcl-3 can enhance p50 or p52 homodimer binding to DNA, without being a stable component of the complex\(^{(23,24)}\).

We have previously reported that the up-regulation of PTHrP in ATLL and HTLV-1-infected T-cells is mediated by the PTHrP P2 and P3 promoters and that the P3 promoter is regulated by the ETS signaling pathway. The relative PTHrP P2 promoter usage was determined to be 16, 4, 7, and 40 copies (per \(10^4\) copies of β2M) in HT-1RV, SLB-1, MT-2 and RV-ATL cells, respectively, compared to the 47, 86, 104, 644 copies (per \(10^4\) copies of β2M) of total PTHrP\(^{(25)}\). Since ATLL cells display constitutive expression of NF-κB and PTHrP and since sequence analysis of the PTHrP P2 promoter revealed that it contained potential binding sites for NF-κB, we tested in this report the hypothesis that PTHrP gene is a direct target of the transcription factor, NF-κB. We identified an NF-κB binding site within the human PTHrP P2 promoter region that is responsible for NF-κB-mediated stimulation of PTHrP promoter activity. We also demonstrated and characterized the formation of different protein/DNA complexes on the NF-κB-binding site located within the second promoter (P2) of the human PTHrP gene in HTLV-1-infected and ATLL cells. We present evidence that transactivation of the PTHrP P2 promoter can occur in a subunit-dependent manner by p50/c-Rel and p50/Bcl-3. Finally, we showed that inhibition of NF-κB by the Bay 11-7082 decreased P2 promoter-initiated transcription.
Our data demonstrated that transactivation of the PTHrP P2 promoter in HTLV-1-infected and ATLL cells occurs by activation of NF-κB in a Tax-independent manner.

3.3 MATERIALS AND METHODS

**Sequence Analysis:** Human, mouse, rat, and dog upstream PTHrP P2 promoter sequences (GenBank accession: NM 002820, NM 008970, NM 012636, and NM 001003303, respectively) were analyzed for potential NF-κB binding sites using MatInspector (Genomatix Software GmbH, Munchen, Germany), with ‘weight matrices’ as the search parameter.

**Animals and inoculation.** Immunodeficient SCID-NOD (NOD CB17-PRKDC-SCID/J) mice (Jackson Lab, Bar Harbor, ME) were maintained under specific pathogen-free conditions in the animal facility of the College of Veterinary Medicine at The Ohio State University. Male mice aged 5 weeks were used as recipients and injected intraperitonially with 4x10⁷ RV-ATL cells or 3x10⁷ Met-1 cells suspended in RPMI 1640 medium. The source of the RV-ATL and Met-1 cells (a kind gift of Dr. Feuer and Dr. Waldman respectively) was previously described(26,27). RV-ATL cells were harvested by peritoneal lavage 28 days post-inoculation. Met-1 cells were harvested from peritoneal tumors approximately 60 days post-inoculation.
**Cell lines.** MT-2 and SLB-1 are in-vitro transformed HTLV-1-positive cell lines that were obtained by co-cultivating lymphocytes from healthy donors with leukemic cells from ATLL patients\(^{(28)}\). RV-ATL and Met-1 cells are leukemic cells derived from ATLL patients. HT-1RV cells were obtained by superinfecting RV-ATL cells with HTLV-1\(^{(29)}\). IMM-1 cells are IL-2-dependent immortalized cells obtained by *in vitro* co-cultivation of peripheral blood mononuclear cells from a healthy human donor with lethally-irradiated SLB-1 cells. MT-2 and SLB-1 cells were cultured in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS) and the HT-1RV and RV-ATL cells in RPMI 1640 with 20% heat-inactivated (56°C, 30 min) FBS, l-glutamine (2 mM), penicillin (50 U/ml) and streptomycin (50 µg/ml) (Invitrogen, Carlsbad, CA) at 37°C and 5% CO\(_2\). NIH 3T3 cells were grown in DMEM supplemented with 10% FBS and 10 mM L-glutamine.

**Long-term Immortalization Assay.** Human peripheral blood mononuclear cells (PBMCs) were isolated from the blood of healthy donors by centrifugation over Ficoll-Paque (Amersham, Piscataway, NJ) and cultured in RPMI 1640 supplemented with 20% FBS, 10 U/mL IL-2 (Boehringer Mannheim, Mannheim, Germany), 2 mM glutamine, and antibiotics. Irradiated SLB-1 cells (10\(^6\)) were co-cultivated with 2x10\(^6\) freshly isolated PBMCs with 10 U/mL IL-2 in 24-well culture plates. The presence of HTLV-1 expression was confirmed by detection of p19 Gag protein in the culture supernatant at weekly intervals using a commercially-available ELISA kit (Zeptometrix, Buffalo, NY). Cells from a donor that continued to proliferate after 30
weeks of co-culture in the presence of exogenous interleukin-2 (IL-2) were identified as to be HTLV-1-immortalized cells and were named IMM-1 cells.

**EMSAs and Supershifts.** Nuclear extracts were prepared from RV-ATL, MET-1, HT1-RV, MT-2, SLB-1, IMM-1, and Jurkat cells using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Pierce). For NF-κB binding activity on the PTHrP P2 promoter, 1-5 µl of each nuclear extract (7.5 µg of protein) were incubated in 18 µl total reaction volume, containing 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, 1 µg of poly(dI-dC).poly(dI-dC) (Amersham Biosciences, Piscataway, NJ), and 0.4 µg/µl bovine serum albumin for 15 minutes at room temperature. The reaction mixture was then incubated with a Cy5 5’-end labeled double-stranded PTHrP P2 promoter wild-type oligonucleotide (sense strand: 5’- TCATTCCCGCTCGGGGCTCCCCCTCCACTCGCTCG-3’; κB site is underlined) alone or with 25-fold excess of unlabeled wild-type or mutant P2 oligonucleotides for 15 minutes at room temperature. Samples were analyzed by electrophoresis using 4% non-denaturing polyacrylamide gels with 1X TGE buffer (25 mM Tris, 189 mM glycine, and 1 mM EDTA) containing 5% glycerol. The gels were scanned with a Typhoon 9410 Variable Mode Imager (Amersham Biosciences) to detect the Cy5 fluorescence. Consensus NF-κB binding activity was measured using 5 µg of the nuclear extracts with 2x10^4 cpm of a ^32^P-labeled oligonucleotide probe containing a κB site from the class I MHC promoter (5’- CAGGGCTGGGATTCCTCCATCTCCAC AGTTTCACTTC-3’; κB site is
underlined), as described previously\(^\text{(30)}\). For supershift experiments, 4 \(\mu l\) each of p50 (H-119; sc-7178), p50 NLS (sc-114), P65 (sc-109), c-Rel (sc-71), Bcl-3 (C-14; sc-185) antibodies (Santa Cruz) was incubated with 7.5 \(\mu g\) nuclear extract for 10 minutes at room temperature prior to addition of the buffers and oligonucleotides.

**Chromatin immunoprecipitation (ChIP)-PCR and quantitative PCR.** ChIP was performed with antibodies against Bcl-3 (C-14), c-Rel (C) and p50 (H-119) using ChIP assay kit (Upstate, Charlottesville, VA). The specific sequence from immunoprecipitated and input DNA was detected by PCR using primers for PTHrP P2 promoter region: (forward, 5'-GCCACCTCTTTGCGACTAGCT-3') and (reverse, 5'-GGTTGGAGGCGAGTTGAAAAC-3'). The annealing temperature was 58\(^\circ\)C and the amplicon size was 91-bp. Quantitative PCR monitored with SYBRGreen was performed using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) as described previously\(^\text{(31)}\). Experimental ChIP-PCR values were normalized against values obtained by a standard curve constructed by input DNA (5% to 0.008%, 5-fold dilution, \(R^2 >0.99\) with the same primer set. Quantitation of transcription factor binding was expressed as enrichment ratio of antibody over IgG control, and each error bar represents standard deviation calculated from triplicates.

**Plasmids and site-directed mutagenesis.** A human PTHrP P2-luciferase reporter gene construct was derived by cloning the human P2 promoter -1030 (Sma I) to -611 (Sau3A) fragment into pGL-2 basic vector (Promega, Madison, WI). For site directed
mutagenesis, a Sac I-Apa I restriction fragment from the human PTHrP P2 promoter was sub-cloned into the Sac I-Apa I sites of pCR2.1TOPO plasmid (Invitrogen). The NF-κB site within the PTHrP P2 promoter was mutated by PCR-amplifying the entire PTHrP-P2/pCR2.1TOPO plasmid using the Expand Long Template PCR System (Roche) and sense (5’-CTCACATCCACTCGCTCG-3’) and anti-sense (5’-CACAGAGCCGGAATGAG-3’) oligonucleotides that contained the desired mutations (mutated nucleotides are underlined), as described previously(32). A PCR product of the appropriate size (~3.9 kb) was obtained. This PCR product was blunted-ended with T4 DNA Polymerase (New England Biolabs), kinased with T4 polynucleotide kinase (New England Biolabs), circularized with T4 DNA Ligase (New England Biolabs) and transformed into DH5α cells. Once clones containing the desired mutations were obtained and confirmed by DNA sequencing, the mutated P2 promoter was sub-cloned back into pGL2/PTHrP-P2/Luc plasmid.

**Western Blotting:** Approximately 40 µg of the nuclear and cytoplasmic lysates were separated on a 12% tris-glycine SDS-PAGE gel. Protein was transferred to a nylon membrane and then probed with primary antibodies specific for Tax (168A51-42 (Tab 176; NIH AIDS Research & Reference Reagent Program), NF-κB p50 (Epitomics, Burlingame, CA), p65, c-Rel, IκB-α, Bcl-3 (same as used in supershifts), and β-Actin (Sigma, St. Louis, MO) followed by incubation with goat anti-mouse or goat anti-rabbit (Promega, Madison, WI) horseradish peroxidase-conjugated secondary
antibodies. The signal was detected by chemiluminescence using Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Sciences, Boston, MA).

