Animal Model

Humoral Hypercalcemia of Malignancy

Severe Combined Immunodeficient/Beige Mouse Model of Adult T-Cell Lymphoma Independent of Human T-Cell Lymphotropic Virus Type-1 Tax Expression

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The majority of patients with adult T-cell leukemia/lymphoma (ATL) resulting from human T-cell lymphotropic virus type-1 (HTLV-1) infection develop humoral hypercalcemia of malignancy (HHM). We used an animal model using severe combined immunodeficient (SCID)/beige mice to study the pathogenesis of HHM. SCID/beige mice were inoculated intraperitoneally with a human ATL line (RV-ATL) and were euthanized 20 to 32 days after inoculation. SCID/beige mice with engrafted RV-ATL cells developed lymphoma in the mesentery, liver, thymus, lungs, and spleen. The lymphomas stained positively for human CD45RO surface receptor and normal mouse lymphocytes stained negatively confirming the human origin of the tumors. The ATL cells were immunohistochemically positive for parathyroid hormone-related protein (PTHrP). In addition, PTHrP mRNA was highly expressed in lymphomas when compared to MT-2 cells (HTLV-1-positive cell line). Mice with lymphoma developed severe hypercalcemia. Plasma PTHrP concentrations were markedly increased in mice with hypercalcemia, and correlated with the increase in plasma calcium concentrations. Bone densitometry and histomorphometry in lymphoma-bearing mice revealed significant bone loss because of a marked increase in osteoclastic bone resorption. RV-ATL cells

contained 1.5 HTLV-1 proviral copies of the *tax* gene as determined by quantitative real-time polymerase chain reaction (PCR). However, *tax* expression was not detected by Western blot or reverse transcriptase (RT)-PCR in RV-ATL cells, which suggests that factors other than Tax are modulators of PTHrP gene expression. The SCID/beige mouse model mimics HHM as it occurs in ATL patients, and will be useful to investigate the regulation of PTHrP expression by ATL cells *in vivo*. (Am J Pathol 2001, 158:2219–2228)

Humoral hypercalcemia of malignancy (HHM) is an important paraneoplastic syndrome occurring in humans with a wide variety of cancers.¹ Parathyroid hormone-related protein (PTHrP) was originally isolated from specific tumors as the primary cause of HHM² and is overexpressed by many types of neoplasms.³ PTHrP is a polypeptide hormone discovered in 1987 and is structurally similar to parathyroid hormone (PTH).^{4–6} 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) and resulting in hypercalcemia.^{7–9} The primary mechanism for bone loss and hypercalcemia in patients with lymphoma is increased osteoclastic bone resorption induced by humoral mediators produced by neoplastic cells.¹⁰ It has been demonstrated that expression of PTHrP mRNA in both humans and animals correlates well with the occurrence of hypercalcemia and hypercalcemia in tumor-

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bearing animals is corrected by a neutralizing antibody to PTHrP.^{11,12}

Adult T-cell leukemia/lymphoma (ATL) is an aggressive and often fatal malignancy of helper/inducer T lymphocytes (CD4) caused by infection with a complex retrovirus, human T-cell lymphotropic virus type 1 (HTLV-1).^{13,14} Hypercalcemia is frequently observed in ATL patients and represents a life-threatening complication of this disease.¹⁵ The virus contains, in addition to *gag*, *pol*, and *env* genes, a regulatory gene region (pX) that encodes several proteins from four open reading frames, including Tax.^{16,17} Tax is a 40-kd nuclear-localizing phosphoprotein that increases viral transcription from the HTLV-1 LTR, as well as many cellular genes including interleukin (IL)-2, IL-2 receptor (IL-2R) α chain,¹⁸ and PTHrP.¹⁹ It also has been reported that Tax transactivates the PTHrP gene promoter *in vitro*.²⁰ In contrast, little is known about regulation of the PTHrP gene in HTLV-1 lymphoma cells *in vivo*. Moreover, very low levels of tax mRNA are present in ATL cells *in vivo*.^{21,22} Thus, factors other than Tax are likely responsible for transcription of the PTHrP gene in ATL cells *in vivo*. ATL cells from patients can secrete other humoral factors such as IL-1 β ,²³ transforming growth factor- β (TGF- β),²⁴ and tumor necrosis factor- β (TNF- β).²⁵ These cytokines have been shown to induce bone resorption in humans.¹ Moreover, IL-2 increases PTHrP production and secretion in HTLV-1-infected T cells.^{26,27} In addition, PTHrP and IL-6 act synergistically in the development of hypercalcemia in patients with hematological malignancies.²⁸ Other cytokines, such as TGF- β , TNF- α , and IL-1, up-regulate PTHrP gene expression in a variety of non-lymphoid cell lines and tissues.^{29,30} Nevertheless, little is known about their action on PTHrP expression in HTLV-1-infected lymphocytes.

