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Effects of parathyroid hormone-related protein and macrophage inflammatory protein-1 α in Jurkat T-cells on tumor formation *in vivo* and expression of apoptosis regulatory genes *in vitro*

Sherry T. Shu¹, Wessel P. Dirksen², Lisa G. Lanigan², Chelsea K. Martin², Nanda K. Thudi^{2,*}, Jillian L. Werbeck³, Soledad A. Fernandez⁴, Blake E. Hildreth III², and Thomas J. Rosol²

¹Department of Microbiology and Molecular Genetics, School of Medicine, University of Pittsburgh, Pittsburgh, PA 15219

²Department of Veterinary Biosciences, The Ohio State University, Columbus, OH, USA

³Cancer Biology and Genetics, Memorial Sloan-Kettering Cancer Center, New York, NY 10065

⁴Center for Biostatistics, The Ohio State University, Columbus, OH, USA

Abstract

Parathyroid hormone-related protein (PTHrP) and macrophage inflammatory protein-1 α (MIP-1 α) have been implicated in the pathogenesis of adult T-cell leukemia/lymphoma, but their effects on T-cells have not been well studied. Here we analyzed the functions of PTHrP and MIP-1 α on T-cell growth and death both *in vitro* and *in vivo* by overexpressing either factor in human Jurkat T-cells. PTHrP or MIP-1 α did not affect Jurkat cell growth *in vitro*, but PTHrP increased their sensitivity to apoptosis. Importantly, PTHrP and MIP-1 α decreased both tumor incidence and growth *in vivo*. To investigate possible mechanisms, polymerase chain reaction (PCR) arrays and real-time reverse transcription (RT)-PCR assays were performed. Both PTHrP and MIP-1 α increased the expression of several factors including signal transducer and activator of transcription 4, tumor necrosis factor α , receptor activator of nuclear factor κ B ligand and death-associated protein kinase 1, and decreased the expression of inhibitor of DNA binding 1, interferon γ and CD40 ligand in Jurkat cells. In addition, MIP-1 α also increased the expression of transcription factor AP-2 α and PTHrP increased expression of the vitamin D3 receptor. These data demonstrate that PTHrP and MIP-1 α exert a profound antitumor effect presumably by increasing the sensitivity to apoptotic signals through modulation of transcription and apoptosis factors in T-cells.

Keywords

Lymphocytes; cell lines and animal models; cytokine production and paraneoplastic conditions

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Correspondence: Dr. Thomas Rosol, Ohio State University, 1925 Coffey Road, Columbus, Ohio 43210. Tel: (616) 292-4265. Fax: (614) 292-3544. rosol.1@osu.edu.

*Current affiliation for N. K. Thudi: Department of Radiation Oncology, University of Alabama, Birmingham, AL, USA.

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Introduction

Adult T-cell leukemia/lymphoma (ATLL) is caused by human T-lymphotropic virus type 1 (HTLV-1) [1]. HTLV-1 is a *Deltaretrovirus* that transforms mainly human CD4+ T-lymphocytes. Only about 5% of HTLV-1-infected individuals develop ATLL after a long incubation time (about 20 years). Patients with ATLL have a high incidence of humoral hypercalcemia of malignancy (HHM) induced by increased osteoclastic bone resorption. We and others have shown that osteoclast stimulating factors expressed from HTLV-1-infected T-cells play a major role in the HHM that develops in patients with ATLL [2–8]. Parathyroid hormone-related protein (PTHrP) and macrophage inflammatory protein-1 α (MIP-1 α) are two important proteins that have been found in the serum of patients with ATLL and have been implicated in the development of HHM and osteolytic bone lesions [3,9,10].

PTHrP is a polyhormone that increases osteoclastic bone resorption by inducing the expression of osteoclast activator, receptor activator of nuclear factor κ B (NF- κ B) ligand (RANKL) in osteoblasts [11]. MIP-1 α increases osteoclast activity through mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways in osteoblasts and bone marrow stromal cells [12]. Although the pathogenic effects of these two factors on bone resorption have been demonstrated, their effects on T-cells specifically, if any, have not been well studied.

Previously, we have shown that the expression of PTHrP and MIP-1 α was increased by HTLV-1 in human peripheral blood mononuclear cells in an *ex vivo* transformation and immortalization assay [13], suggesting that these two factors may play a role in the early stages of HTLV-1 infection in T-cells. In fact, PTHrP has been shown to regulate cell growth, differentiation and survival [14]. Up-regulation of PTHrP in cancer cells has been demonstrated in breast, prostate and lung cancer, neuroblastic tumors, ATLL and other cancers [15]. PTHrP functions mainly by binding to its membrane receptor, PTH1R. It has been shown that a neutralizing antibody against PTHrP and an antagonist to PTH1R both inhibit renal cell carcinoma growth *in vitro* and *in vivo* by promoting cell death [16,17]. The up-regulation of MIP-1 α has been reported in multiple myeloma [18], and it has been shown to increase the proliferation, migration and survival of plasma cells and multiple myeloma cells [19,20]. However, its effect on T-cell fate is currently unknown.

In this study, we used overexpression methodology to investigate the role of PTHrP and MIP-1 α on T-cell growth both *in vitro* and *in vivo*. PTHrP increased the sensitivity of T-cells to an apoptosis-inducing agent *in vitro*. Using a xenograft mouse model, we found that both factors significantly decreased tumor formation *in vivo*. We also identified several potential candidates that may be the downstream targets of PTHrP and MIP-1 α in T-cells that led to their distinct phenotypes. Thus, PTHrP and MIP-1 α not only cause HHM in patients with ATLL in the later stages of the disease, but also affect T-cell signaling and growth, which may be important in the early stages of disease pathogenesis.