**Transfections:** To investigate the effect of NF-κB on PTHrP transcriptional regulation, NIH3T3 cells at 60% confluence were co-transfected with 1 µg of either wild-type pGL2/PTHrP-P2/Luc, mutant pGL2/PTHrP-P2/Luc or pGL2 constructs in the presence or absence of expression vectors for NF-κB p50 (50 to 500 ng), p65 (500 ng), c-Rel (50 to 500 ng) or Bcl-3 (10 to 500 ng) using Lipofectamine Plus reagent (Invitrogen, Carlsbad, Ca) and harvested after 48 h of transfection. pcDNA-3.1 was used as a “filler” plasmid so that the total amount of DNA would be the same in all transfection groups. The plasmid pβgal-Control Vector (250 ng) was included in each transfection and served as an internal control to correct for transfection efficiency. Luciferase activity was measured with the Luciferase Assay System (Promega) using 40 µl of lysate. Simultaneously, β-galactosidase activity was measured with the Luminescent β-Galactosidase Detection Kit II (BD Biosciences).

**RT-PCR, Bay 11-7082 Treatment and Real Time RT-PCR:** Total RNA was extracted using TRIZOL® Reagent (Invitrogen, CA). Approximately 2.5 µg of RNA was reverse transcribed with Superscript™ Reverse Transcriptase (Invitrogen, CA). The cDNA was amplified with specific oligonucleotide primers for Bcl-3, and β2-microglobulin using Platinum Taq DNA polymerase (Invitrogen), as described previously[33,34]. The PCR products were analyzed by electrophoresis on a 1% agarose
gel. For Bay 11-7082 treatment, 2x10^6 cells were treated with Bay 11-7082 (Alexis Biochemical Corporation, CA) or vehicle control (DMSO). To measure the total PTHrP and P2 promoter-initiated transcripts, 1 µg RNA was reverse-transcribed and submitted for real-time RT-PCR analysis using TaqMan® Gene Expression assays (4331182 and 364171-B4 respectively, Applied Biosystems, CA). β2M (4333766, Applied Biosystems) was used as a reference gene.

**Statistical analysis:** Pooled-variance t-tests were used to analyze the data from transfection assays. ANOVA with Dunnett’s tests were used to analyze the data from titration with p50, c-Rel, Bcl-3, and Bay 11-7082 treatment and vehicle control groups. Means and standard deviations are plotted in the figures. The mean differences and 95% confidence intervals are available upon request. A p value of <0.05 was considered significant.

### 3.4 RESULTS

**Identification of NF-κB Sites in the PTHrP P2 Promoter:** Following sequence analysis of the human PTHrP gene (described in the methods section), we identified two putative NF-κB binding sequences in the 5’-regulatory region beginning at positions -1558 to -1548 (GGAAATTC CC) and -152 to -142 (GGGGCTCCCC) nucleotides from the transcription start site for exon 3 (Figure 3.1). These sites represented excellent matches to the 5’-GGGRNNYYCC-3’ consensus NF-κB
sequence\(^{(35)}\). Putative κB sites almost identical to the human PTHrP κB sites were also present in the mouse and the rat PTHrP promoter regions (Fig 3.1). The κB binding sequence (-1513 to -1503) was present in dog but the sequence proximal to the transcription start site (-152 to -142) was a partial consensus. In this study, we focused on the κB1 sequence (-152 to -142) due to its proximity to the transcription start site for exon 3 in the human PTHrP gene.

**Tax and IκB-α expression in HTLV-1-infected T-cells and ATLL cells.** In resting cells, NF-κB is retained in the cytoplasm due to binding to specific NF-κB inhibitors of the IκB family. Following activation of cells by various stimuli, signal transduction cascades lead to the degradation of IκB-α and translocation of NF-κB into the nucleus. Since Tax is known to activate the NF-κB pathway, we measured the levels of Tax expression in various cell lines (Figure 3.2). Three of the HTLV-1-infected cell lines (MT-2, SLB-1 and HT-1RV) have very high levels of Tax expression. In addition to the 40-kDa Tax band a 69-kDa band, which is a fusion between the envelope and the Tax-coding sequence, is seen in MT-2 cells as described previously\(^{(36)}\). IMM-1 cells have a lower Tax protein expression compared to the other HTLV-1-infected cell lines. There was no detectable Tax protein expression in RV-ATL and MET-1 cells. A canine prostate carcinoma cell line, Ace-1, was used as a negative control for Tax. Also, in agreement with prior studies (Mori et al. 1999), the protein level of IκB-α was significantly lower in three of the four HTLV-1-infected cell lines (SLB-1, MT-2, and HT-1RV) compared to ATLL cells (MET-1 and
RV-ATL) (Figure 3.2). Imm-1 cells have a higher level of IκB-α protein compared to the other HTLV-1 infected cell lines, indicating a lower IκB-α turnover in these cells and likely lower NF-κB activation. These results suggested a direct relationship between Tax, IκB-α expression and NF-κB activity in T-cells.

**Constitutive NF-κB binding activity in HTLV-1-infected T-cell lines and ATLL cells.** To measure the NF-κB binding activity in HTLV-1-infected and ATLL cells, nuclear extracts from these cells were subjected to electrophoretic mobility shift assays (EMSA) with a $^{32}$P-labeled, double-stranded oligonucleotide probe containing a κB site from the class I MHC promoter. No NF-κB-specific protein DNA complexes were detected in HTLV-1-negative Jurkat T-cells. In contrast, enhanced NF-κB binding activity was detected in the nuclear extracts from HTLV-1-infected T-cell lines and ATLL cells (Figure 3.3A, lanes 3-8). The NF-κB binding activity in MT-2 and SLB-1 extracts consisted of a single complex (C1), while there were two complexes in MET-1, RV-ATL, HT-1RV and IMM-1 extracts (C2 & C3). The complexes in MT-2 cells extracts were composed predominantly of p50 and c-Rel as antibodies against p50 and c-Rel produced a shifted complex (Figure 3.3B, lanes 3, 4 and 6). The lower complex (C2) in MET-1, RV-ATL, HT-1RV, and IMM-1 extracts consisted of p50/p50 (Fig. 3B, lanes 3-4). The upper complex (C3) in RV-ATL and IMM-1 extracts contained p50 and p65 (Figure 3.3B lanes 3, 4, 5) while the HT-1RV extracts contained p50, p65 and c-Rel (Figure 3.3B, lanes 3-6).
**NF-κB binds to the sequences in the 5' regulatory region of PTHrP:** To determine if the sequences in the PTHrP P2 promoter region were authentic NF-κB binding sites, a Cy5-labeled, double-stranded oligonucleotide probe spanning the putative NF-κB binding site was tested by EMSA for binding of NF-κB proteins (Figure 3.4). A single complex (C1) formed in MT-2 and SLB-1 extracts, whereas two complexes (C2 & C3) formed in MET-1, RV-ATL, HT-1RV and IMM-1 extracts (Figure 3.4A). The complexes in MT-2 cells were composed of p50 and c-Rel since antibodies specific to these proteins produced a supershift (Fig. 4B, lanes 5, 6 and 8). The lower complex (C2) in RV-ATL, HT-1RV, MET-1 (data not shown), and IMM-1 extracts contained only p50. The upper complex (C3) consisted predominantly of p50 and c-Rel in HT-1RV extracts, while the complexes in RV-ATL, MET-1 and IMM-1 extracts did not supershift with antibodies to p50, p52 (not shown), p65, c-Rel, RelB (not shown) or Bcl-3. All of the above complexes formed specifically on the NF-κB binding site, since competition with an excess of unlabeled wild-type probe eliminated the complex (Figure 3.4B, lanes 3), but an excess of an unlabeled probe containing a mutated NF-κB site did not eliminate the complex (Fig 3.4B, lanes 4). Additional complexes observed are non-specific since they were out-competed by the mutated competitor.

**Chromatin immunoprecipitation (ChIP) demonstrated binding of NF-κB p50 and c-Rel to the PTHrP P2 promoter in vivo.** To determine if NF-κB transcription factors bind to the PTHrP P2 promoter in vivo, we performed a ChIP assay with
antibodies against p50, c-Rel, and Bcl-3. IgG was used as a negative control. As shown in Figure 3.5a, the PTHrP P2 promoter was occupied by NF-κB p50 and c-Rel in MT-2 cells as measured by ChIP with real-time PCR quantification. As an additional measure, gel electrophoresis of the PCR product after a limited number of cycles gave an independent visual confirmation of the binding activity on the PTHrP P2 promoter (Figure 3.5b).

Transactivation of the PTHrP κB site by the NF-κB family: To determine which NF-κB subunits transactivated the PTHrP κB site, expression plasmids for four κB proteins, p50, p65, c-Rel and Bcl-3 were co-transfected in NIH3T3 cells with either wild-type pGL2/PTHrP-P2/Luc, mutant pGL2/PTHrP-P2/Luc or pGL2 reporter plasmids. As shown in Fig. 6A, expression of p65, c-Rel, Bcl-3 or Tax by themselves did not increase the expression of luciferase activity. Interestingly, p50 alone or in combination with c-Rel or Bcl-3 strongly transactivated the PTHrP P2 promoter. Mutation of the κB binding sequence significantly reduced luciferase activity compared to the wild-type, showing that the transactivation was occurring through the κB site. The effect of p50 and c-Rel were directly proportional to their concentration (Figure 3.6B & C). Interestingly, the cooperative effect of Bcl-3 with p50 was inversely related to Bcl-3 concentration (Figure 3.6D&E). While low levels of Bcl-3 co-operated with p50 in transactivating PTHrP, higher levels of Bcl-3 nullified the effect.
HTLV-1-infected T-cells and ATLL cells expressed Bcl-3 and other NF-κB family members. p50 is known to transactivate genes in association with Bcl-3. Following our observations that p50 alone and p50/Bcl-3 up-regulated PTHrP we measured the levels of Bcl-3 expression in HTLV-1-infected and ATLL cells. As shown in Figure 3.7A, Tax-expressing cells have higher levels of Bcl-3 mRNA expression compared to the ATLL cells. To determine the sub-cellular localization of Bcl-3 and other NF-κB members in these cells, western blotting was performed on cytoplasmic and nuclear extracts (Figure 3.7B). Cytoplasmic and nuclear fractions of MT-2, SLB-1, HT-1RV and IMM-1 cells had high levels of Bcl-3 protein. In contrast, lower levels of Bcl-3 protein were present in MET-1 and RV-ATL cells, but it was predominantly localized in the nucleus. This was in agreement with the RT-PCR data. The presence of other NF-κB family members was confirmed by western blot analysis of the cytoplasmic and nuclear fractions, with β-actin serving as a loading control. p50 and c-Rel were present in similar amounts in all the cells, while p65 was expressed in the highest levels in SLB-1, MT-2, MET-1 and RV-ATL cells.