We have previously reported development of lymphoma in severe combined immunodeficient (SCID) mice inoculated with peripheral blood lymphocytes from ATL patients.³¹ However, SCID/beige (bg) mice were chosen in the present study because they previously have been shown to have a greater efficiency for xenografting neoplastic human lymphoid tissue in comparison with SCID mice. SCID/bg mice lack natural killer (NK) cell activity and have macrophage defects in addition to lack of functional B and T lymphocytes.

There is little new information on the pathogenesis of HHM in humans because of the lack of relevant *in vivo* models. We have demonstrated that ATL cells develop lymphoma in SCID/beige mice, and animals consequently develop HHM as observed in human patients. This new model will permit mechanistic studies on the interrelationships between cytokines and PTHrP in the pathogenesis of HHM.

Materials and Methods

Animals and ATL Cell Inoculation

Immunodeficient SCID/bg (C.B-17/lcrCrl-scid-bgBR) mice (Charles River Laboratories, Inc., Wilmington, MA) were maintained under specific pathogen-free conditions

in the animal facility of the College of Veterinary Medicine at The Ohio State University (Columbus, Ohio). Male mice (5 weeks-of-age) were used as recipients, anesthetized with xylazine-ketamine, and injected intraperitoneally with 4×10^7 RV-ATL cells suspended in RPMI 1640 medium. Controls were inoculated with medium alone. The source of the RV-ATL cell line was previously described.³²

Histology and Immunohistochemistry

Animals from the early control group (animals C1 to C7) and early ATL group (animals L1 to L7, L26, and L28) were sacrificed at day 20. Animals from late control group (animals C8 to C14) and late ATL group (animals L8 to L25, L27, L29, L30, and L31) were sacrificed between days 29 and 32 after inoculation. A complete necropsy was performed on each animal. Heart, lungs, thymus, kidneys, liver, stomach, small intestine, colon, mesentery, liver, spleen, vertebrae, and pancreas were fixed immediately after removal from the sacrificed animals in 10% neutral-buffered formalin, embedded in paraffin, cut into 5- μ m-thick sections, and stained with hematoxylin and eosin. Immunohistochemistry was performed on paraffin sections with the following primary antibodies: polyclonal rabbit anti-PTHrP (PTHrP amino acids 34 to 53) (1:100, Ab-2, Oncogene Research Products, Cambridge, MA), and monoclonal mouse anti-CD45RO (1:100; DAKO, Carpinteria, CA). Visualization was achieved using an avidin-biotin complex (Pierce, Rockford, IL), color development with diaminobenzidine (Research genetics, Huntsville, AL), and hematoxylin counterstain. Negative control slides were stained with omission of the primary antibody. Normal human skin sections served as positive controls.

Measurement of Plasma Calcium and PTHrP Concentrations

Calcium and PTHrP concentrations were measured in early control (C2 to C7) and late control (C8 to C14) animals, as well as, in lymphoma-bearing animals from early ATL (L1, L3 to L5, L7, L26, and L28), and late ATL (L14, L15, L17, L20, L21, L23, L25, L27, and L29 to L31) groups. Blood was obtained from the femoral artery at necropsy. Ionized calcium concentrations were measured with a Nova 8 electrolyte/chemistry analyzer (Nova Biomedical, Waltham, MA). Total calcium concentrations were measured by colorimetric assay (Sigma Chemical Co., St. Louis, MO). Plasma PTHrP concentrations were determined by 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).

Bone Histomorphometry and Mineral Densitometry

Lumbar vertebrae were collected and fixed in 10% neutral-buffered formalin for 24 hours 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 (Poly-science Inc., Warrington, PA) for 10 days at 4°C, and embedded in glycol methacrylate at 4°C. Sections were cut at 5 μ m, histochemically stained for tartrate-resistant acid phosphatase (Sigma Co.), and counterstained with hematoxylin. Histomorphometry of bones was completed in mice with lymphoma from early ATL (L1, L3 to L5, L7, and L26) and late ATL (L8, L11, L12, L13, L14, L15, L17, L19, L20, L23, L27, and L29 to L31) groups, as well as, age-matched control mice (C1 to C3, C5, C7, C8, and C10 to C14) with Bioquant Nova Image Analysis Software (R&M Biometrics Inc., Nashville, TN). Measurement of osteoclastic bone resorption was completed on trabecular bone and osteoclasts were identified as cells lining trabecular bone that stained intensely fuchsia for tartrate-resistant acid phosphatase. Measurements included total bone area, trabecular bone area and perimeter, osteoclast number/mm trabecular bone, and percent osteoclast perimeter.

