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Enhancing Effect of Interleukin-2 on Production of Parathyroid Hormone-related Protein by Adult T-Cell Leukemia Cells

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Leukemic cells from patients with adult T-cell leukemia (ATL) can produce a calcium-regulating protein, parathyroid hormone-related protein (PTHrP). Moreover, it has been reported that ATL cells produce some cytokines besides PTHrP and that these cells respond to the T-cell growth factors, interleukin-2 (IL-2) and interleukin-4 (IL-4). To elucidate whether PTHrP produced by ATL cells is regulated by IL-2 or IL-4, we investigated the *in vitro* effects of IL-2 and IL-4 on the release of PTHrP. IL-2 increased the release of PTHrP into the conditioned medium from leukemic cells in some, but not all, ATL patients; however, IL-4 did not affect the PTHrP release. PTHrP messenger RNA (mRNA) levels were increased in ATL cells cultured in the presence of IL-2. These data suggest that IL-2 plays a role in the regulation of hypercalcemia by enhancing the production of PTHrP in ATL patients.

Key words: Parathyroid hormone-related protein — Interleukin-2 — Adult T-cell leukemia

Adult T-cell leukemia (ATL) is a leukemia of human T-cell leukemia virus type I (HTLV-I)-infected peripheral mature T cells.[1] Almost all patients with ATL show hypercalcemia, which often results in death. Parathyroid hormone-related protein (PTHrP) is a newly isolated protein with calcium-elevating activity in some solid tumors,[2] fresh leukemic cells from ATL patients, and HTLV-I-infected cell lines.[3,4] In our laboratory, clinical studies on serum levels of PTHrP from ATL patients indicate that PTHrP is the factor most likely to be involved in the development of hypercalcemia.[5] Although PTHrP synthesis, secretion or both has been reported to be regulated by several different agents, including cyclic AMP, phorbol esters, growth factors, steroid hormones, and 1,25-dihydroxyvitamin D_3[1,25$(OH)_2D_3$] in a number of tumor cell lines[6–9] or normal human keratinocytes,[10] the mode of regulation of PTHrP in leukemic cells from ATL patients is not known. It has been reported that the *tax* protein encoded by the pX gene of HTLV-I transactivates several cytokine genes, including interleukin-2 (IL-2), transforming growth factor-β, and IL-2 receptor α chain genes.[11–13] Furthermore, PTHrP gene expression is reportedly induced by HTLV-I infection through transactivation by the *tax* gene *in vitro*.[14] However, the production of PTHrP in T cells is not always associated with HTLV-I infection, as reported previously.[15] These observations led us to investigate whether IL-2 regulates the production of PTHrP in ATL patients by examining the effect of IL-2 on the production of PTHrP by ATL cells.

MATERIALS AND METHODS

Patients Fourteen patients with ATL were studied. The clinical features of the patients are summarized in Table I. More than 80% of the peripheral white blood cells were leukemic cells, as determined by Wright-Giemsa staining.

Cells and cell culture The mononuclear cells were separated from blood of fourteen ATL patients and a lymph node of one ATL patient by centrifugation on LSM solution (Litton Bionetics, Kensington, MD). Cells were further incubated for 2 h in plastic culture dishes to remove adherent cells. The cells in suspension were used for the experiments. ATL cells were cultured at a concentration of 5×10^6 cells/ml in RPMI 1640 medium (Nissui Seiyaku Co., Ltd., Tokyo) containing 10% fetal calf serum (FCS, GIBCO, Grand Island, NY) in the presence or absence of recombinant IL-2 (provided by Shionogi Pharmaceutical Co., Ltd., Osaka) or IL-4 (provided by Ono Pharmaceutical Co., Ltd., Osaka). After 72 h of culture, the conditioned medium was collected and PTHrP was measured by radioimmunoassay (RIA). Cell viability determined by trypan blue exclusion always exceeded 90%.

Proliferative response ATL cells (1×10^5) were cultured in 96-well flat-bottomed plates with various concentrations of IL-2 or IL-4 in 200 μl of RPMI 1640 medium containing 10% FCS in triplicate for 72 h. Proliferation

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was measured by incorporation of 0.5 μCi of [^{3}H]TdR, which was added to the cultures after 48 h of culture.

Assay for immunoreactive (IR)-PTHrP in the medium The IR-PTHrP secreted into the medium was measured by RIA for the C-terminal region of PTHrP. The anti-C-terminal region of PTHrP antiserum was produced by immunization of rabbits with [Tyr^{126}]-PTHrP (127–141) conjugated to porcine thyroglobulin. ^{125}I-[Tyr^{126}]-PTHrP (127–141) was iodinated by the chloramine T method. The RIA incubation mixture consisted of 0.1 ml of standard PTHrP (109–141) (Peninsula Institute, Belmont, CA) or samples diluted in assay buffer, and 0.1 ml of antiserum diluted in assay buffer. The mixture was incubated for 20 h at 4°C, then 0.1 ml of ^{125}I-[Tyr^{126}]-PTHrP (127–141) was added and it was further incubated for 20 h at 4°C. The antibody-bound tracer peptide and free tracer peptide were separated by adding 0.1 ml of goat anti-rabbit gamma globulin antiserum. The minimum concentration of IR-PTHrP detected in the cultured medium was 20 pg/ml. Data were expressed as pg/ml equivalent to PTHrP (109–141).