Inhibition of NF-κB by Bay 11-7082 decreased PTHrP mRNA expression in HTLV-1 infected T-cells. Since NF-κB is constitutively activated in HTLV-1 infected T-cells, we wanted to determine the effect of inhibition of NF-κB on PTHrP expression. For this purpose we used Bay 11-7082, a specific IκB phosphorylation inhibitor, which is known to reduce NF-κB activity. As shown in Figure 3.8a and c, inhibition of NF-κB activity by Bay 11-7082 significantly decreased PTHrP mRNA
expression in MT-2 and SLB-1 cell lines at 3 and 6 hrs respectively. Analysis of the promoter-initiated transcripts revealed that Bay 11-7082 reduced P2 promoter-initiated transcripts as expected (Figure 3.8b and d). Bay 11-7082 treatment reduced PTHrP starting at 1 hr following treatment (data not shown). Reduction of PTHrP expression occurred at higher concentrations (20µM) and at a later time point (24hrs) in RV-ATL cells (data not presented). Inhibition of NF-κB activity with PS-341, a potent proteasome inhibitor, inhibited PTHrP P2-promoter initiated transcripts and the total PTHrP expression in RV-ATL cells (data not shown).

3.5 DISCUSSION

PTHrP is expressed in a wide variety of tissues and is known to be dysregulated in several cancers. Increasing evidence suggests a role for PTHrP in proliferation and apoptosis besides its central role in the pathogenesis of HHM(37). However, transcriptional regulation of this complex gene is poorly understood. In this study, we tested the hypothesis that the PTHrP gene is a direct target for NF-κB and report three major observations: (1) Sequence, EMSA, and competition analyses of the PTHrP P2 promoter sequences identified NF-κB binding sequences in the human PTHrP P2 promoter in vitro that was further confirmed as an authentic NF-κB binding site in vivo by chromatin immunoprecipitation assay; (2) NF-κB activated the human PTHrP P2 promoter in a subunit-specific manner as determined by an NF-κB over-expressing
model in 3T3 cells; (3) Inhibition of NF-κB by Bay 11-7082 in HTLV-1 infected T-cell decreased P2 promoter-initiated transcripts.

The GC-rich PTHrP P2 promoter has been shown to be active in a variety of cells, including HTLV-1-infected and ATLL cells\(^{38}\). We identified two putative NF-κB-binding sequences in the human PTHrP P2 promoter that are located 1558 (κB2) and 152 (κB1) nucleotides upstream from the transcription initiation start site. Vasavada et al have shown that the Sma I/Sau3A fragment of the P2 promoter had significant activity in renal carcinoma cells\(^{39}\). We identified and characterized the κB binding site (κB 1) within this fragment of the promoter. In addition, both the κB1 and κB2 sites are also present in the P2 promoter regions of mice and rats, although their order is reversed compared to humans. While the κB1 sequence appears to have two point mutations in the dog, the κB2 sequence is present and the location of both these elements is the same as in humans. The presence of these transcription elements across different species is consistent with the concept that NF-κB is important in modulating the expression of PTHrP.

Although tightly regulated in normal physiologic cellular responses, NF-κB is constitutively activated in various malignancies such as solid tumors, lymphomas and leukemias, including ATLL\(^{40}\). HTLV-1 Tax, in addition to mediating transcription from the viral promoter, activates numerous cellular genes by interacting with NF-κB, CREB/ATF, SRF, and NF-AT pathways. Tax-mediated NF-κB activation is crucial for transformation of T-cells by HTLV-1\(^{41}\). However, the presence of constitutively activated NF-κB pathway in ATLL cells, despite the lack of detectable Tax
expression, suggests that Tax may be needed to initiate but not to maintain NF-κB activation. Although several studies have shown that Tax transactivates PTHrP, particularly the P3 promoter\(^\text{(42-44)}\), it is not clear whether Tax is required for PTHrP expression because ATLL cells that do not express detectable Tax have high levels of PTHrP. In this study, we have compared a variety of cell lines with different levels of Tax expression to understand the role of Tax and NF-κB in the regulation of PTHrP.

In vitro transformed MT-2 and SLB-1 cells express high tax and low PTHrP. ATLL cells (RV-ATL and MET-1) express PTHrP in the absence of Tax. HT-1RV cells, obtained by superinfecting RV-ATL cells with HTLV-1, express very high levels of Tax and low PTHrP\(^\text{(45)}\). Our newly developed \textit{in vitro} immortalized cells (IMM-1) express low levels of Tax and moderate levels of PTHrP (data not shown). Our data are in agreement with previous studies where Tax-expressing T-cells have a very low level of \(\text{IκB}\alpha\) compared to ATLL cells (RV-ATL and MET-1) indicating that Tax significantly accelerated loss of I-κBα.

EMSA and competition analyses of the PTHrP \(\kappa\text{B1}\) sequence showed specific complexes formed between NF-κB transcription factors and P2 oligonucleotides in extracts from ATLL and the HTLV-1 cell lines tested. It is a well known feature of NF-κB signaling that different NF-κB dimers can preferentially bind functionally distinct DNA binding sites. Tax has been shown to activate the c-Rel gene and induce predominantly c-Rel-containing complexes\(^\text{(46)}\). Consistent with these observations, the complex formed in extracts from SLB-1 and MT-2 cells, which express high levels of Tax, contained p50 and c-Rel as the major DNA binding components. This finding
was confirmed by the presence of a similar complex with a known κB containing probe (from MHC class I gene promoter) in these cells. Interestingly, there were two distinct complexes in the ATL, HT-1RV and surprisingly even in IMM-1 cells. The lower complexes (C1) in extracts from ATL, HT-1RV and IMM-1 cells were composed of p50 alone. The upper complex (C2) in extracts from in HT-1RV cells was composed of p50/c-Rel, confirming our previous observation that Tax induces the formation of p50/c-Rel-containing complexes. On the other hand, the upper complex in extracts from ATL and IMM-1 cells is intriguing since it did not supershift with antibodies against p50, p65, c-Rel, Bcl-3, RelB (data not shown) or p52 (data not shown). Our attempts to identify these proteins by proteomics and mass spectrometry have been unsuccessful; therefore, the proteins in this complex remain unidentified. Chromatin immunoprecipitation assays confirmed binding of NF-κB p50 and c-Rel on the PTHrP P2 promoter \(\textit{in vivo}\). Finally, the differences in the complexes formed in these cells potentially explain the differences in the level of PTHrP P2 promoter transactivation in these cells.

Our transfection studies showed that the human PTHrP P2 promoter can be activated in a subunit-dependent manner by p50/p50 alone, or by a combination of p50/c-Rel or p50/Bcl-3, but not by p65 or p50/p65. Most of the transcriptional effect was attributable to an intact NF-κB site as demonstrated by a significant decrease in luciferase activity in 3T3 cells transfected with constructs bearing a disrupted κB sequence. We were surprised by the activation of PTHrP by p50/50 alone since p50 does not have a transactivation domain. There have been conflicting reports regarding
the role of p50/p50 in transcription. While some studies support the ability of p50 to transactivate, particularly in the presence of Bcl-3, others have shown that p50 homodimers act as repressors by binding κB sites that would normally be activated by p50/p65 heterodimers. Mori et al have shown that leukemic cells from ATL patients have abundant p50/p50 homodimers; however, p50 was not able to activate transcription from a p50/p65-inducible construct containing five repeats of a κB motif from the IL-2Rα gene. Interestingly, p50 homodimers are capable of binding all κB binding motifs with similar efficiency but can probably transactivate only a subset of these such as those found in the Bcl-2 and H-2K genes\textsuperscript{(47-49)}. It is possible that κB binding motifs like those found in Bcl-2, H-2K and PTHrP promoters may provide a sequence with which p50 forms an activating rather than a repressing complex. Evidence in support of this hypothesis was provided recently by Schreiber et al showing that p50 homodimers can interact with the RNA polymerase II at the promoters for genes such as CSF2, ICAM1, and TNF and likely maintain basal level of target gene activity in unstimulated cells\textsuperscript{(50)}. Alternatively, transcriptional activity of p50 may be cell type-dependent or dependent on undefined cofactors.

Although p50 and p52 have no defined transactivating domains, the association with Bcl-3 can lead to the conversion of these homodimers to positive regulators of gene expression. In addition, it has been shown that the formation of Bcl-3-p50 homodimer-DNA complexes depends on Bcl-3 concentration and phosphorylation\textsuperscript{(51)}. Furthermore, it has been shown that Bcl-3 can increase the quantity of p50 homodimers that translocate to the nucleus\textsuperscript{(52)}. In light of the observations that p50
activation can be induced by Bcl-3, we tested the possible association of Bcl-3 with p50 in transactivating PTHrP. First, we studied the expression of Bcl-3 in HTLV-1-infected and ATLL cells. Interestingly, Tax-expressing cells have higher levels of Bcl-3 compared to ATLL cells. Our data from transfection experiments have shown that a higher p50/Bcl-3 ratio resulted in activation of the promoter, while a lower p50/Bcl-3 ratio (higher concentrations of Bcl-3) resulted in a dramatic reduction of transactivation. This correlated well with our data where ATLL cells had lower Bcl-3 levels expressed high levels of PTHrP and Tax-expressing cells that had higher Bcl-3 levels expressed low levels of PTHrP. These data suggest that Bcl-3 may mediate its effect on PTHrP indirectly or at higher concentrations Bcl-3 dissociates from complexes with p50. Based on the results from Bcl-3 expression analysis and transfection assays, it is likely that the low level of Bcl-3 expression in ATLL cells contributed to the transactivation of the promoter while the high levels of Bcl-3 in Tax-expressing cells inhibited the promoter. This may help explain the differences in the transactivation of the PTHrP P2 promoter in ATL and Tax-expressing T-cells.