Bone mineral density (BMD) was measured in early control (C1 to C7), late control (C8 to C14), early ATL (L1, L3 to L5, and L7), and late ATL (L8 to L15, L17, L19 to L21, L23, and L25) groups using dual-energy X-ray absorptiometry on an Eclipse peripheral DEXA Scanner (Norland, Ft. Atkinson, WI) using research software. To measure femoral BMD, the right femur was excised from soft tissue and placed on the scanner in lateral position. The femur was scanned at 5 mm/s with a resolution of 0.1 mm $\times$ 0.1 mm. Total femoral BMD was determined in a window that encompassed the entire femur. Distal metaphyseal BMD was measured in a window that originated 0.25-cm proximal from the distal epiphysis and extended proximally for 0.25 cm.

Northern Blot Analysis of PTHrP Expression

Mesenteric lymphomas were snap-frozen in liquid nitrogen. Total RNA was isolated using TRIzol (Life Technologies, Inc., Grand Island, NY). Total RNA (40 μ g) was separated on a 1.2% agarose-formaldehyde gel, transferred to Duralon UV membranes (Stratagene, La Jolla, CA), and crosslinked using a UV Stratalinker 1800 (Stratagene). The membranes were hybridized for 3 hours at 68°C with a ³²P-dATP-labeled probe for human PTHrP (clone 661).³³ The membranes were exposed to Kodak X-OMAT AR film (Kodak, Rochester, NY) for 24 hours at -80°C for autoradiography.

HTLV-1 Provirus Copy Number in RV-ATL Cells

Genomic DNA from cells was obtained using the Qiaamp Blood Kit (Qiagen, Valencia, CA). Genomic DNA (50 ng) was amplified using a Roche Molecular Biochemicals Light Cycler in triplicate samples (Roche Molecular Biochemicals, Indianapolis, IN). Amplification was performed in the presence of 4 mmol/L of MgCl₂ using primer pairs specific for the *tax* (670/671) and *gag* (SG166/SG296) genes³⁴ of HTLV-1. Cycling conditions

were as follows: 2 minutes at 94°C for denaturation, and 60 cycles of 94°C for 1 second, 55°C for 1 second, and 72°C for 10 seconds. After the last amplification cycle, a melting curve analysis was performed to determine the specificity of the PCR reaction. Proviral copy number was determined using a standard curve obtained by amplification of a serially diluted plasmid (ACH) representing an infectious molecular clone of HTLV-1³⁵ or Styl Δ 28 that contains a 588-bp fragment of the *gag* gene.³⁶

Western Blot Analysis of HTLV-1 Gene Products

Approximately 1×10^7 RV-ATL cells were collected by peritoneal lavage from lymphoma-bearing SCID/bg mice. Other HTLV-1 infected and noninfected cells (MT-2, HT1-RV, Jurkat, and SLB-1 cell lines) were maintained in culture in RPMI 1640 medium supplemented with 10% fetal bovine serum. Cells were lysed (0.15 mmol/L NaCl, 10 mmol/L sodium pyrophosphate, 10 mmol/L NaF, 0.5% deoxycholate, 50 mmol/L Tris, pH 8.0, 0.1% sodium dodecyl sulfate, 10% glycerol, 1% Nonidet P-40) on ice for 10 minutes. Cell lysates were centrifuged at $17,000 \times g$ for 10 minutes at 4°C. Lysate protein concentrations were determined using the BioRad (Hercules, CA) microassay. Equivalent amounts of protein (40 μ g) were mixed with 2 $\times$ sample buffer (0.08 mol/L Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate, 0.1 mol/L dithiothreitol, 10% glycerol, 0.1% bromophenol blue). The samples were boiled for 3 minutes and separated on a 10% sodium dodecyl sulfate-polyacrylamide gel. The proteins were transferred to a nitrocellulose membrane (BA-79; Schleicher & Schuell, Keene, NH) for 2 hours at 40 V. The membrane was washed three times with TBS-T (9% NaCl, 0.1 mol/L Tris base, 0.1% Tween 20, pH 7.2) and blocked in a 5% powdered milk/TBS-T solution overnight at 4°C. The membrane was washed three times and incubated with rabbit polyclonal anti-Tax (1:500; AIDS Reagent Program, Rockville, MD), mouse monoclonal anti-p19 (1:100; Zep-toMatrix Corporation, Buffalo, NY), mouse monoclonal anti-p24 (1:100; Genzyme Corporation, Cambridge, MA), and mouse monoclonal anti-gp46 (1:500)³⁷ overnight at 4°C. The membrane was washed three times with TBS-T, and incubated with rabbit horseradish peroxidase-labeled secondary antibody (Amersham Pharmacia, Piscataway, NJ) for 1 hour. After a final wash with TBS-T, the HTLV-1 proteins were detected by enhanced chemiluminescence detection reagents (Amersham Pharmacia) and developed on BioMax MR Film (Kodak, Rochester, NY).