Methods

Cells

HTLV-1-infected cell lines and the HTLV-1-negative T-cell Jurkat line were cultured as previously described [21].

RNA extraction, reverse transcription, RT-PCR and real-time RT-PCR

RNA extraction, reverse transcription (RT), RT-polymerase chain reaction (PCR) and real-time RT-PCR were performed as previously described [22–24]. In brief, total RNA was

extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) and DNase-treated using TURBO DNA-free™ (Applied Biosystems, Carlsbad, CA). Approximately 0.5 µg of RNA was reverse transcribed with SuperScript II Reverse Transcriptase (Invitrogen) and submitted for real-time RT-PCR analysis using primers for human β_2 -microglobulin (HB2M): forward GAT GAG TAT GCC TGC CGT GTG and reverse CAA TCC AAA TGC GGC ATC T; CD40 ligand (CD40LG): forward ATC AGA ATC CTC AAA TTG CGG CAC A and reverse CGG TCA GCT GTT TCC CAT TTT CCA; death-associated protein kinase 1 (DAPK1): forward CGT GGT GGC CCT CTG TGA AGC and reverse CGT TGT TGG CCG CAA GGT GC; tumor necrosis factor α (TNF α): forward CCC TCT GGC CCA GGC AGT CA and reverse CGG CGG TTC AGC CAC TGG AG; interferon γ (IFN γ): forward GGC TGT TAC TGC CAG GAC CCA and reverse TCC GCT ACA TCT GAA TGA CCT GCA T; RANKL: forward AGA GAA AGC GAT GGT GGA TG and reverse TTT ATG GGA ACC AGA TGG GA; vitamin D3 receptor (VDR): forward CTG TGA AGG CTG CAA AGG CTT CTT CA and reverse GGT TGT CCT TGG TGA TGC GGC A; interleukin 4 (IL-4): forward GAC TGC ACA GCA GTT CCA CAG GC and reverse CTG GTT GGC TTC CTT CAC AGG ACA G; interleukin 12 α (IL-12A): forward TGC CGG CTC AGC ATG TGT CC and reverse GCC ACG GGG AGG TTT CTG GC; inhibitor of DNA binding 1 (ID1): forward CCT CAA CGG CGA GAT CAG and reverse CGC TTC AGC GAC ACA AGA T; paired box 6 (PAX6): forward TAA GCG GGA GTG CCC GTC CA and reverse GCC GTC TGC GCC CAT CTG TT; signal transducer and activator of transcription 4 (STAT4): forward CTC TCA GCC TTG CGA AGT TT and reverse TGG AGA ATG TGG CTC TGT TG; transcription factor AP-2 α (TFAP2A): forward CGC GCT CAG CTC AGG ACT CG and reverse CTT GGA CAG GGA CAC GGG GC. Real-time RT-PCR for CD40LG, DAPK1, RANKL, ID1 and TNF α was repeated twice on four replicate samples. Real-time RT-PCR for VDR, PAX6, TFAP2A, STAT4, IFN γ and IL-12A was repeated twice on five replicate samples. Real-time RT-PCR for PTHrP and MIP-1 α was performed in duplicate and repeated three times. Representative data are shown in Fig. 1.

Overexpression of PTHrP, MIP-1 α and luciferase in Jurkat T-cells

Full-length human PTHrP (–36–141) and human MIP-1 α cDNAs were cloned into pcDNA3.1 vector [25]. Jurkat cells were transfected with the pcDNA3.1 vector alone (Jurkat-pcDNA), or pcDNA3.1 containing PTHrP (Jurkat-PTHrP) or MIP-1 α (Jurkat-MIP-1 α) cDNA sequences. Stably transfected cells were isolated using G418 (1 mg/mL) selection for 2 weeks. The cell lines were transduced with a lentiviral vector containing the yellow fluorescent protein-luciferase fusion gene [4] to generate the Jurkat-pcDNA-luc, Jurkat-PTHrP-luc and Jurkat-MIP-1 α -luc cell lines.

Measurement of PTHrP and MIP-1 α protein concentrations

Human MIP-1 α levels in culture media were measured using a Human CCL3/MIP-1 α Quantikine enzyme-linked immunosorbent assay (ELISA) kit (R&D systems, Minneapolis, MN). PTHrP concentrations in culture media were measured using an immunoradiometric assay (IRMA) kit (Diagnostic Systems Laboratories, Webster, TX). MIP-1 α ELISA was performed in quadruplicate. PTHrP IRMA was performed in duplicate and the assay was repeated twice with similar results. Representative results are shown in Figs. 1(C) and 1(D), respectively.

Cell proliferation and viability assays

For cell proliferation assays, 12 500 cells in 100 µL of culture medium were seeded in 96-well plates and cultured for up to 5 days. Cell proliferation was measured daily using a CellTiter 96® non-radioactive cell proliferation assay kit (Promega, Madison, WI). Data were normalized to the data obtained immediately after seeding the cells and were represented as ratios. For cell viability assays, 20 000 cells in 2 mL of culture medium were

seeded in 24-well plates and cultured for up to 4 days. Total number of viable cells were counted by the trypan blue exclusion method immediately after seeding and daily afterward. Data were normalized to the data obtained immediately after seeding and were represented as ratios. Assays were performed in quadruplicate and repeated three times. The average ratios from three experiments are shown in Fig. 2.