Bay 11-7082 has been shown to be a specific NF-κB inhibitor by inhibiting the phosphorylation of the inhibitor IκB\textsuperscript{(53)}. Treatment of HTLV-1 infected T-cells not only reduced the P2 promoter-initiated transcripts but also total PTHrP expression providing additional support to our hypothesis that PTHrP is a direct target of NF-κB signaling. PS-341, a potent and selective proteasome inhibitor reduced NF-κB activity and total PTHrP expression and the P2 promoter-initiated transcripts in ATLL cells.
(data not shown). These results support for attempting to reduce NF-κB levels in ATLL patients.

In conclusion, our results support a direct role for NF-κB in the regulation of PTHrP. Since NF-κB is known to often mediate its effects through other transcription factors including AP-1, SP-1, and the Ets family of proteins and because binding sites for these factors and others exist within the P2 promoter, it will be important to establish the functional interaction between these factors and NF-κB in the regulation of PTHrP.
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Figure 3.1: Schematic representation of human, mouse, rat and dog PTHrP P2 promoter regions. The diagram illustrates the highly conserved nature of the NF-κB sequences in the PTHrP P2 promoter of humans, mice, rats, and dogs. The NF-κB binding sequence in the human promoter studied in this report was found at -152 to -142 nucleotides upstream from transcriptional start site (indicated by arrows) in exon 3.
Figure 3.2: HTLV-1 Tax expression was inversely proportional to IκB-α. Western blot analysis was performed on cytoplasmic (C) and nuclear (N) lysates from HTLV-1-infected and ATLL cells with antibodies against Tax and IκB-α proteins. Ace-1, a canine prostate carcinoma cell line was used as a negative control. β-actin was used as loading control.
Figure 3.3: NF-κB binding activity on a consensus κB binding site in various cell lines. a. Approximately 5 µg of nuclear lysates from the cells were incubated with a [\(^{32}\)P]-radiolabeled oligonucleotide corresponding to MHC class 1 gene κB sequence and analyzed by EMSA. The complexes are indicated as C1, C2 and C3. b. In super-shift assays, antibodies specific for each NF-kB subunit (indicated above the lane) were incubated with the nuclear extracts from MT-2, RV-ATL, HT-1RV and IMM-1 cells before addition of the probe.
Figure 3.4: Nuclear NF-κB proteins specifically bound to an oligonucleotide containing the NF-κB binding sequences from the human PTHrP P2 promoter.

a. A Cy5 5’-end labeled double-stranded PTHrP P2 promoter wild-type oligonucleotide was analyzed by EMSA using 7.5 µg of protein from nuclear extracts of various cell lines. The complex formation is indicated as C1, C2, C3 and the specificity of binding was evaluated using a mutant -κB binding site as described in materials and methods. The additional bands in the gel were not characterized and considered non-specific (NS). b. Antibody-mediated shift interference or supershifts were performed using 7-15 µg of protein from the nuclear extracts with specific NF-κB antibodies as indicated.
Figure 3.5: NF-κB p50 and c-Rel bound to the PTHrP P2 promoter in vivo: Chromatin immunoprecipitation was performed in MT-2 cells with antibodies to p50, c-Rel, Bcl-3 and IgG. a. Real-time PCR quantification of transcription factor binding was expressed as enrichment ratio of antibody over IgG control, and each error bar represents standard deviation calculated from triplicates. b. PCR amplification of a 91-bp product from the PTHrP P2 promoter genomic DNA is shown on a 2% agarose gel.
Figure 3.6: PTHrP P2 promoter can be transactivated by NF-κB in a sub-unit dependent manner: Relative luciferase activity in NIH3T3 cells transfected with: a. 1 μg of either wild-type pGL2/PTHrP-P2/Luc, mutant pGL2/PTHrP-P2/Luc or pGL2 constructs in the presence or absence of expression vectors for NF-κB p50, p65, c-Rel, Bcl-3, p50/p65, p50/c-Rel, or p50/Bcl-3 or Tax. b. 1 μg wild-type pGL2/PTHrP-P2/Luc with or without 0.5 μg of NF-κB c-Rel expression vector and 0.1 to 0.5 μg of p50 expression vector. c. 1 μg wild-type pGL2/PTHrP-P2/Luc with or without 0.5 μg of NF-κB p50 expression vector and 0.05 to 0.25 μg of c-Rel expression vector. d. 1 μg wild-type pGL2/PTHrP-P2/Luc with or without 0.2 to 0.5 μg of NF-κB p50 expression vector and 0.25 to 0.5 μg of Bcl-3 expression vector. e. 1 μg wild-type pGL2/PTHrP-P2/Luc with or without 0.05 to 0.5 μg of NF-κB Bcl-3 expression vector and 0.5 μg of p50 expression vector. The quantity of the expression plasmids is indicated in (μg). Bars represent the mean ± SD of 3 independent samples. (***) indicates significant differences between various groups compared to PTHrP P2 Luc alone (p<0.05). (*) indicates significant differences between mutant and wild-type (p<0.05).
Figure 3.6: PTHrP P2 promoter can be transactivated by NF-κB in a sub-unit dependent manner.
Figure 3.7: Bcl-3 expression was high in HTLV-1-infected T-cells compared to ATLL cells: a. RT-PCR was performed on total RNA isolated from various cells as indicated on the top row using primers specific for Bcl-3. β2 microglobulin was used as a loading control. b. Western blot analysis was performed on the cytoplasmic and nuclear lysates of various cells and probed with antibodies specific for Bcl-3, p50, p65, and c-Rel. β-actin was used as protein loading control.
Figure 3.8: Inhibition of the NF-κB activity in HTLV-1-infected T-cells by Bay 11-7082 decreased PTHrP mRNA expression. Total RNA was extracted from MT-2 cells treated with 5, 10, and 20µM for 3 hrs and SLB-1 cells treated with 7.5µM Bay 11-7082 or vehicle alone for 6hrs. Quantitative real time RT-PCR was performed using primers specific for PTHrP all transcripts and PTHrP P2 promoter-initiated transcripts using TaqMan® Gene Expression Assays. In MT-2 cells, Bay 11-7082 decreased a. total PTHrP b. and the P2 promoter-initiated transcripts in a dose dependent manner. In SLB-1 cells 11-7082 significantly decreased c. total PTHrP and d. the P2 promoter-initiated transcripts at 6hrs of treatment. The data were normalized to β2-microglobulin (β2-M). Bars represent the mean ± standard deviation of 3 independent samples (* P<0.05).
CHAPTER 4

NOD/SCID MOUSE MODEL OF CANINE T-CELL LYMPHOMA WITH HUMORAL HYPERCALCEMIA OF MALIGNANCY: CYTOKINE GENE EXPRESSION PROFILING AND IN VIVO BIOLUMINESCENT IMAGING

4.1 ABSTRACT

Lymphoma is a malignant neoplasm arising from B or T lymphocytes. In dogs, one-third of lymphomas are highly aggressive multicentric T-cell lymphomas that are often associated with humoral hypercalcemia of malignancy (HHM). There are no cell lines or animal models to investigate the pathogenesis of T-cell lymphoma and HHM in dogs. We developed the first xenograft model by injecting lymphoma cells from an Irish Wolfhound intraperitoneally into NOD/SCID mice. The mice developed multicentric lymphoma along with HHM and increased parathyroid hormone-related protein (PTHrP) as occurs in dogs with T-cell lymphoma. Using cytokine cDNA arrays we identified genes that have potential implications in the pathogenesis of T-cell lymphoma. Quantitative RT-PCR of T-cell lymphoma samples from hypercalcemic canine patients showed that PTHrP likely plays a central role in the pathogenesis of HHM and that hypercalcemia is the result of a combinatorial effect of different hypercalcemic factors. Finally, we monitored in vivo tumor progression and metastases in the mouse model by transducing the lymphoma cells with a lentiviral vector that encodes a luciferase-yellow fluorescent protein reporter and showed that in
vivo trafficking patterns in this model were similar to that seen in dogs. This unique mouse model will be useful for translational research in lymphoma and for investigating the pathogenesis of T-cell lymphoma and HHM in the dog.

4.2 INTRODUCTION

Non-Hodgkin’s lymphoma (NHL) includes several malignant lymphoproliferative diseases that originate from lymphocytes and represents the fifth most common cause of cancer-related deaths in humans in the United States\(^1\). Lymphoma is the third most common neoplasm in dogs \(^2\) with an estimated annual incidence of approximately 33 per 100,000 dogs \(^3\). In the absence of chemotherapy, survival beyond one month after diagnosis of lymphoma is uncommon \(^4\). The most common anatomic presentation of canine lymphoma is the multicentric form which usually presents as superficial lymphadenopathy \(^5\) with or without hepatosplenomegaly. About 26-38% of the lymphomas in dogs \(^6;7\) and 15% of the lymphomas in humans\(^8\) are T-cell in origin. Humoral hypercalcemia of malignancy (HHM) is a frequent paraneoplastic syndrome seen in approximately 40% of the dogs with T-cell lymphoma\(^9\) and in 15% of humans with NHL\(^10\). In addition, 70% of human patients with adult T-cell leukemia/lymphoma caused by human T-cell lymphotropic virus type-1 (HTLV-1)\(^11\) develop HHM. HHM is a multifactorial syndrome due to the action of multiple factors produced by neoplastic cells affecting bone, kidney and intestine that disrupt normal calcium homeostasis \(^12;13\). The primary mechanism for
hypercalcemia in patients with lymphoma is increased osteoclastic bone resorption induced by humoral mediators\(^{(14;15)}\). Factors produced by lymphoma implicated in the pathogenesis of HHM include parathyroid hormone-related protein (PTHrP), calcitriol, transforming growth factors (TGF), tumor necrosis factors (TNF), interleukin-1 (IL-1), interleukin-6 (IL-6), macrophage inflammatory protein-1 alpha (MIP-1\(\alpha\)), and granulocyte colony stimulating factor (GCSF)\(^{(16-21)}\). Calcitriol, the active product of vitamin D metabolism, enhances gastrointestinal calcium absorption and also mobilizes calcium from bone. Increased calcitriol production was reported in human NHL patients with hypercalcemia and was implicated as a major humoral mediator in the pathogenesis of HHM in lymphoma; however, dysregulated calcitriol production was also observed in normocalcemic patients with NHL\(^{(22;23)}\). Firkin et al reported that NHL patients with hypercalcemia had elevated circulating levels of PTHrP with no increase in the levels of calcitriol\(^{(24)}\).