RT-PCR for HTLV-1 Tax/Rex RNA

Total cellular RNA was extracted from 729, HT1-RV, SLB-1, MT-2 cells, and from xenografted RV-ATL cells using the Tri Reagent (Molecular Research Center Inc., Cincinnati, OH) procedure.³⁸ All RNA was digested three times with RNase-free DNase (Boehringer Mannheim, Indianapolis, IN), precipitated, and quantified by absorbance at 260 and 280 nm. Approximately 600 ng of RNA

Table 1. Gross Pathology and Histopathology in SCID/bg Mice Xenografted with Human ATL Cells

Group	Gross pathology				Histopathology						
	Mesenteric lymphoma	Splenomegaly	Thymus enlargement	Pericardial mineralization	Lymphoma						Pericardial mineralization
	Mesentery	Spleen	Liver	Thymus	Lungs	Bone					
Early Lymphoma (day 20)	7/9	3/9	0/9	0/9	7/9	3/9	0/9	3/9	0/9	0/9	0/9
Late lymphoma (day 29 to 32)	19/22	8/22	2/22	9/22	19/22	5/22	17/22	17/22	2/22	5/22	13/22
Total	26/31	11/31	2/31	9/31	26/31	8/31	17/31	20/31	2/31	5/31	13/31

was amplified by a coupled primer extension-30 cycle PCR reaction containing HTLV-1-specific oligonucleotide primer pairs. The coupled primer extension-PCR reaction (50 μ l) contained RNA, 0.25 mmol/L deoxynucleoside triphosphates, 50 mmol/L KCL, 10 mmol/L Tris (pH 8.0), 1.5 mmol/L MgCl₂, 0.01% gelatin, 100 ng 3' (antisense) oligonucleotide, 50 ng 5' (sense) oligonucleotide end-labeled with T4 DNA kinase to a specific activity of $\sim 2 \times 10^8$ cpm/ μ g, and 2.5 U of *Taq* DNA polymerase (Promega, Madison, WI) in the presence (+) or absence (–) of 5 U of murine leukemia virus reverse transcriptase (Amersham Pharmacia). The reaction was performed in a Perkin Elmer thermal cycle 9600: 65°C for 10 minutes, 50°C for 8 minutes, and 95°C for 5 minutes followed by 30 cycles of 95°C for 1 minute, 55°C for 2 minutes, and 72°C for 2 minutes. PCR-amplified products were separated on a 6% polyacrylamide gel and visualized by autoradiography. The sequence of the HTLV-1-specific oligonucleotides were; LA79 HTLV-1–5' GTC CAA ACC CTG GGA AGT GG 3'; LA78 HTLV-1–5' CCA GTG GAT CCC GTG GAG AT 3'. The primer pair was designed to amplify spliced *tax*/*rex*-specific RNA (117 bp). A second primer pair (670/671) was used, as previously described³⁹, to amplify a region of the *tax*/*rex* gene, but it also detects all species (spliced and nonspliced) of viral RNA (159 bp).

Statistical Analysis

Numerical data were expressed as means $\pm$ SD. Statistical differences between means for the different groups were evaluated with InStat 3.01 (GraphPAD software) using one-way analysis of variance, Bonferroni multiple comparisons test, and paired *t*-test with the level of significance at *P* < 0.05. Correlation coefficients were determined by linear regression.

Results

Xenografted ATL Cells Grow in SCID/bg Mice

ATL cells inoculated intraperitoneally into 5-week-old SCID/bg mice produced diffuse lymphoma in the mesentery of 84% (26 of 31) of the mice between 20 to 32 days after inoculation (Table 1). At necropsy, mice in the early ATL group (day 20) had mild to moderate ascites (3 to 4 ml) containing a suspension of ATL cells recovered by abdominal lavage. Small tumors were present at the root of the mesentery in 78% (7 of 9) of the animals sacrificed. Eighty-six percent (19 of 22) of the animals

necropsied between day 28 and day 32 after inoculation had severe ascites and larger tumors in the mesentery that extended to the pelvic cavity. In addition, 47% of the lymphoma-bearing mice (9 of 19) in the late ATL group had mild to marked white striations on the right ventricular epicardium (mineralization). Mild to marked splenomegaly was present in 35% (11 of 31) of the mice inoculated with ATL cells. The thymus was 5 to 10 times larger than controls in 2 of 22 mice in the late ATL group.