Cell apoptosis assays

Four million cells in 4 mL of culture medium were incubated with 2 or 10 μ M of camptothecin or vehicle control overnight in six-well plates. Apoptotic cells were measured using an *in situ* cell death detection kit (Roche Applied Science, Indianapolis, IN). Camptothecin (Sigma-Aldrich, St. Louis, MO) was used as an apoptosis-inducing agent. Assays were performed in triplicate and repeated twice. Representative data are shown in Fig. 3.

In vivo tumor growth and bioluminescent imaging

Four- to six-week-old male NOD/SCID (non-obese diabetic/severe combined immunodeficiency; NOD.CB17-PRKDCSCID/J) mice (The Jackson Laboratory, Bar Harbor, ME) were housed and treated in accordance with the University Laboratory Animal Resources guidelines, and experimental protocols were approved by the Institutional Laboratory Animal Care and Use Committee (IACUC protocol number 2004A0191). A total of 5×10^6 Jurkat-pcDNA-luc, Jurkat-PTHrP-luc or Jurkat-MIP-1 α -luc cells were injected into mice (10 mice/group) subcutaneously between the shoulders. Mice were weighed weekly. Bioluminescent imaging was performed using an *in vivo* imaging system (IVIS 100; Caliper Life Sciences, Hopkinton, MA) as previously described [26] after the inoculation of cells at 1, 2, 3, 5 and 7 weeks. Photon signals were quantified using LivingImage software version 2.2 (Caliper Life Sciences). Data were normalized to the signal obtained immediately after tumor cell injection and were represented as ratios. Mice were sacrificed 7 weeks after injection of tumor cells. A complete necropsy was performed and the subcutaneous tumors were weighed and measured using an electronic caliper. Tumor volumes were calculated using the formula $L \times W^2/2 = \text{mm}^3$, where L is the length and W is the width of the tumor [27]. The tumors were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned, stained with hematoxylin and eosin (H&E) and evaluated histopathologically. The H&E stained slides were scanned using an Aperio ScanScope XT Scanner (Vista, CA) at $\times 400$ and tumor areas were measured using Image Scope viewing software.

PCR arrays

RNA from Jurkat-pcDNA-luc, Jurkat-MIP-1 α -luc and Jurkat-PTHrP-luc cells was DNase-treated using the Turbo DNA-free kit (Applied Biosystems) and reverse transcribed using the RT² First Strand Kit (SABioscience, Frederick, MD). The cDNA was mixed with RT² SYBR Green qPCR Master Mix (SABioscience) and used for a Transcription Factor PCR array and Human Apoptosis PCR array (SABioscience). Data analysis was performed using PCR Array Data Analysis Software (SABioscience).

Statistical analysis

Results are displayed as mean $\pm$ standard deviation (SD). A natural logarithm transformation was used to normalize all non-normally distributed data, which allowed for parametric statistical analyses; however, data that had unequal variances after transformation were compared with non-parametric statistical analyses. Apoptosis ratios were compared between (1) Jurkat-pcDNA-luc and Jurkat-PTHrP-luc and (2) Jurkat-pcDNA-luc and Jurkat-MIP-1 α -luc cell lines after treatment with vehicle, 2 μ M and 10 μ M of camptothecin with a two-way

analysis of variance (ANOVA) and a Bonferroni *post hoc* analysis. Apoptosis ratios were similarly compared within each cell line between all treatment groups. Photons and percent change in mouse body weight from the time of tumor cell injection to the time of sacrifice were compared between all cell lines with a one-way ANOVA and a Tukey's *post hoc* analysis. Tumor cross-sectional areas were compared between all cell lines with a Kruskal–Wallis test and a Dunn's multiple comparison test. Cell proliferation and viability at each time point and relative gene expression were compared between (1) Jurkat-pcDNA-luc and Jurkat-PTHrP-luc and (2) Jurkat-pcDNA-luc and Jurkat-MIP-1 α -luc cell lines with a one-way ANOVA and a Dunnett's *post hoc* analysis. All analyses were performed using SAS 9.2 (SAS Institute Inc., Cary, NC) and Prism ver. 5.00 (GraphPad Software, Inc., San Diego, CA) software with the level of statistical significance established at $p < 0.05$.

Results

Overexpression of PTHrP and MIP-1 α in Jurkat cells

To investigate the effects of PTHrP and MIP-1 α on T-cells, Jurkat cells, a CD4⁺ T-cell leukemia line that does not express or secrete PTHrP and MIP-1 α , were stably transfected with a pcDNA3.1 vector containing either full-length PTHrP or MIP-1 α cDNA. Jurkat cells transfected with an empty pcDNA3.1 vector were used as controls. In order to visualize *in vivo* tumor growth, cells were also transduced with lentiviral vectors expressing luciferase. Real-time RT-PCR was performed to measure the mRNA expression of PTHrP and MIP-1 α . PTHrP mRNA and protein was expressed and secreted at a high level in Jurkat-PTHrP-luc cells, but was near background levels in Jurkat-pcDNA-luc and Jurkat-MIP-1 α -luc cells [Figs. 1(A) and 1(C)]. It has been shown that PTHrP mRNA expression levels in leukemic cells from patients with ATLL were up-regulated 10-fold compared to peripheral blood mononuclear cells (PBMCs) [28]. PTHrP mRNA expression ranged from undetectable to a ratio of 0.075 PTHrP/HB2M in HTLV-1⁺ cell lines *in vitro* [29,30]. Similar to PTHrP, MIP-1 α mRNA and protein was expressed and secreted at a high level in Jurkat-MIP-1 α -luc cells, but was near background levels in Jurkat-pcDNA-luc and Jurkat-PTHrP-luc cells [Figs. 1(B) and 1(D)]. Messenger RNA expression of MIP-1 α in HTLV-1⁺ T-cell lines *in vitro* ranged from undetectable to a ratio of 0.11 MIP-1 α /HB2M [30]. These data suggest that Jurkat-PTHrP-luc and Jurkat-MIP-1 α -luc lines are clinically relevant.