PTHRP originally was isolated from specific tumors as the primary cause of HHM\(^{(25)}\) and is over-expressed by many different types of neoplasms\(^{(26)}\). Studies over the past several years have shown that PTHrP plays a primary role in HHM\(^{(27)}\) and hypercalcemia in tumor-bearing animals could be corrected using a neutralizing antibody to PTHrP\(^{(28)}\). Amino-terminal peptides of PTHrP have been shown to exert PTH-like actions in bone and kidney by binding to a common receptor for PTH/PTHrP (PTH-1 receptor) resulting in hypercalcemia\(^{(29;30)}\). Our laboratory previously reported that dogs with lymphoma and hypercalcemia have elevated levels of plasma PTHrP, but that these levels were lower than in dogs with carcinomas and
hypercalcemia. Moreover, there was no significant correlation between serum calcium and PTHrP concentrations in dogs with lymphoma and hypercalcemia, suggesting a role for other cytokines in this syndrome \(^{(31)}\). Factors such as TGF\(\alpha\), IL-1, IL-6, and TNF have been shown to enhance the hypercalcemic effects of PTHrP \(^{(32)}\). Furthermore, TGF\(\beta\), TNF\(\alpha\), and IL-1 have been reported to up-regulate PTHrP gene expression in a variety of nonlymphoid cell lines and tissues \(^{(33,34)}\). We hypothesized that PTHrP plays a central role in the pathogenesis of HHM in dogs with T-cell lymphoma and acts synergistically with other cytokines produced by the tumor cells.

Canine lymphoma is a spontaneous disease which has a clinical presentation and biologic behavior that closely resembles the human disease \(^{(35)}\). Furthermore, canine lymphoma is a useful translational model to study the pathogenesis and treatment of lymphoma because dogs share extensive genome homology and a common environment with humans \(^{(36,37)}\). The value of the canine model also depends on the availability of rodent models that can reproduce the disease as it occurs in dogs. Development of animal models that recapitulate the natural history of cancers and their clinical response to therapy is an important prerequisite for rapid bench-to-bedside translation of anticancer therapies \(^{(38)}\). Moreover, the pathogenesis of HHM in dogs with T-cell lymphoma has not been investigated due to the lack of relevant \textit{in vivo} models and little is known about PTHrP expression and its interrelationship with other cytokines. In this study, we report the development and characterization of a NOD/SCID mouse model of canine T-cell lymphoma with HHM that closely resembles the disease as it occurs in dogs and humans.
The study of animal models has been limited by the difficulty of accurately assessing disease burden and response to therapy. Measurement of tumor volume using calipers is limited to tumors that occur at accessible sites \(^{(39)}\). Some of the available *in vivo* models of hematological malignancies do not readily allow for sensitive, real-time detection of tumors, or for serial measurements of tumor progression \(^{(40)}\). For this purpose, we have developed canine lymphoma cells that stably express luciferase and yellow fluorescent protein (YFP), which allows imaging of tumor growth and metastasis *in vivo* in real time. Bioluminescent imaging, a non-invasive imaging technique, can be used to monitor the growth of luciferase-expressing lymphoma cells.

In this study, we demonstrated that NOD/SCID mice injected intraperitoneally with canine lymphoma cells develop multicentric lymphoma and HHM as observed in canine patients. The bioluminescent mouse model recapitulates the multicentric anatomical distribution of lymphoma, confirmed by histopathological analyses, and is consistent with the distribution of tumors in dogs and humans with lymphoma. Cytokine gene expression profiling revealed several genes that may contribute to tumorigenesis and metastasis in lymphoma. Furthermore, analysis of expression of potential hypercalcemic factors in xenograft tumors and tumors from dogs with T-cell lymphoma and HHM using quantitative real-time RT-PCR showed that distinct hypercalcemic factors were produced by these cells.
Therefore, this study not only provides a clinically relevant *in vivo* model for more accurate preclinical evaluation of investigational therapies against lymphoma, but also will permit mechanistic studies on the interrelationships between cytokines and PTHrP in the pathogenesis of HHM.

### 4.3 MATERIALS AND METHODS

**Animals and Lymphoma Cell Inoculations**

Immunodeficient SCID-NOD (NOD CB17-PRKDC-SCID/J) mice (Jackson Lab, Bar Harbor, ME) were maintained under specific pathogen-free conditions in the animal facility of the College of Veterinary Medicine at The Ohio State University, Columbus, OH. Lymphoma cells were obtained from a 4-year-old intact female Irish Wolfhound that was presented to the Veterinary Teaching Hospital, University of Wisconsin-Madison, Madison, WI with generalized lymphadenopathy, hepatosplenomegaly and hypercalcemia (total calcium - 15.4 mg/dL). The lymphoma was CD3 positive and CD79a negative as determined by routine immunohistochemistry and T-cell lymphoma was diagnosed. One of the popliteal lymph nodes from the dog was surgically excised and the cells were passed through a #100 mesh stainless steel tissue sieve (Bellco, Vineland, NJ) after dissecting away the capsule. The cells were washed three times with cold HBSS/5µM EDTA and clumps were removed with 40-µm nylon filter. The RBCs were lysed with ACK lysing buffer (BioWhittaker, Walkersville, MD) for 5 min at room temperature followed by brief
vortexing. The cells were kept frozen (95% fetal bovine serum and 10% DMSO) in liquid nitrogen until use. Male mice (5 weeks old) were used as recipients and were injected intraperitoneally with $1.5 \times 10^7$ cells suspended in RPMI 1640 medium. Controls were inoculated with medium alone. The xenografted mice were sacrificed, the tumors from the spleen and mesenteric lymph nodes were removed, minced, washed three times with RPMI 1640 medium containing 10% fetal bovine serum, resuspended in RPMI 1640 medium and were serially passaged into SCID-NOD mice approximately every 6-8 weeks.

**Patient Samples and Normal Canine Lymph Nodes**

T-cell lymphomas (confirmed by immunohistochemistry, CD3$^+$/CD79a$^-$) were collected from patients presented to the Veterinary Teaching Hospital at The Ohio State University, Columbus, OH. A serum calcium concentration of greater than or equal to the laboratory reference value of 11.6 mg/dL was considered hypercalcemic. The samples are represented as dog numbers with the total calcium (mg/dL) indicated in parenthesis: 1(20.5), 2(14.9), 3(11.6), 4(14.2), 5(11.9), 6(17.9). Two popliteal lymph nodes were collected at necropsy from three control (healthy) experimental female Beagle dogs each and used as controls.

**Histopathology, Immunohistochemistry and Immunofluorescence**

Complete necropsies were performed on all mice. Tissues were fixed in 10% neutral-buffered formalin, embedded in paraffin, cut into 5-µm sections, and stained with
hematoxylin and eosin. Immunohistochemistry was performed on paraffin sections with the following antibodies: rabbit anti-human CD3 (1:100; DAKO, Carpinteria, CA) and biotinylated goat anti-rabbit antibody (1:200; DAKO); mouse anti-human CD79αcy (1:25; DAKO, Carpinteria, CA) and biotinylated horse anti-mouse antibody (1:200; DAKO). Visualization was achieved using diaminobenzidine (DAKO) and hematoxylin counterstain. Immunofluorescence staining was performed on a cytospin preparation of cells from ascites fluid. Briefly, the cells were fixed for 10 min in PBS containing 4% paraformaldehyde. The fixed cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min and blocked with 5% bovine serum albumin in PBS for 1 h at room temperature. The cells were stained for 1 h at room temperature with polyclonal rabbit anti-PTHrP (PTHrP amino acids 34 to 53) (1:200, Ab-2, Oncogene Research Products, Cambridge, MA) followed by incubation for 1 h with goat anti-rabbit IgG conjugated with FITC (1:1000, Molecular Probes, Eugene, OR). Confocal microscopy was performed using a Leica TCS SP2 AOBS Confocal Laser Scanning Microscope (Germany).

**Bone Histomorphometry**

Proximal tibiae were collected and fixed in 10% neutral-buffered formalin for 24 h at 4°C, decalcified in 10% ethylenediaminetetraacetic acid (pH 7.4) at 4°C, dehydrated in graded series of ethanol for 5 days at 4°C, infiltrated in two changes of glycol methacrylate (Polysciences Inc., Warrington, PA) for 10 days at 4°C, and embedded in glycol methacrylate at 4°C. Sections were cut at 5 μm, histochemically stained for
tartrate-resistant acid phosphatase (Sigma Co., St. Louis, MO), and counterstained with hematoxylin. Measurements were performed using Image-Pro Plus version 5.0 (Media Cybernetics, Silver Spring, MD) image-analysis software. Histomorphometry was performed on 12 mice xenografted with canine T-cell lymphoma and compared to 6 age-matched control mice. Osteoclastic bone resorption was measured in trabecular bone and osteoclasts were identified as cells lining trabecular bone that stained positive (red) for tartrate-resistant acid phosphatase. Measurements included total bone area, trabecular bone area and perimeter, osteoclast number/mm trabecular bone, and percent osteoclast perimeter.

**PCR Amplification of Canine Microsatellites**

Genomic DNA was isolated using DNeasy Tissue Kit (Qiagen, Valencia, CA) from control mouse spleen, whole blood from a dog (canine WB), Ace-1 cells (canine prostate cancer cell line), and mesenteric lymph nodes from the xenografted mice. DNA (100 ng) was amplified with primers specific for canine microsatellites FH2365, FH2356 \(^{41,42}\) and C36672 as described \(^{43}\) using PCR Master Mix (Promega, Madison, WI). The products were separated by electrophoresis on a 1.5% agarose gel for visualization.

**Measurement of Plasma Calcium and PTHrP Concentrations**

Calcium and PTHrP plasma concentrations were measured in control and lymphoma-bearing mice. Blood was obtained from the femoral artery at necropsy. Total calcium
concentrations were measured in 10 µL of heparinized plasma with a Vitros DT-60 II clinical chemistry analyzer (Johnson & Johnson, Cornilla, GA). Plasma PTHrP concentrations were measured using a two-site immunoradiometric assay (DiaSorin, Stillwater, MN) specific for the PTHrP N-terminal region (amino acids 1 to 40) and mid-region (amino acids 57 to 80).