Histopathology

Microscopic evaluation (Table 1) of mice inoculated with ATL cells revealed lymphoma in the mesentery (26 of 31), thymus (20 of 31), liver (17 of 31), spleen (8 of 31), lungs (2 of 31), and bone marrow (5 of 31) in both early and late ATL groups. In the mesentery, spleen (Figure 1j), and thymus (Figure 1i), ATL cells formed large solid sheets or nodules replacing most of the normal tissue. In the liver, lymphoma was limited to the hilus region of the hepatic lobes, and was interpreted to be a direct extension of the mesenteric neoplasm. In the lungs, ATL cells formed large peribronchial cuffs (Figure 1h). Sixty-eight percent of the late ATL mice (13 of 19) had mild to marked mineralization in the epicardium of the right ventricle.

Plasma Concentrations of Calcium and PTHrP

Lymphoma-bearing mice in the late ATL group had a statistically significant (*P* < 0.001) increase in both ionized (6.7 ± 1.0 mg/dl) and total (15.8 ± 2.8 mg/dl) calcium concentrations compared to control mice (4.9 ± 0.2 and 8.7 ± 0.4 mg/dl, respectively) (Figure 2). Total plasma calcium concentrations were as great as 19.7 mg/dl in the SCID/bg mice engrafted with RV-ATL cells. A slight increase in calcium concentrations was observed in early ATL mice (day 20) when compared to age-matched control mice, although the difference was not statistically significant. Plasma PTHrP concentrations (Figure 3) were markedly increased in mice with lymphoma (12 to 140 pmol/L compared to <1.5 pmol/L in control mice). A strong positive correlation between plasma total calcium and PTHrP concentrations was present in SCID/bg mice engrafted with RV-ATL cells from both early (*r* = 0.85, *P* < 0.001) and late ATL (*r* = 0.90, *P* < 0.001) groups, respectively.

Figure 1. Immunohistochemistry, histology, and enzyme histochemistry of SCID/bg mice engrafted with human ATL cells. **a:** PTHrP immunohistochemistry in mesenteric lymphoma (immunoperoxidase; original magnification, $\times 550$). **b:** Negative control for PTHrP immunohistochemistry in mesenteric lymphoma (hematoxylin counterstain; original magnification, $\times 550$). **c:** Small osteoclasts (black arrow) in vertebra from control mouse (tartrate-resistant acid phosphatase stain; original magnification $\times 352$). **d:** Markedly increased number of large osteoclasts (black and white arrows) in a SCID/bg mouse with ATL (day 32) (tartrate-resistant acid phosphatase stain; original magnification, $\times 352$). **e:** Positive CD45RO immunohistochemistry in RV-ATL cells engrafted in the mesentery of a SCID/bg mouse (immunoperoxidase; original magnification, $\times 285$). **f:** Negative CD45RO immunohistochemistry in the thymus of a control mouse (immunoperoxidase; original magnification, $\times 285$). **g:** Epicardial mineralization (white arrows) in a SCID/bg mouse with lymphoma and hypercalcemia (H&E stain; original magnification, $\times 83$). **h:** Peribronchial lymphoma in SCID/bg mouse (H&E stain; original magnification, $\times 22$). **i:** RV-ATL cells forming a nodular mass in the thymus of a SCID/bg mouse (H&E; original magnification, $\times 22$). **j:** Diffuse lymphoma in the spleen of a SCID/bg mouse (top) in comparison with a spleen from a control mouse (bottom) (H&E stain; original magnification, $\times 8$).

Figure 2. Plasma-ionized and total calcium concentrations (mg/dL). Mice engrafted with RV-ATL cells had significantly ($P < 0.001$) greater plasma-ionized calcium levels than controls (6.7 ± 1.0 versus 4.9 ± 0.2). Total calcium levels were also significantly ($P < 0.001$) higher in late lymphoma SCID/bg mice (15.8 ± 2.8) in comparison to age-matched controls (8.7 ± 0.4). Total calcium levels were not statistically different between early control (9.4 ± 0.3) and lymphoma (10.5 ± 1.0) mice. Bars indicate means.