PTHrP and MIP-1 α did not affect Jurkat cell growth but PTHrP increased sensitivity to camptothecin

To investigate the effects of PTHrP and MIP-1 α on T-cell growth, we performed cell proliferation and trypan blue exclusion assays. PTHrP and MIP-1 α did not affect the proliferation rates or viability of Jurkat cells [Figs. 2(A) and 2(B)].

To investigate the effects of overexpressing PTHrP and MIP-1 α on the sensitivity to apoptosis, Jurkat-pcDNA-luc, Jurkat-PTHrP-luc and Jurkat-MIP-1 α -luc cells were treated with the apoptosis-inducing agent, camptothecin (2 μ M or 10 μ M), and the apoptotic cells were measured by TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay. As shown in Fig. 3, camptothecin significantly increased ($p < 0.01$ for 2 μ M; $p < 0.001$ for 10 μ M) apoptosis of the Jurkat-PTHrP-luc cells when compared to Jurkat-pcDNA-luc cells. We also found that live:dead cell ratios were decreased when Jurkat-PTHrP cells were treated with 500 ng/mL anti-Fas antibody ($68 \pm 1.9\%$ vs. $76 \pm 4.1\%$ in control cells) or 1 μ M staurosporine ($56 \pm 1.7\%$ vs. $73 \pm 2.0\%$ in control cells). The effects of the apoptotic agents on Jurkat-MIP1 α -luc cells were variable. The result with PTHrP was unexpected since we have previously shown that PTHrP was up-regulated during human T-cell immortalization by HTLV-1 *in vitro*, suggesting that it might facilitate the transformation of T-cells [13].

PTHrP and MIP-1 α decreased Jurkat cell tumor formation *in vivo*

To investigate the effects of PTHrP and MIP-1 α on tumor formation of Jurkat T-cells *in vivo*, the Jurkat-pcDNA-luc, Jurkat-PTHrP-luc or Jurkat-MIP-1 α -luc cells were injected into mice subcutaneously and tumor formation was monitored by *in vivo* bioluminescent imaging (BLI). There were detectable bioluminescent signals in all mice immediately following injection, which demonstrated successful subcutaneous injections, and these data served as baseline values. In order to control for differences in luciferase gene expression levels between the cell lines and differences in the cell numbers injected into the mice, photon data from each week were normalized to the baseline bioluminescence. One week after injection, a majority of the mice lost much of the photon signal, indicating death of the Jurkat cells. However, tumor formation started to increase over time. Four weeks after injection, visible tumor formation was observed. By week 5, 60% (6/10) of the mice that received Jurkat-pcDNA-luc cells had tumors, whereas only 10% (1/10) of the mice that received Jurkat-PTHrP-luc cells and none of the mice that received Jurkat-MIP-1 α -luc cells formed measurable tumors. Signals from BLI accurately represented the progression and location of tumor growth when compared to visible tumor formation over time. At week 7, there was a significant decrease ($p < 0.05$) in the BLI signals from Jurkat-PTHrP-luc and Jurkat-MIP-1 α -luc mice [Fig. 4(A)]. This represented a 16-fold and 66-fold reduction in viable tumor mass in the Jurkat-PTHrP-luc and Jurkat-MIP-1 α -luc tumors, respectively. There was no significant difference in mouse body weight among the three groups (data not shown). At the time of sacrifice (7 weeks after injection), 10/10 mice with Jurkat-pcDNA-luc, 10/10 mice with Jurkat-PTHrP-luc and 3/10 mice with Jurkat-MIP-1 α -luc formed tumors that were measurable during necropsy [Fig. 4(B)]. Histologically, the tumors consisted of solid sheets of round lymphoma cells with prominent mitoses and apoptosis of the tumor cells. Many of the tumors had large regions of necrosis, which ranged from a few percent to 80% of the tumor. Note that even some of the smaller tumors had large regions of central necrosis [Fig. 4(B)]. This was especially striking in the Jurkat-PTHrP-luc tumors. The cross-sectional areas of the tumors were significantly decreased ($p < 0.05$) seven- and 18-fold, respectively, in mice that received Jurkat-PTHrP-luc ($22 \pm 8 \text{ mm}^2$) and Jurkat-MIP-1 α -luc ($8.8 \pm 2.8 \text{ mm}^2$) when compared to mice that received Jurkat-pcDNA-luc cells ($156 \pm 38 \text{ mm}^2$) [Fig. 4(B)]. These data demonstrated that expression of PTHrP and MIP-1 α had dramatic antitumor effects *in vivo*.