**Cytokine Gene Expression Profiling**

Panorama Human Cytokine Gene Arrays (Sigma-Genosys, St. Louis, MO) were used to identify the cytokines involved in the pathogenesis of T-cell lymphoma. Total RNA was isolated using TRIZOL (Invitrogen, Carlsbad, CA). Briefly, $^{32}$P-radiolabeled cDNA probes were prepared from 2.5 µg of pooled total RNA using oligo dT and AMV reverse transcriptase at 42°C, and were purified on a Sephadex G-25 spin column (Sigma-Genosys). The arrays were hybridized overnight at 60°C, washed, and subjected to autoradiography for 12-72 h. The intensity of hybridization signals was quantified using ArrayVision software version 6.0 (Imaging Research Inc., Haverhill, UK). The intensity of each spot was corrected for background levels and was normalized for differences in labeling using the average values of the housekeeping genes ($\beta$2-Microglobulin, $\beta$-Actin, GAPDH, Cyclophilin A, HPRT, HLA-A0201, L19, Transferrin R and $\alpha$-Tubulin). For each gene, the normalized array data was compared as the ratio between lymphoma versus normal canine lymph node. Genes that were up-regulated or down-regulated at least 2.5-fold or more were considered dysregulated.
Quantitative Real-Time RT-PCR

Total RNA was isolated from mouse lymph nodes with canine lymphoma, primary dog lymphomas, and normal canine lymph nodes (NCLN) using TRIZOL (Invitrogen). Total RNA (5 µg) was reverse transcribed using the Superscript II First Strand cDNA synthesis kit (Invitrogen) and the final cDNA was diluted to 200 µL with RNase- and DNase- free water. qRT-PCR was performed with 5 µL of the cDNA in a 25 µl volume in triplicate using ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) with a standard temperature protocol and 2X SYBR Green PCR Master Mix reagent (Applied Biosystems, Foster City, CA). As a control, the mRNA level of GAPDH was determined in the real time PCR assay for each RNA sample and used to normalize the data. Fold changes were calculated by the $2^{-\Delta\DeltaCT}$ method as described by the manufacturer (Applied Biosystems). Canine-specific primers for IL-6, TNFα, TGFβ, GAPDH (44,45) and IL-1α (46) were used as described. Primers for IL-1β, TGFα, TNFβ, PTHrP, RANKL, and MIP-1α were designed by aligning the human sequence with the dog genome at http://genome.ucsc.edu/cgi-bin/hgBlat and are shown in Table 1.

Generation of Lentiviral Vectors and Transduction of Lymphoma Cells

Retroviral transduction was performed using vectors encoding luciferase/yellow fluorescent protein, generated as described previously(47). The xenografted lymph
nodes were minced into 0.25-0.05 mm³ pieces and transduced with 7.2 × 10^6 infectious viral particles by spin-inoculation at 2700 rpm for 1 h at 32°C. Polybrene was added at a final concentration of 8 µg/ml. Following transduction, the tissues were incubated in a cell culture incubator at 37°C for 1 h and washed twice with RPMI 1640 medium before injection into mice.

**In Vivo and Ex Vivo Imaging**

Mice were injected intraperitoneally with 150 µl (40 mg/ml) luciferin (Xenogen, Alameda, CA) dissolved in PBS. Anesthesia was induced using a 3% isoflurane-air mixture and maintained at 1.5% isoflurane. Mice were placed in the light-impermeable imaging chamber of the IVIS imaging system (Xenogen) and the photons emitted from luciferase-expressing cells were quantified and analyzed using LivingImage software version 2.50 (Xenogen). For ex vivo imaging, luciferin was injected into the mice immediately prior to necropsy and tissues were collected after humane euthanasia and imaged.

**Statistical Analysis**

Student’s t-test was performed to compare the plasma calcium, PTHrP, and bone histomorphometric parameters between the control mice and mice bearing canine lymphoma xenografts. T-tests were used to compare the cytokine mRNA expression profiles between NCLN and xenograft lymphomas from the mice. A p value less than 0.05 was considered significant.
4.4 RESULTS

Xenografted Dog Lymphoma Grew in NOD/SCID Mice

Canine lymphoma cells inoculated intraperitoneally into SCID-NOD mice successfully engrafted and produced tumors (Figure 1). The mice (83%; 13 of 16) had diffuse lymphoma in the mesenteric lymph nodes. Moderate to marked splenomegaly was present in 66% (10 of 16) of the mice (Figure 1b). Small, white raised tumors were present in the liver in 66% of the mice (10/16). Tumors also were present in the mandibular lymph nodes in 3 mice (Figure 1c). Moderate amounts of ascites were present in 4/16 mice. The thymus was approximately 6 to 8 times larger than controls in 2 of the 16 mice (Figure 1d). Microscopic evaluation of the mice inoculated with canine lymphoma cells revealed lymphoma in the mesenteric lymph nodes (14 of 16 mice) (Figure 1j), spleen (14 of 16 mice), liver (12 of 16 mice) (Figure 1i), kidney (10 of 16 mice), lung (8 of 16 mice) (Figure 1k) and the thymus (2 of 16 mice). In the mesentery and the spleen solid sheets of large round cells replaced most of the normal tissue. The cells had a large round to oval nucleus with a moderate amount of cytoplasm and a high mitotic index (4 per high power field, 400X).

Lymphomas were T-cell and of Canine Origin

The mesenteric lymph nodes from the xenografted mice demonstrated positive immunoreactivity for CD3 and but not for CD79a confirming the T-cell origin of the lymphomas (Figures 1e-f). Since specific antibodies to detect canine antigens (that do
not cross-react with the mouse) were not available, we used a novel PCR-based technique to amplify canine microsatellites. A specific PCR product was amplified for canine microsatellites FH2365, FH2356 (data not presented) and C36672 from the genomic DNA of the lymphoma xenografts, normal canine tissue and canine prostate cancer cell line (Ace-1), but not from the control mouse spleen (Figure 1g), confirming canine origin of the lymphoma.

**Plasma Calcium Concentration**

Lymphoma-bearing mice had a statistically significant \( P < 0.005 \) increase in total calcium \( (10.9 \pm 0.8 \text{ mg/dl}) \) concentrations compared to control mice \( (9.2 \pm 0.1 \text{ mg/dl}) \) (Figure 2). Total calcium concentrations were as high as 12.4 mg/dl in mice with lymphoma.

**Hypercalcemic Factors in Lymphoma-Bearing Mice**

Plasma PTHrP concentrations were increased in mice with lymphoma \( (3.2 \pm 2.0 \text{ pM/L}) \) compared to the control mice \( (<1.5 \text{ pM/L, Figure 3a}) \). PTHrP was present predominantly and diffusely in the cytoplasm of neoplastic xenograft cells with some nuclear staining as evaluated by immunofluorescence microscopy (Figure 1-l). The xenografted canine lymphomas expressed PTHrP mRNA at significantly higher level (30 fold) than NCLN. In addition to PTHrP, the xenografted cells also expressed moderately increased levels of TNF\( \alpha \) mRNA (3- to 5-fold). There was mild (but not statistically significant) up-regulation of TGF\( \beta \) (2-fold) and RANKL (3-fold) mRNA.
in the xenografted canine lymphoma cells compared to normal canine lymph nodes (Figure 3b). There was a significant down-regulation of IL-6 mRNA (data not shown) in the xenografted lymphoma cells compared to the normal canine lymph nodes. Although not statistically significant, the xenografted cells had a trend towards down-regulation of IL-1β, TNFβ and TGFα mRNA compared to NCLN.

**Bone Histomorphometry**

To examine parameters of osteoclastic bone resorption, bone histomorphometric analysis was performed. There was no significant difference in the trabecular bone volume in the xenografted mice with lymphoma when compared to controls (Figure 4a). The perimeter of trabecular bone lined by osteoclasts and the number of osteoclasts/mm of trabecular bone, were increased approximately 2-fold (Figure 4b and c respectively) in NOD/SCID mice xenografted with canine T-cell lymphoma compared to controls ($p < 0.05$).

**Microarray Analysis of Cytokines**

To allow rapid screening of cytokines potentially involved in the pathogenesis of lymphoma, we compared cytokine gene expression profiles between lymphoma xenografts and normal canine lymph nodes using the Panorama Human Cytokine Array. Of 865 different cytokines and chemokines present, 34 genes were significantly up-regulated and 18 genes down-regulated in the xenografts compared to the normal canine lymph nodes. The list of genes dysregulated in xenografts
compared to the normal lymph nodes is shown in Table 3. The up-regulated genes included those involved in signal transduction, cell surface proteins, integrins, and G-protein coupled receptors. The down-regulated genes included FGF receptor-1, binding proteins and interleukin receptors. Quantitative real-time RT-PCR for integrin α4, integrin β9 (data not presented), and IL-1 receptor like 2 (IL-1RL2) genes validated the microarray findings (Figure 5).

**Canine T-cell Lymphomas Express Distinct Hypercalcemic Factors**

In order to identify the osteoclastogenic factors involved in the pathogenesis of HHM in dogs with T-cell lymphoma, we compared the mRNA levels of various hypercalcemic cytokines using quantitative real-time RT-PCR in canine T-cell lymphoma samples from hypercalcemic dogs (Figure 6). The samples from hypercalcemic T-cell lymphoma dogs expressed very high levels of PTHrP mRNA compared to lymph nodes from normal dogs (69 ± 71 fold). The expression of PTHrP mRNA levels from lymphoma samples ranged from 2- to 194-fold higher than normal lymph nodes. While the average expression of TNFα mRNA was moderately up-regulated (13 ± 23) in lymphomas compared to normal canine lymph node, there was decreased TNFα expression in two lymphoma samples and no TNFα expression in two other lymphoma samples (#6 and 8) compared to NCLN. There was a mild up-regulation of IL-1β mRNA (3.7 ± 8.6) in the lymphoma samples compared to NCLN. There was a mild up-regulation of RANKL mRNA (2.3 ± 1.2) in the lymphoma samples compared to NCLN. There was moderate down-regulation of IL-6 mRNA (-
10 ± 10) and IL-1α mRNA (-6.0 ± 7.6) in the lymphoma samples compared to NCLN. There was no difference in the expression of TNFβ, TGFα and MIP-1α mRNA between the lymphoma samples and NCLN as shown in Figure 6.