PTHrP mRNA and Protein Expression by Xenografted RV-ATL Cells

Northern blot analysis revealed that RV-ATL cells forming mesenteric lymphoma in SCID/bg mice expressed PTHrP mRNA at very high levels (see Figure 5A). PTHrP mRNA expression in RV-ATL cells was greater than MT-2 cells, a well-established HTLV-1-infected cell line, that has been reported to secrete PTHrP⁴⁰ and was greater than SCC 2/88 cells, a squamous carcinoma cell line, that expresses PTHrP mRNA.⁴¹ No PTHrP mRNA was detectable by Northern blot analysis in normal human peripheral blood lymphocytes, Jurkat cells, and HT1-RV, a cell line immortalized *in vitro* by superinfection of RV-ATL cells with HTLV-1. PTHrP protein expression in RV-ATL cells xenografted in SCID/bg mice was evaluated by immunohistochemistry. The RV-ATL cells xenografted in the mesentery (Figure 1a), liver, spleen, and thymus of the lymphoma-bearing SCID/bg mice were strongly positive for PTHrP protein. PTHrP protein was identified both in the cytoplasm and the nucleus of the RV-ATL cells. The

Figure 3. Plasma PTHrP (1-80) concentrations (pmol/L). Both early and late lymphoma mice had markedly increased circulating PTHrP levels (16.8 ± 11.9 and 61.3 ± 41.8 , respectively) in comparison to age-matched controls (<1.5). Bars indicate means.

Figure 4. Femoral BMD (mg/cm²). BMD was significantly ($P < 0.001$) lower in late lymphoma SCID/bg mice (0.048 ± 0.006) in comparison to age-matched controls (0.059 ± 0.005). BMD was not statistically different between early control (0.049 ± 0.005) and lymphoma (0.051 ± 0.005) mice.

cytoplasmic:nuclear ratio of PTHrP immunohistochemical staining was ~4:1 based on histological examination of 200 positive RV-ATL cells. Other HTLV-1 cell lines were not examined immunohistochemically for PTHrP.

BMD and Histomorphometry

Femur BMD was significantly decreased ($P < 0.001$) in the late ATL group when compared with the control mice, whereas there was no significant difference observed between early ATL and control mice (Figure 4). Also, the late control group mice had a significant increase ($P < 0.01$) in BMD when compared to early control group mice. PTHrP concentrations and BMD were strongly negatively correlated ($r = -0.93$, $P < 0.005$). In addition, there was no significant difference (paired *t*-test, $P = 0.90$) in the BMD of the late ATL animals with bone marrow infiltration by lymphoma cells ($n = 5$) when compared to mice without bone marrow lymphoma ($n = 9$).

To examine osteoclastic bone resorption in this model, bone histomorphometric analysis was performed (Figure 6). Total bone volume (trabecular and cortical) was significantly decreased in both early ($P < 0.05$) and late ATL ($P < 0.001$) mice when compared to age-matched controls. There was no significant difference in percent trabecular bone volume. In contrast, osteoclastic bone resorption, percent osteoclast surface on trabeculae and number of osteoclasts/mm of trabecular bone, were increased two to fourfold in both early and late ATL-bearing SCID/bg mice in comparison to age-matched controls ($P < 0.001$).

Immunophenotypic Characterization of RV-ATL Cells

Analysis of cell surface antigens of the xenografted RV-ATL cells by flow cytometry revealed that CD4 and CD25 were expressed in 98 and 91% of the cells, respectively. CD3 and CD8 antigen expression was absent in 100% of the cells. Cell surface antigen expression in RV-ATL cells

Figure 5. A: Northern blot showing PTHrP mRNA expression in xenografted lymphoma (RV-ATL) from five SCID/bg mice (L13 to L25) in the late ATL group in comparison to HT1-RV and MT-2 cells. Squamous cell carcinoma 2/88 line (SCC) served as positive control. Jurkat (Jrk) and normal human peripheral blood lymphocytes served as negative controls. **B:** Western blot for HTLV-1 Tax expression. Tax (40 kD) was not detected in xenografted lymphoma (RVATL). In comparison, Tax was highly expressed in other HTLV-1-lymphoma cell lines tested (Hut102, SLB-1, HT1-RV, and MT2). Tax also was detected as a fusion protein with gp46 (66 kD) in Hut102 and MT2 cells. Jurkat cells (Jrk) served as the negative control. **C:** RT-PCR for HTLV-1 mRNA expression in RV-ATL cells. **Top:** HTLV-1 total RNA (159 bp) was not detected in RV-ATL cells with 670/671 primers. HTLV-1 total RNA was detected in other HTLV-1 cell lines tested (SLB-1, HT1-RV, and MT-2). Negative control consisted of 729 B cells. **Bottom:** Doubly-spliced Tax/Rex (117 bp) also was not detected in RV-ATL cells with LA78/LA79 primers. Tax/Rex was detected in SLB-1, HT1-RV, and MT-2. Negative control consisted of 729 B cells.

was consistent with that observed in human patients with ATL, and indicates expansion of CD4⁺ cells expressing high levels of IL-2R α (CD25). Immunohistochemical phenotyping of xenografted RV-ATL cells demonstrated cell surface expression of CD45RO (an antigen expressed in most human thymocytes and mature activated T cells) in lymphoma of the mesentery (Figure 1e) and the thymus. Whereas there was absence of CD45RO expression in the spleen and thymus of control SCID/bg mice confirming the human origin of the lymphoma (Figure 1f).