PTHrP and MIP-1 α regulated expression of apoptosis genes and transcription factors in Jurkat cells

To identify possible mechanisms by which PTHrP and MIP-1 α affect apoptosis, we measured the expression of apoptosis-related genes in Jurkat-PTHrP-luc and Jurkat-MIP-1 α -luc cells compared to Jurkat-pcDNA-luc cells using a Human Apoptosis PCR array. The expression of CD40LG mRNA was markedly decreased (77-fold in Jurkat-PTHrP-luc cells and 41-fold in Jurkat-MIP-1 α -luc cells) when compared to Jurkat-pcDNA-luc cells. The expression of DAPK1 mRNA was markedly increased (48-fold in Jurkat-PTHrP-luc cells and 26-fold in Jurkat-MIP-1 α -luc cells). TNF α mRNA was moderately increased, 1.2-fold in Jurkat-PTHrP-luc cells and 4.1-fold in Jurkat-MIP-1 α -luc cells.

Real-time RT-PCR confirmed the relative changes observed with the PCR array. As shown in Figs. 5(A)–5(C), overexpression of PTHrP and MIP-1 α greatly decreased the expression of CD40LG ($p < 0.01$), and increased the expression of DAPK1 and TNF α in Jurkat cells ($p < 0.01$ and < 0.05 , respectively). It has been reported that CD40LG blocks apoptosis [31,32], DAPK1 induces apoptosis in the presence of apoptosis-stimulating agents [33,34] and TNF-mediated apoptosis is involved in the deletion of mature T-cells after antigenic stimulation [35]. Our data support a role for these three factors as mediators of apoptosis in Jurkat cells transfected with PTHrP or MIP-1 α .

As mentioned, CD40LG was dramatically down-regulated in the Jurkat-PTHrP-luc and Jurkat-MIP-1 α -luc cells. Regulators of CD40LG expression include IFN γ , IL-12 and IL-4. IFN γ has been shown to increase CD40LG expression in endothelial cells, smooth muscle cells and macrophages [36,37], but inhibited CD40LG expression in T-lymphocytes [38,39]. CD40LG expression was down-regulated by IL-4 and up-regulated by IL-12 in T-cells [40]. Therefore, we measured the mRNA expression of IFN γ , IL-4 and IL-12 in Jurkat-pcDNA-luc, Jurkat-PTHrP-luc and Jurkat-MIP-1 α -luc cells by real-time RT-PCR [Fig. 5(D) and data not shown]. IL-4 expression was decreased 3.1- and 2.2-fold in Jurkat-PTHrP-luc and Jurkat-MIP-1 α -luc cells, respectively ($p < 0.001$), indicating that decreased CD40LG expression in these cells was not mediated through IL-4. IL-12A was up-regulated 4.3-fold, but only in Jurkat-PTHrP-luc cells ($p < 0.01$), while IL-12B was unchanged in both cell lines (data not shown). IFN γ is the major proinflammatory cytokine in a T-helper type 1 (TH1) response. Its expression is regulated by a number of genes. Surprisingly, we found a significant decrease in IFN γ expression in both Jurkat-PTHrP-luc and Jurkat-MIP-1 α -luc cells ($p < 0.01$) [Fig. 5(D)] that was similar to the decrease in CD40LG mRNA [Fig. 5(A)], suggesting that IFN γ may positively regulate CD40LG expression in Jurkat cells.

It has been previously shown that RANKL increases the expression of TNF α and DAPK1 [41–43], while calcitriol (active form of vitamin D) inhibits IFN γ expression through its receptor VDR [44,45]. Therefore, we measured their level of expression by real-time RT-PCR. RANKL was not expressed in Jurkat-pcDNA-luc cells, but it was induced in both Jurkat-PTHrP-luc and Jurkat-MIP-1 α -luc cells ($p < 0.05$) [Fig. 5(E)]. The VDR was up-regulated in Jurkat-PTHrP-luc cells ($p < 0.01$), but not in Jurkat-MIP-1 α -luc cells [Fig. 5(F)].

In order to examine the effects of PTHrP and MIP-1 α overexpression on the expression of transcription factors in Jurkat T-cells, a Transcription Factor RT² Profiler^{TM} PCR array was used. The expression of four transcription factors was increased, including PAX6, STAT4 and TFAP2A (or activating enhancer binding protein 2 α) by 6.4-, 4.1- and 32-fold in Jurkat-PTHrP-luc cells when compared to Jurkat-pcDNA-luc cells, respectively. The expression of ID1 was decreased 7.2-fold in Jurkat-PTHrP-luc cells. To validate the results from the PCR array, real-time RT-PCR for these transcription factors was performed. As shown in Fig. 6(A), ID1 expression was significantly decreased in Jurkat-PTHrP-luc and Jurkat-MIP-1 α -luc cells ($p < 0.01$). The expression of PAX6 and STAT4 was significantly increased in both Jurkat-PTHrP-luc and Jurkat-MIP-1 α -luc cells ($p < 0.01$) [Figs. 6(B) and 6(C)]. The expression of TFAP2A was significantly increased only in Jurkat-MIP-1 α -luc cells ($p < 0.01$) [Fig. 6(D)]. These data suggest that the effects of PTHrP and MIP-1 α are mediated through several transcription factors.

HTLV-1-infected T-cells expressed MIP-1 α receptor CCR1

MIP-1 α functions through the binding of membrane receptors, including CCR1. In order to understand whether MIP-1 α can function in an autocrine and/or paracrine fashion in T-cells, we measured CCR1 mRNA levels in Jurkat and several HTLV-1-infected T-cell lines using RT-PCR. As shown in Fig. 7, CCR1 expression was detected in Jurkat, MT2, Hut102, MET-1, C8166 and RV-ATL cells. RNA from PBMCs obtained from healthy donors served as positive controls. These data suggested that MIP-1 α may regulate T-cells through a paracrine and/or autocrine pathway. In contrast, it has been previously shown that Jurkat cells do not express PTH1R, the major receptor for PTHrP [13]. This suggests that PTHrP functions through an intracrine pathway in Jurkat cells, which is the pathway that has been shown to regulate cell proliferation and apoptosis [46,47].