**Tumors Were Detected by In Vivo and Ex Vivo Imaging**

Since the canine lymphoma cells could not be maintained in culture, a retroviral transduction system was used for the delivery of a luciferase/yellow fluorescent protein fusion gene. The time course of engraftment of canine lymphoma cells after intraperitoneal injection into SCID-NOD mice is shown in Figure 7a. The recipients had detectable tumor growth one week after the injection of lymphoma cells. Repetitive imaging of mice at weekly intervals demonstrated lymphoma growth in the abdominal viscera. Tumor engraftment and progressive tumor growth was readily demonstrable by bioluminescent imaging (BLI) several weeks before the animals appeared sick as monitored by dehydration and depression. At 7-8 weeks, BLI revealed widespread lymphoma growth in the mesenteric lymph nodes, with metastasis to the spleen, liver, lungs which was confirmed by *ex vivo* imaging of these organs as shown in Figure 7b.
Lymphoma is a common malignancy in humans and dogs. Animal models that can be easily evaluated are critically important to enhance our understanding of the pathogenesis of lymphoma and for the development of more effective therapies. There are few animal models that closely recapitulate the disease as it occurs in both species, and there are no animal models that are useful for investigating the molecular pathogenesis of lymphoma and hypercalcemia in dogs. We report the establishment of a model of canine T-cell lymphoma and HHM in NOD/SCID mice with lymphoma cells from a dog with HHM. The primary lymphoma cells produced multicentric lymphoma in NOD/SCID mice and maintained their morphological, immunohistochemical, and molecular features. The xenografted lymphoma cells in NOD/SCID mice induced hypercalcemia by up-regulation of PTHrP and TNFα that stimulate osteoclastic bone resorption. Histomorphometric analysis showed that the HHM in mice bearing canine lymphoma was due to increased osteoclastic bone resorption as seen in human and canine patients with lymphoma and HHM.

There is little information on the pathogenesis of HHM in humans with lymphoma due to the lack of in vivo models. To our knowledge, the only available mouse models of lymphoma with HHM are for adult T-cell leukemia/lymphoma in which hypercalcemia occurs with a very high frequency. We established a SCID/beige mouse model of ATLL with HHM and have shown that hypercalcemia in those mice was mediated primarily by increased levels of PTHrP. Similarly, increased PTHrP
is associated with HHM in this canine T-cell lymphoma xenograft mouse model and will be very useful for investigating the pathogenesis of HHM in lymphoma.

DNA microarrays represent a powerful tool for rapid screening of expression of many genes in parallel and have been used extensively for the study of gene expression in a variety of lymphoid malignancies\(^{(49)}\). To identify the molecular basis underlying the pathogenesis of T-cell lymphoma, we used a cDNA microarray to characterize the cytokine gene expression profiles of the canine lymphoma xenograft. Human cDNA arrays could be used to study comparative cytokine gene expression in canine lymphoma due to the extensive genetic homology between the two species. Using this technique, we identified a variety of genes that have potential implications in the tumorigenesis and metastases of canine lymphoma. For example, the up-regulation of cell cycle regulator $cdk4$ has been reported in a variety of solid tumors such as breast carcinoma\(^{(50)}\), mantle cell lymphoma\(^{(51)}\) and diffuse large B-cell lymphoma\(^{(52)}\). Histone deacetylase (HDAC) has been shown to be up-regulated in several solid tumors such as prostate\(^{(53)}\), breast\(^{(54)}\) and gastric cancer\(^{(55)}\). Up-regulation of MMP-9 has been reported to be a key player in the dissemination of T-cell lymphoma cells to peripheral tissues\(^{(56)}\). Integrins have been a subject of extensive research and several antagonists of integrins are currently being evaluated as anticancer therapeutics. Integrins have been shown to promote cellular migration and survival in tumors and primary cells\(^{(57)}\) and have been shown to be involved in specific homing of lymphocytes to various tissues\(^{(58)}\). For example, it has been shown that integrin $\alpha 4\beta 7$ targets T-cells to the GALT\(^{(59)}\). Interestingly, up-regulation of
 integrin α4 and β7 might explain the predominance of mesenteric lymphomas seen in our model. Genes such as the interleukin-1 receptor-like 2 (IL-1RL2) have been shown to be down-regulated several-fold in mantle cell lymphoma\(^\text{(60)}\), consistent with our observations; however, instead of tumor necrosis factor receptor superfamily-1A (TNFRSF1A), rather TNFRSF1B was down-regulated in our lymphoma model.

Many dogs with T-cell lymphoma develop HHM, a paraneoplastic syndrome that significantly contributes to morbidity and mortality when it occurs\(^\text{(61)}\). PTHrP has been shown to be a causative factor of hypercalcemia in several epithelial cancers in humans and in animals bearing human solid tumors\(^\text{(62;63)}\). A previous investigation by our laboratory showed that dogs with lymphoma and hypercalcemia had increased concentrations of plasma PTHrP, but there was no correlation between serum calcium and PTHrP levels\(^\text{(31)}\). It was suggested that PTHrP induced HHM in synergy with tumor-related cytokines. Kuboto et al reported increased plasma PTHrP in one dog with lymphoma and hypercalcemia, and no detectable PTHrP in dogs with lymphoma and normocalcemia and healthy dogs. They also detected PTHrP mRNA expression in one dog with lymphoma and hypercalcemia and four dogs with lymphoma and normocalcemia\(^\text{(64)}\). In this study, we examined the expression profiles of various cytokines capable of mediating bone resorption in dogs with T-cell lymphoma and hypercalcemia. Comparison of the mRNA levels in six hypercalcemic dogs with T-cell lymphoma revealed a distinct set of hypercalcemic factors in each case. PTHrP mRNA was up-regulated in all hypercalcemic dogs and ranged from 3- to 194-fold. TNFα mRNA was either moderately up-regulated or down-regulated in
hypercalcemic dogs. TNFα, a pro-inflammatory cytokine, is known to stimulate osteoclastogenesis by RANKL-dependent and independent mechanisms. Also, TNFα has been shown to be involved in the pathogenesis of several inflammatory and metabolic bone diseases such as rheumatoid arthritis, periodontitis, and/or postmenopausal osteoporosis. The up-regulation of TNFα mRNA in some of the lymphoma samples suggests a potential role for TNFα in the pathogenesis of HHM. RANKL, secreted by activated T lymphocytes, has been shown to promote differentiation and fusion of osteoclast precursors and activate mature osteoclasts by binding to its specific receptor, RANK. There was a mild to moderate up-regulation of RANKL in some of the hypercalcemic lymphoma samples (dog # 2, 4, and 5) which likely contributed to the pathogenesis of HHM in those dogs. Although IL-6 has been shown to be up-regulated in some cancers with HHM, we found down-regulation of IL-6 in all the samples examined. For example in dog # 1, there was mild to moderate up-regulation of PTHrP with a moderate to marked up-regulation of TNFα and IL-1β suggesting a possible synergistic role of TNFα and IL-1β with PTHrP in causing HHM. In summary, as indicated by Cox et al, because of the heterogeneity of the lymphomas and particularly T-cell lymphomas, a common cause of hypercalcemia is unlikely. Inspite of these differences, our study has revealed that PTHrP likely plays the central role in the pathogenesis of HHM in dogs with T-cell lymphoma and it is likely that hypercalcemia in each case is the result of a combinatorial effect of different sets of cytokines and in this context the roles of various cytokines must be evaluated.
The investigation of mechanisms of tumor growth, metastasis, and response to therapy requires animal models capable of detecting small numbers of cells and assessing disease burden noninvasively and quantitatively. Since lentiviral vectors can infect non-dividing cells, and because our xenografted lymphoma cells do not grow \textit{in vitro}, we used a novel lentiviral transduction technique. The lentiviral vectors were very useful and efficient for delivering the reporter transgene into tumor cells that do not grow \textit{in vitro}. Using BLI, we were able to detect and monitor lymphoma cell growth in locations that were not easily monitored grossly such as the abdominal cavity and viscera. Tumor cell trafficking, engraftment in different organs, and metastasis could be visualized without perturbing intact organs. BLI showed that trafficking of lymphoma cells in this NOD/SCID mouse model has similarities to that in dogs and humans with lymphoma involving the lymph nodes, liver and spleen. The abdominal/mesenteric distribution of the tumor seen in this model is not a common clinical presentation in dogs. This could either be due to the intraperitoneal route of injection of tumor cells, or specific host (species) factors that influence tumor cell trafficking and anatomic distribution of the tumors in dogs and mice.