HTLV-1 Provirus Copy Number and Tax Expression in Xenografted RV-ATL Cells

To accurately determine the number of integrated viral genomes in the RV-ATL and HT1-RV cell lines we performed quantitative real-time PCR. The RV-ATL line had 1.5 copies of provirus. The HT1-RV cell line, which was created by superinfection of the RV-ATL line with HTLV-1, had ~10 copies per cell. By Western blot analysis, RV-ATL cells did not express Tax (Figure 5B) or any structural HTLV-1 proteins, p19, p24, or gp46. Furthermore, *tax/rex* doubly spliced or total viral mRNA was not de-

Figure 6. Histomorphometry of lumbar vertebrae in SCID/bg mice engrafted with RV-ATL cells (*, $P < 0.05$ different from age-matched control; **, $P < 0.001$ different from age-matched controls).

tected by RT-PCR (Figure 5C). The oligonucleotide primers, LA78/LA79 and 670/671, designed to amplify doubly spliced *tax/rex* RNA or all species of viral RNA, respectively, did not detect any expression of *tax*. In contrast, as demonstrated by Western blot analysis, HT1-RV, MT-2 and SLB-1 cells expressed high levels of Tax, p19, p24, and gp46. RT-PCR also demonstrated *tax/rex* mRNA in all three lines with the oligonucleotide primers.

Discussion

We report the establishment of a model of human T-cell lymphoma and HHM in SCID/bg mice with leukemic cells from a patient with HTLV-1-induced ATL. The primary ATL cells xenografted into SCID/bg mice maintained their morphological, immunohistochemical, and molecular features. In addition, xenografted ATL cells in SCID/bg mice induced hypercalcemia by secretion of PTHrP, which stimulated osteoclastic bone resorption.

SCID mice have been used as xenograft recipients for a variety of human neoplastic cells including hematological malignancies.^{32,42-46} The *scid* mutation was first described in 1983 in C.B-17 mice,⁴⁷ and is associated with a disrupted gene that encodes a DNA-dependent protein kinase, identified as *Prkdc*,⁴⁸ which is involved in immunoglobulin and T-cell receptor gene rearrangement. The mutation prevents T- and B-lymphocyte maturation. Despite this defect, engraftment of human hematological cells in SCID mice has been reported to be only modestly successful.^{49,50} NK-cell activity has been shown to play a key role in the rejection of human lymphoid neoplasms in SCID mice.^{31,51} We have previously used different methods to improve xenograft efficiency of ATL cells in SCID mice including γ -irradiation and administration of α -AGM₁ antiserum (anti-NK-cell antibodies).³¹ These methods were relatively efficient at reducing NK cell activity, but cumbersome to perform. To circumvent these difficulties we used SCID/bg mice in this investigation. As described, the *beige* mutation is responsible for a selective defect in the NK-cell immune response.⁵¹ When combined with the *scid* mutation, the *beige* mutation creates more favorable conditions for engraftment of human lym-

phoid neoplasms in mice, consequently allowing the establishment of a reliable animal model.

Most patients with ATL will develop hypercalcemia, which represents a life-threatening condition.¹⁵ PTHrP has been shown to be a causative factor of hypercalcemia in animals bearing human solid tumors.^{12,52} In ATL patients, PTHrP also has been identified as an important mediator of hypercalcemia, but other humoral factors such as IL-1 β and TGF- β have also been proposed to be involved.^{23,24} Previous studies examining the regulation of PTHrP in ATL cells were conducted *in vitro*, because of the lack of a reproducible animal model of ATL-associated HHM. Consequently, little is known about the specific mechanisms regulating PTHrP expression in ATL cells *in vivo*, and the interactions between PTHrP and other humoral factors in the development of hypercalcemia in humans with ATL.

A previous investigation³¹ by our laboratory reported the tumorigenic potential of ATL cells (RV-ATL) and HTLV-transformed cell lines (SLB-1 and JLB-II) by comparing the engraftment efficiency in SCID mice. In the present study, we examined and confirmed the central role played by PTHrP in the induction of hypercalcemia and increased osteoclastic resorption leading to bone loss in SCID/bg mice xenografted with ATL cells. An alternative model of HHM using SCID mice was reported by Takaori-Kondo and colleagues.⁵³ In contrast to our study, the authors showed increased levels of C-terminal PTHrP, but did not measure circulating levels of N-terminal PTHrP, the region of PTHrP that contains PTH-like activity, and lymphoma-bearing mice did not have increased osteoclastic bone resorption. Both increased circulating N-terminal PTHrP and excessive osteoclastic bone resorption are known to be important characteristic features of HHM observed in human patients with ATL.