Discussion

ATLL is caused by HTLV-1 infection and the average incubation time is more than 20 years. During the multi-stage carcinogenesis of ATLL, T-cell transformation, proliferation and progression require the effects of humoral and local factors. In previous studies, we and others have shown that both PTHrP and MIP-1 α are important for HHM development in ATLL [3,4,7,8,48,49]. Interestingly, we also found that both factors were expressed in the early stages of *ex vivo* T-cell transformation and immortalization [13]. We hypothesized that both PTHrP and MIP-1 α not only induce HHM development during the later disease stages, but also play important roles in T-cell transformation and proliferation at the early stages of ATLL.

The functions of PTHrP and MIP-1 α have been studied in a number of cell types. Overexpression of PTHrP inhibited pancreatic beta cell death *in vitro* and *in vivo* [50] and increased cell survival, migration and invasion of colon cancer cells [51]. PTHrP is a mitogenic factor for renal [52] and rat intestinal cells [53]. PTHrP may play a role in colon tumor invasion and metastasis by enhancing cell adhesion to specific extracellular matrix proteins [53]. In vascular smooth muscle cells and MCF-7 breast cancer cells, PTHrP stimulated mitogenesis after nuclear localization, but inhibited proliferation after binding to surface receptors [46,54,55]. Therefore, the paracrine/autocrine functions of PTHrP may counteract its intracrine functions within the same cells. In addition, PTHrP is able to promote apoptosis in rat intestinal cells induced by serum deprivation through an intracrine mechanism, possibly through the Bcl-2 family of proteins [52]. Similar to PTHrP, MIP-1 α has been shown to enhance the proliferation, migration and survival of plasma cells and multiple myeloma cells through the AKT/protein kinase B (PKB) and MAPK pathways, but was independent of the extracellular-regulated kinase (ERK) pathway *in vitro* [20,56]. *In vivo*, mice receiving myeloma cells transfected with an antisense construct of MIP-1 α had decreased tumor growth and a prolonged survival [57]. Administration of neutralizing anti-MIP-1 α antibodies decreased tumor load and prevented splenomegaly in mice with myeloma [25]. The mechanisms for the increased survival of myeloma cells may include an autocrine effect induced by direct receptor binding and/or an indirect effect caused by the increased adherence to bone marrow stromal cells and subsequent local IL-6 production, which is a mitogenic factor for myeloma cells [20].

In this study, we generated T-cells that overexpressed PTHrP and MIP-1 α to investigate the roles of these two factors on T-cell growth *in vivo* and *in vitro*. In Jurkat T-cells, we found that both PTHrP and MIP-1 α did not affect cell proliferation *in vitro*, but PTHrP increased the sensitivity to apoptosis-inducing agents, without increasing the basal level of Jurkat cell apoptosis (Fig. 3 and data not shown). Both PTHrP and MIP-1 α significantly decreased tumor formation of Jurkat cells *in vivo*. The similar *in vivo* phenotype caused by PTHrP and MIP-1 α suggests that there may be crosstalk between their downstream pathways. Although the *in vitro* effects of MIP-1 α on cell growth and apoptosis were inconclusive, it is possible that *in vivo* MIP-1 α inhibits cell growth and induces apoptosis. *In vivo*, the apoptotic signal likely comes from natural killer (NK) cells (NOD/SCID mice do not have cytotoxic T-cells) that recognize the Jurkat cells. NK cells may have had a greater effect on the Jurkat-PTHrP-luc and Jurkat-MIP-1 α -luc cells due to the dramatic decrease in CD40LG in both cells. The CD40LG-CD40 interaction is a co-stimulatory pathway that activates T-cells and antigen-presenting cells (APCs) [58,59], and CD40LG blocks apoptosis [31]. The decrease in CD40LG expression would reduce the CD40LG-CD40 interaction between the Jurkat T-cells and NK cells, allowing inhibitory pathways, such as PDL1 and PDL2 [60,61], to shift the balance in favor of T-cell apoptosis and decreased tumor burden.

Jurkat cells do not express the PTHrP receptor, PTH1R [13], suggesting that PTHrP plays an intracrine role in these cells. In contrast, there was expression of CCR1, one of the MIP-1 α receptors, suggesting an autocrine and/or paracrine role for MIP-1 α in Jurkat cells. The sensitization to apoptosis *in vivo* by PTHrP may have been the primary reason for decreased tumor burden. Alternatively, local secretion of PTHrP or MIP-1 α could have stimulated the microenvironment to produce antitumor factors to decrease tumor growth. Unfortunately, the tumors formed in mice with Jurkat-PTHrP-luc and Jurkat-MIP-1 α -luc cells were small or absent, and it was not possible to measure local concentrations of PTHrP or MIP-1 α *in vivo* or perform gene analyses on the tumor samples. Therefore, we utilized high-throughput gene arrays on cell line samples to investigate possible mechanisms.