In conclusion, this bioluminescent NOD/SCID mouse model of canine T-cell lymphoma provides a clinically relevant \textit{in vivo} model to study the regulation of PTHrP and its interrelationships with cytokines produced by lymphoma cells in the induction of HHM. This new tool will improve our understanding of tumor biology and facilitate drug discovery and evaluation.
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Table 4.1: Primers used for Q-RT-PCR with accession numbers for each gene
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<td>8/16</td>
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<td>Thymus</td>
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Table 4.2. Histopathology in NOD/SCID mice xenografted with canine T-cell lymphoma
Table 4.3 List of genes up-regulated in the xenografted canine T-cell lymphoma compared to normal canine lymph nodes (NCLN)
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Genes down-regulated in lymphoma compared to normal canine lymph node | Fold decrease
--- | ---
1. TNF Superfamily:  
   TNFRSF1B | 20.2 |
   EDAR | 10.4 |
2. Adhesion Molecules:  
   BCAM | 4.4 |
3. Protease or Related Factor:  
   ADAM 12 | 6.2 |
4. Other Factors:  
   NMA | 10.2 |
5. Binding Protein:  
   LBP | 18.2 |
6. Interleukin Receptors:  
   IL-1 RL2 | 8.2 |
   IL-18 RAP | 4.8 |
7. Cell Surface Proteins:  
   RP105 | 4.8 |
   SLAM | 6.2 |
8. FGF Family:  
   FGFR1 | 22.3 |
9. Neurotrophic group:  
   Ret | 5.2 |
10. Chemokines  
    MCP-1 | 2.6 |

Table 4.4 List of genes down-regulated in the xenografted canine T-cell lymphoma compared to normal canine lymph nodes (NCLN)
Figure 4.1. Characterization of canine T-cell lymphoma xenografted in NOD/SCID mice. Photomicrographs of a: control NOD/SCID mouse. NOD/SCID mouse with canine lymphoma xenograft (shown by arrows) in b: the spleen c: the mandibular lymph nodes and d: the thymus e: Negative immunohistochemistry for CD79α (pan B-cell marker) in lymphoma of the mesenteric lymph node (immunoperoxidase; final magnification, x200). f: Positive immunohistochemistry for CD3 (pan T-cell marker) in lymphoma of the mesenteric lymph node (immunoperoxidase; final magnification, x200). g: PCR amplification of canine microsatellites. The specific products of 520 bp (FH2365) and 180 bp (C36672) were amplified from canine whole blood (WB), xenografted canine lymphoma, a canine prostate cancer cell line (Ace-1), but not from control mouse spleen. h: Lymphoma cells in the ascites fluid from the xenografted mouse (Wright’s stain; magnification:200X). i: Lymphoma in the liver of a NOD/SCID mouse (H&E, original magnification, x100) j: Diffuse lymphoma in the mesenteric lymph node of a NOD/SCID mouse (H&E, original magnification, x200) k: Lymphoma in the lungs of a NOD/SCID mouse (H&E, original magnification, x200) l: PTHrP immunofluorescence in the cytoplasm and nucleus of lymphoma cells (FITC, confocal imaging, 400 X)
Figure 4.1. Characterization of canine T-cell lymphoma xenografted in NOD/SCID mice.
Figure 4.2. Plasma total calcium concentrations (mg/dL). Mice engrafted with canine T-cell lymphoma had significantly ($P < 0.005$) greater plasma total calcium levels ($10.9 \pm 0.8 \text{ mg/dl}$) than controls ($9.2 \pm 0.13 \text{ mg/dl}$). Bars indicate means.
Figure 4.3. Hypercalcemic factors in the NOD/SCID mice xenografted with canine lymphoma. a: Plasma PTHrP concentrations (pM). The xenografted NOD/SCID mice have increased concentrations of circulating PTHrP (3.2 ± 2.0 pM/L) (p=0.025) compared to control mice (<1.5). Bars represent averages. b: Q-RT-PCR for expression of mRNA for hypercalcemic factors in the xenografted canine T-cell lymphoma. The mesenteric lymphomas expressed significantly higher levels of PTHrP and TNFα mRNA (p<0.005) compared to normal canine lymph nodes (NCLN). No significant differences were observed in other cytokines examined. Bars represent the mean ± SD of 3 independent samples.
Figure 4.4. Histomorphometry of tibiae of NOD/SCID mice xenografted with canine T-cell lymphoma. **a.** There was no significant difference in the trabecular bone volume between the control and lymphoma bearing NOD/SCID mice. **b.** Percent osteoclast perimeter was significantly increased (*p<0.005) in the lymphoma-bearing mice compared to the controls. **c.** Osteoclast number/mm trabecular bone was significantly increased in lymphoma-bearing mice compared to controls *p<0.05. Bars represent the mean ± SE.
Figure 4.5. Q-RT-PCR verification of microarray data. a. Up-regulation of integrin alpha-4 mRNA in the primary tumor and the mesenteric lymphomas from the xenografted mice compared to the normal canine lymph nodes (NCLN). b. Downregulation of IL-1RL-2 mRNA in the primary tumor and the mesenteric lymphomas from the xenografted mice compared to NCLN.
Figure 4.6. Hypercalcemic factors in canine T-cell lymphoma patients with hypercalcemia. Q-RT-PCR analyses for mRNA expression for IL-6, IL-1α, IL-1β, TNFα, TNFβ, TGFα, TGFβ, RANKL, MIPα and PTHrP from dogs with T-cell lymphoma and hypercalcemia normalized to normal canine lymph nodes (NCLN). Moderate to markedly elevated PTHrP and mild up-regulation of RANKL mRNA expression was present in most of the dogs with T-cell lymphoma and hypercalcemia.
Figure 4.7. Bioluminescent Imaging in mouse model of canine T-cell lymphoma

a. Serial non-invasive bioluminescent imaging (BLI) of canine T-cell lymphoma in NOD/SCID mice following intraperitoneal injection of Luc/YFP transduced lymphoma cells. Note the progressive increase in the photon intensities reflecting the progression of the lymphoma from week 1 to week 8. The BLI signal intensity was measured as photons/sec/cm$^2$. The color bar to the right of the images represents the range of photon flux.

b. Ex vivo imaging of mesenteric lymphomas, liver, spleen, heart and lungs, and the gastrointestinal tract after euthanasia. Note the high photon intensities in the mesenteric lymphomas and along the mesentery.
A central role for PTHrP in the pathogenesis of HHM associated with breast, renal and squamous cell carcinomas in humans has been well established\(^1\). PTHrP has been identified as one of the important factors involved in osteoclastic bone resorption and hypercalcemia associated with lymphoma and ATLL\(^2\). A weaker correlation between PTHrP expression and hypercalcemia was observed in these patients. Moreover, constitutive PTHrP expression has been reported in HTLV-1-carriers and ATLL patients without hypercalcemia\(^3\). Research over the past several years has shown that ATLL and lymphoma cells secrete several factors that can induce osteoclastic bone resorption suggesting a multifactorial etiology for HHM in these patients. Circulating PTHrP levels were generally found to be low or undetectable in normal individuals. However, there is widespread expression of PTHrP gene in various normal tissues and cell types indicating several roles for PTHrP in normal physiology as a local or paracrine factor rather than as a systemic factor. Investigations over the past several decades have shown that PTHrP affects tumor cell proliferation and apoptosis and has transforming growth factor like properties\(^4,5\).

Experiments presented in this dissertation demonstrated that PTHrP is expressed during immortalization of lymphocytes due to HTLV-1 infection, likely in a Tax-independent manner. HTLV-1 infection also markedly up-regulated the PTH1R
suggesting a potential autocrine role for PTHrP during the immortalization and subsequent transformation of lymphocytes by HTLV-1 infection. PTHrP is up-regulated by the P2 and P3 promoters in HTLV-1-infected and ATLL cells. Data presented in the third chapter showed that the PTHrP P2 promoter can be transactivated by the NF-κB pathway.

Finally, data presented in the fourth chapter demonstrated that hypercalcemia in dogs with T-cell lymphoma is mediated by several osteoclastogenic factors confirming the multifactorial etiology for HHM in lymphoma patients. In addition, dogs can also be used as comparative animal models to study the pathogenesis of HHM associated with lymphoma.

Our observations raised an array of questions as outlined below.

**What is the role of PTHrP in transformation of lymphocytes due to HTLV-1 infection?**

Data presented in the second chapter showed that PTHrP and its receptor were highly up-regulated during immortalization and the subsequent transformation of lymphocytes due to HTLV-1 infection. However, a definitive role for PTHrP in this process has not been determined. A few reports have shown that PTHrP can cooperate with oncogenes such as Ras or EGFR in the transformation process. It has been reported that HTLV-1 Tax can cooperate with Ras in the transformation of fibroblasts. The Ras-MAP-Kinase pathway can increase PTHrP expression. Hence, additional experiments will be required to understand the role of PTHrP, Ras and Tax in the transformation of lymphocytes due to HTLV-1 infection. The role of PTHrP in
transformation can be better understood by knocking-down PTHrP using siRNA constructs specific for PTHrP. However, primary lymphocytes derived from the peripheral blood are fragile and hard to transfect. This can be circumvented by designing retroviral or lentiviral vectors to infect cells for effective delivery of the SiRNA constructs. We recently developed such retroviral vectors and showed that they were effective in reducing PTHrP expression in a human pulmonary squamous cell carcinoma cell line (Nadella and Rosol; unpublished data). These vectors have yet to be tested in primary lymphocytes for efficacy. Secondly, since the PTHrP receptor was up-regulated during the immortalization process, antibodies towards PTHrP and/or PHT1R can be used in the long-term co-culture assays to determine if PTHrP functions through the receptor during the transformation process. Proliferation and apoptosis of lymphocytes can be measured at various time points. Finally, additional experiments knocking out Tax and Ras expression will provide further evidence for roles of these factors in transformation.

**What are the complexes formed on the PTHrP P2 promoter in ATLL cells?**

Data presented in the third chapter show that the PTHrP P2 promoter is activated by NF-κB pathway in HTLV-1-infected and ATLL cells. We have shown that a single complex consisting of p50/c-Rel forms in high Tax-expressing T-cells. In contrast two distinct complexes formed on the P2 promoter in ATLL cells. We have identified one of the complexes to be composed of p50/p50 homodimers, but we were unable to identify the protein involved in the second complex formed in ATLL cells. So far our approaches using proteomics and mass spectrometry have been unsuccessful because
of high level of contaminating proteins. Nuclear lysates can be passed through a hydroxyapatite column to decrease the level of contaminating proteins and the filtered lysates can be subjected to mass spectrometry to identify the potential proteins present in the lysates. This data can be subsequently confirmed by super-shift assays using specific antibodies against the proteins identified by mass spectrometry.

**What are the hypercalcemic factors in lymphoma and ATLL patients?**

HHM in lymphoma and ATLL patients is likely multifactorial. Although several studies have shown that PTHrP plays an important role in the development of HHM in these patients, it is becoming obvious that it needs additional factors acting synergistically to induce hypercalcemia. Studies in the past have analyzed single factors in hypercalcemic lymphoma and ATLL patient samples. To better answer this question, a comprehensive approach has to be undertaken to analyze the levels of several hypercalcemic factors in a large number of samples from hypercalcemic patients compared to normocalcemic lymphoma and ATLL patients. Additionally development of new xenograft animal models will be useful in mechanistic investigations on HHM associated with lymphoma and ATLL.

In conclusion, a variety of questions regarding the role of PTHrP in transformation of lymphocytes and development of HHM remain to be addressed. More comprehensive studies integrating various HTLV-1 viral proteins, PTHrP and other hypercalcemic factors is essential to understand the pathogenesis of ATLL and to better treat it.
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