PTHrP gene expression has been shown to be up-regulated by the HTLV-1 oncoprotein Tax *in vitro*.¹⁹ Dittmer and colleagues²⁰ have demonstrated that Tax interacts with the transcription factors Ets1 and Sp1 to transactivate the PTHrP P2 promoter when transfected in osteosarcoma OsA-CL cells. Tax can also mediate its effects on the PTHrP gene by the cellular transcription factors AP-1 and AP-2 as proposed by Prager and colleagues.⁵⁴ We were not able to detect Tax and other HTLV-1 viral proteins by Western blot analysis or HTLV-1 *tax/rex* mRNA by RT-PCR in ATL cells xenografted in SCID/bg. These data are consistent with the literature regarding the expression of HTLV-1 *tax/rex* mRNA in ATL cells from human patients.²¹ In contrast, HTLV-1 Tax protein and *tax/rex* mRNA were easily detectable in HT1-RV cells, a cell line produced by superinfection of RV-ATL cells with HTLV-1. Interestingly, no PTHrP mRNA was detectable by Northern blot analysis in HT1-RV cells. We determined the number of HTLV-1 proviral copy by quantitative real-time PCR using primers specifically designed to amplify the *tax* gene. We established that RV-ATL cells had 1.5 copies of provirus, and our results are consistent with published data for primary lymphocyte cell lines transformed by HTLV-1.⁵⁵ In contrast, the HT1-RV cells had 10 copies of HTLV-1 provirus. As we have previously reported, RV-ATL cells, as other ATL cell

lines, carry deletions of the provirus while retaining the *tax/rex* sequence.³²

Primary cell lines from human patients with ATL were shown to produce a wide variety of cytokines, including IL-2, TGF- β , TNF- β , and IL-1. IL-2 can up-regulate PTHrP gene expression in ATL cells by increasing its mRNA stability.²⁶ However, others have reported that IL-2 mRNA was not detectable by RT-PCR in ATL cells xenografted into SCID mice.⁵⁶ Our laboratory and others have previously demonstrated that TGF- β up-regulates PTHrP gene expression in multiple normal and neoplastic tissues,^{29,57–60} but there is no report addressing the effect of TGF- β on PTHrP secretion and expression in ATL cells. It will be useful to study the effect of TGF- β on both PTHrP gene transcription and mRNA stability *in vivo* using the SCID/bg model of human ATL.

Our laboratory has reported that PTHrP can bind to the human MT-2 cells (presumably by interacting with the PTH-1 receptor), and PTHrP inhibited cell growth of the MT-2 cells.⁶¹ Several reports have demonstrated that PTHrP modulates growth in different normal and cancer cells.^{62–67} The proliferative *versus* antiproliferative effects of PTHrP are dependent on the cell type. Falzon and Du⁶⁷ have shown that PTHrP had an antimitogenic effect in the human breast cancer cell line, MCF-7, using an autocrine/paracrine pathway mediated through the cell surface PTH-1 receptor. They also have shown that PTHrP exerted a mitogenic effect through the intracrine pathway, which correlated with nuclear accumulation of PTHrP. The exact mechanism by which nuclear localization of PTHrP stimulates cell growth is unknown, but nuclear PTHrP can inhibit apoptosis.⁶⁸ We examined the expression of the human PTH-1 receptor in four human ATL cell lines (RV-ATL, SLB-1, MT-2, and HT1-RV) by RT-PCR, and our data (not shown) demonstrated that the PTH-1 receptor was expressed in MT-2 cells (moderate), HT1-RV cells (low), and SLB-1 cells (very low), but was not expressed in RV-ATL cells. Interestingly, immunohistochemistry was strongly positive for PTHrP both in the cytoplasm and nucleus of RV-ATL cells xenografted into SCID/bg mice. The exact role of nuclear PTHrP on regulation of proliferation or apoptosis in ATL cells remains to be determined both *in vitro* and *in vivo*. Our model will enable us to examine the effect of PTHrP on ATL cell growth *in vivo*.

In conclusion, the SCID/bg mouse model of human ATL will be useful to study the regulation of PTHrP and the interrelationships between PTHrP and cytokines produced by ATL cells in the induction of HHM *in vivo*. The model is reproducible and mimics the disease observed in human patients with ATL. In addition, the model will be useful to develop new therapeutic strategies for the treatment of hypercalcemia observed in patients with ATL.

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