The dramatic decrease in CD40LG mRNA may have been mediated by IL-12, IL-4 and/or IFN γ . IFN γ increases CD40LG expression in a number of cell types [36,37], but inhibits CD40LG expression in T-lymphocytes [38,39]. CD40LG expression is down-regulated by IL-4 and up-regulated by IL-12 in T-cells [40]. However, the two subunits of IL-12 were unchanged (except for IL-12A, which was up-regulated only in the Jurkat-PTHrP-luc cells), and IL-4 was down-regulated in the PTHrP and MIP-1 α overexpressing cells, which is in contrast to what would be expected if they were regulating CD40LG expression as previously reported. Furthermore, IFN γ was down-regulated proportionally to the decrease in CD40LG mRNA. Therefore, either IFN γ has a unique positive regulatory role for CD40LG expression in Jurkat T-cells or some other unknown pathway affected CD40LG expression in Jurkat cells.

IFN γ can also play a role in T-cell apoptosis. In some cases it induces apoptosis in T-cells [62,63], while in other cases it protects T-cells against apoptosis [64,65]. Interestingly, 1 α , 25-dihydroxyvitamin D₃ (calcitriol) down-regulates IFN γ expression through the VDR [44,45]. PTHrP, but not MIP-1 α , induces VDR expression [66], which is consistent with the fact that we observed up-regulation of the VDR and 1 α -hydroxylase (CYP27B1, which synthesizes 1 α ,25-dihydroxyvitamin D₃; data not shown) in Jurkat-PTHrP-luc, but not in Jurkat-MIP-1 α -luc cells. Therefore, PTHrP may decrease IFN γ expression through downstream signaling of the VDR.

Two additional factors that may have contributed to apoptosis of the Jurkat cells were DAPK1 and TNF α , both of which were up-regulated in Jurkat-PTHrP-luc and Jurkat-MIP-1 α -luc cells [Figs. 5(B) and 5(C)]. DAPK1 induces apoptosis in the presence of apoptosis-stimulating agents [33,34], while TNF α directly induces apoptosis in T-cells [35,67,68]. Both DAPK1 and TNF α can be up-regulated by the osteoclast activating factor, RANKL [41–43], and both PTHrP and MIP-1 α induce RANKL expression [3,6,25,69]. Consistent with this, RANKL was not expressed in control Jurkat cells, but was induced in both Jurkat-PTHrP-luc and Jurkat-MIP-1 α -luc cells. Therefore, PTHrP and MIP-1 α may have induced DAPK1 and TNF α expression through RANKL.

The effects of PTHrP and MIP-1 α on Jurkat cells may also have been mediated through different transcription factors. In order to address this question, a PCR array focusing on transcription factors was used to identify potential candidates. We found that several factors, including PAX6, STAT4, TFAP2A and ID1, were regulated by PTHrP and/or MIP-1 α overexpression. PAX6 is one of many human homologs of the *Drosophila melanogaster* gene, *prd*. PAX6 is expressed in the developing eyes and nervous system. It has been shown that PAX proteins are aberrantly expressed in cancers, and downregulation of PAX leads to differentiation in pancreatic cancer [70]. Overexpression of PAX6 decreased cell proliferation, and PAX6-null retinal spheres had marked proliferation, with reduced expression of several cell cycle negative regulators, such as *p16^{Ink4a}*, *p19^{Arf}*, *p27^{Kip1}*, *p57^{Kip2}*, and *p21^{cip1}* [71]. Full-length PAX6 also induced caspase-3-independent apoptosis

in corneal epithelial cells [72]. We found increased expression of PAX6 in Jurkat T-cells that overexpressed PTHrP and MIP-1 α . It is possible that this transcription factor contributed to decreased tumor formation *in vivo* through a decrease in negative cell cycle regulators and/or an increase in caspase 3-independent apoptosis.

STAT is a family of transcription factors that respond to stimulation by cytokines and growth factors through their phosphorylation by Janus kinases (JAKs). It has been shown that PTHrP signaling may crosstalk with JAK/STAT signaling by positively regulating the activity of STAT3-specific promoter-reporter and negatively regulating the activity of STAT5-specific promoter-reporter constructs in chondrocytes [73]. We found that overexpression of PTHrP and MIP-1 α in Jurkat T-cells increased the expression of STAT4, which is important for mediating the responses to IL-12 in lymphocytes, and regulating the differentiation of T-helper cells. STAT4 has also been shown to decrease proliferation of connective tissue-type mast cells [74].

TFAP2A is involved in both development and differentiation. This gene is hypermethylated in renal cell carcinoma [75], and has been shown to be a tumor suppressor [76] that regulates tumor cell growth, apoptosis, invasion and migration [77]. It causes cell cycle arrest at the G1 phase in human lung carcinoma, fibrosarcoma, colon carcinoma, osteosarcoma and cervical cancer-derived cell lines [78]. We found that overexpressing MIP-1 α in Jurkat T-cells increased the expression of TFAP2A, which may have contributed to the MIP-1 α -induced decrease in tumor growth *in vivo*.

ID1 belongs to the inhibitor of DNA binding (ID) family which is a group of helix-loop-helix (HLH) proteins that are considered to be dominant-negative regulators in differentiation pathways [79]. ID proteins play important roles in cell growth, differentiation, angiogenesis, hematopoiesis and neoplastic transformation. Specifically, ID1 has been shown to be a common downstream target of various oncogenic tyrosine kinases. Inhibition of ID1 expression by an antisense ID1 construct resulted in growth inhibition of human leukemia cells, and expression of ID1 protected leukemia cells from apoptosis [80]. Interestingly, it has been shown that ID1 activates the NF- κ B pathway, which is an important regulator of CD40LG expression [81,82]. We showed that overexpression of PTHrP and MIP-1 α in Jurkat T-cells decreased the expression of ID1, which may have contributed to the down-regulation of CD40LG.

To measure early tumor formation non-invasively, we chose BLI technology for our *in vivo* experiment. We found that photon signals could be detected much earlier (1 week after subcutaneous injection of 5×10^6 Jurkat cells in NOD/SCID mice) than visible detection of subcutaneous tumor formation (4 weeks after injection), demonstrating the sensitivity of bioluminescent technology. BLI also allowed us to ensure successful tumor injection and eliminate any difference in the number of cells injected into the mice by normalizing all subsequent photon signals to the signals obtained immediately following injection. As expected, we found that photon levels correlated well with the tumor size and weight (data not shown) at the time of necropsy. It has been shown that Jurkat cells formed tumors when injected subcutaneously into nude mice (5/5 incidence of tumor formation when injecting 10^8 cells) [83]. Jurkat cells could be found in the peripheral blood after intravenous injection (5/6 incidence of blood engraftment when injecting 2×10^7 cells) [84]. However, we and others have found that Jurkat cells did not form tumors after being injected intraperitoneally and intratibially into NOD/SCID mice ([85] and data not shown).

In summary, we found that PTHrP and MIP-1 α exerted profound effects on T-cell growth *in vivo*. Overexpression of both factors caused differential expression of several transcription factors and apoptosis-related genes *in vitro* and significantly decreased tumor growth *in*

in vivo. Our results demonstrate that PTHrP and MIP-1 α function differently in T-cells than in other tumor types by increasing the sensitivity to apoptosis-inducing signals and by regulating transcription and apoptosis factors.

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Figure 1. Overexpression of PTHrP and MIP-1α in Jurkat cells. Real-time RT-PCR of (A) PTHrP and (B) MIP-1α in Jurkat T-cells. Secretion of (C) PTHrP and (D) MIP-1α in Jurkat T-cells.

Figure 2.

PTHrP and MIP-1 α did not affect Jurkat cell growth. (A) Cell proliferation ratios of Jurkat-pcDNA-luc, Jurkat-PTHrP-luc and Jurkat-MIP-1 α -luc cells and (B) cell viability ratios of Jurkat-pcDNA-luc, Jurkat-PTHrP-luc and Jurkat-MIP-1 α -luc cells in 4-day cultures. Rates were normalized to the data from day 1.

Figure 3.

PTHrP increased the sensitivity to an apoptotic agent (camptothecin) in Jurkat cells. Jurkat-pcDNA-luc and Jurkat-PTHrP-luc were treated with vehicle (black column), 2 μ M of camptothecin (open column) or 10 μ M of camptothecin (gray column) overnight and the percentages of apoptotic cells were detected by TUNEL assay using flow cytometry. Symbol * indicates $p < 0.01$ when compared to Jurkat-pcDNA-luc cells treated with 2 μ M of camptothecin. Symbol ** indicates $p < 0.001$ when compared to Jurkat-pcDNA-luc cells treated with 10 μ M of camptothecin.

Figure 4.

PTHrP and MIP-1 α decreased viable Jurkat tumor growth *in vivo*. (A) Tumor burden measured by bioluminescent imaging. Photon signals obtained at week 7 were normalized to day 1. There was a 16- and 66-fold decrease in viable Jurkat-PTHrP-luc and Jurkat-MIP-1 α -luc cells, respectively. Symbol * indicates $p < 0.05$ when compared to Jurkat-pcDNA-luc group. (B) H&E images of Jurkat tumors at week 7. PTHrP and MIP-1 α decreased the incidence and size of Jurkat tumors *in vivo*. Tumors were identified at postmortem in 10/10 Jurkat-pcDNA-luc mice, 10/10 Jurkat-PTHrP-luc mice and 3/10 Jurkat-MIP-1 α -luc mice. Cross-sectional areas of tumors were significantly decreased ($p < 0.05$) seven- and 18-fold, respectively, in mice that received Jurkat-PTHrP-luc and Jurkat-MIP-1 α -luc cells when compared to mice that received Jurkat-pcDNA-luc cells. Note that the pale regions of the tumors represent areas of necrosis. Bar = 10 mm.

Figure 5. PTHrP and MIP-1α regulated the expression of apoptosis and other factors in Jurkat cells *in vitro*. Relative gene expression of (A) CD40LG, (B) DAPK1, (C) TNFα, (D) IFNγ, (E) RANKL and (F) VDR in Jurkat-pcDNA-luc, Jurkat-PTHrP-luc and Jurkat-MIP-1α-luc cells was measured by real-time RT-PCR. Gene expression in Jurkat-pcDNA-luc cells was set as a basal level for comparison. Symbol * indicates $p < 0.05$ when compared to Jurkat-pcDNA-luc group.

Figure 6.

PTHrP and MIP-1α regulated the expression of transcription factors in Jurkat cells *in vitro*. Relative gene expression of (A) ID1, (B) PAX6, (C) STAT4 and (D) TFAP2A in Jurkat-pcDNA-luc, Jurkat-PTHrP-luc and Jurkat-MIP-1α-luc cells was measured by real-time RT-PCR. Gene expression in Jurkat-pcDNA-luc cells was set as a basal level for comparison. Symbol * indicates $p < 0.01$ when compared to Jurkat-pcDNA-luc group.

Figure 7.

HTLV-1-infected T-cells expressed the MIP-1 α receptor CCR1. CCR1 expression of (1) Jurkat; (2) MT2; (3) SLB-1; (4) Hut102; (5) MET-1; (6) C8166; (7) RV-ATL; (8) HT-1RV; (9) positive control (PBMC-1); (10) positive control (PBMC-2); (11) negative control (water) was measured by RT-PCR.