Messenger RNA (mRNA) studies Total RNA was isolated by the single-step method.[16] RNA (20 μg) was electrophoresed on 1% agarose-formaldehyde gels and transferred to nylon filters. Plasmid containing the cDNA probe for PTHrP was provided by Dr. K. Sato (Tokyo Women's Medical College, Tokyo).[17] Probes were excised with *Eco* RI and *Hin*d III, and labeled with ^{32}P. As controls, an HTLV-I-infected T-cell line, MT-2,[18] which produces large amounts of PTHrP and a non-producing T-cell line, MT-1 (provided by the Fujisaki Cell Center, Okayama),[19] were used.[4]

RESULTS

Proliferative response of ATL cells to IL-2 and IL-4 As shown in Fig. 1, ATL cells from peripheral blood of patients No. 1, 4, 5, 6, 11, 12, 13, and 14 and from a lymph node of patient No. 2 proliferated in response to both IL-2 and IL-4 in a dose-dependent manner. ATL cells from peripheral blood of patients No. 2 and 3 responded to IL-2, but did not respond to IL-4. In contrast, ATL cells from patient No. 7 proliferated in response to IL-4, but did not respond to IL-2. In patients No. 8 and 9, ATL cells did not proliferate in response to IL-2 or IL-4. The baseline values of [^{3}H]TdR incorporation varied greatly among these cells, as reported previously.[20,21] Spontaneous proliferation seems to bear some relationship to the clinical phase of the disease.[20] The maximal response was obtained in most patients with 1–10 ng/ml of both IL-2 and IL-4.

Release of IR-PTHrP into the medium As shown in Table I and Fig. 2, IR-PTHrP was detected in the cultured medium conditioned by unstimulated ATL cells

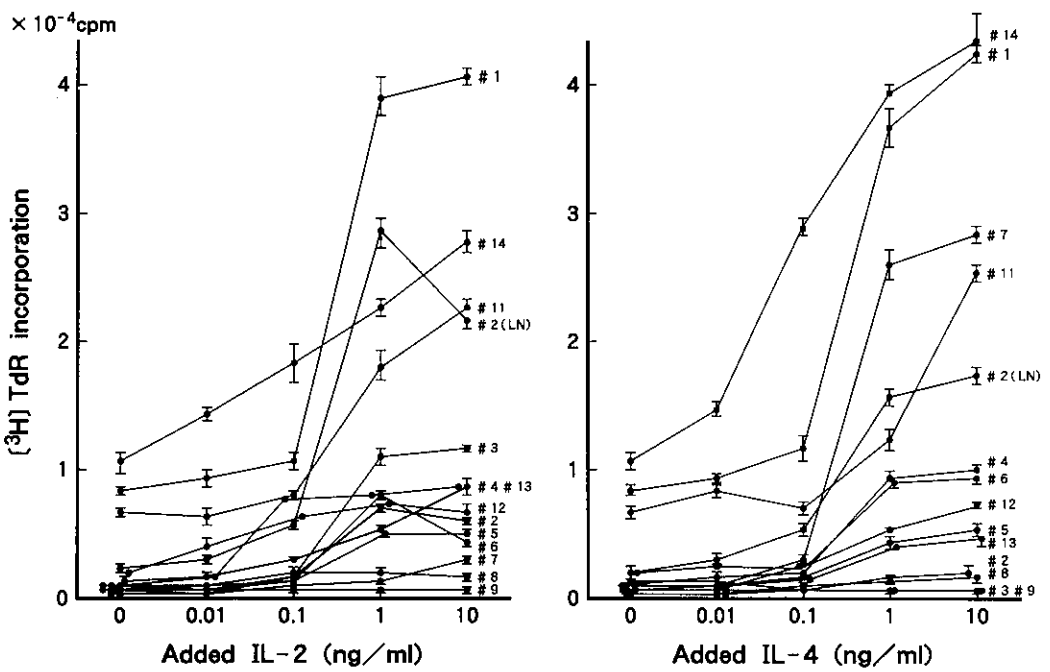

Fig. 1. Proliferative response of ATL cells to IL-2 and IL-4. Data are means±SE of triplicate cultures. #, patient No.; LN, lymph node.

Table I. Profile of Patients with Adult T-Cell Leukemia

No.	Age/Sex[a]	Type[b]	WBC[c] (/μl)	Serum Ca[d] (mg/dl)	IR-PTHrP in conditioned medium (pg/ml)[e]
1	64/M	A	43,300	10.9	<20
2	76/F	A	17,000	9.6	49 (40)[f]
3	74/F	A	104,000	10.7	123
4	65/M	A	242,900	13.3	21
5	45/M	A	13,600	8.5	28
6	49/F	A	6,600	9.6	<20
7	61/M	A	12,400	12.5	79
8	75/F	A	73,300	10.2	32
9	58/F	C	15,900	9.6	<20
10	81/M	A	5,100	10.5	<20
11	66/M	A	14,600	10.2	47
12	60/M	A	60,400	9.2	66
13	60/M	A	21,700	9.0	76
14	45/M	A	16,000	12.6	157

a) M; male, F; female.
b) Clinical type of the patients. A; acute type, C; chronic type.
c) White blood cell count of peripheral blood.
d) Normal range is 8.0–10.2 mg/dl.
e) ATL cells were cultured with RPMI 1640 medium containing 10% FCS. After 72 h culture, immunoreactive PTHrP (IR-PTHrP) concentration was determined by a radioimmunoassay specific for the C-terminal region of PTHrP. IR-PTHrP was expressed as pg/ml equivalent to PTHrP (109–141).
f) Lymph node cells.

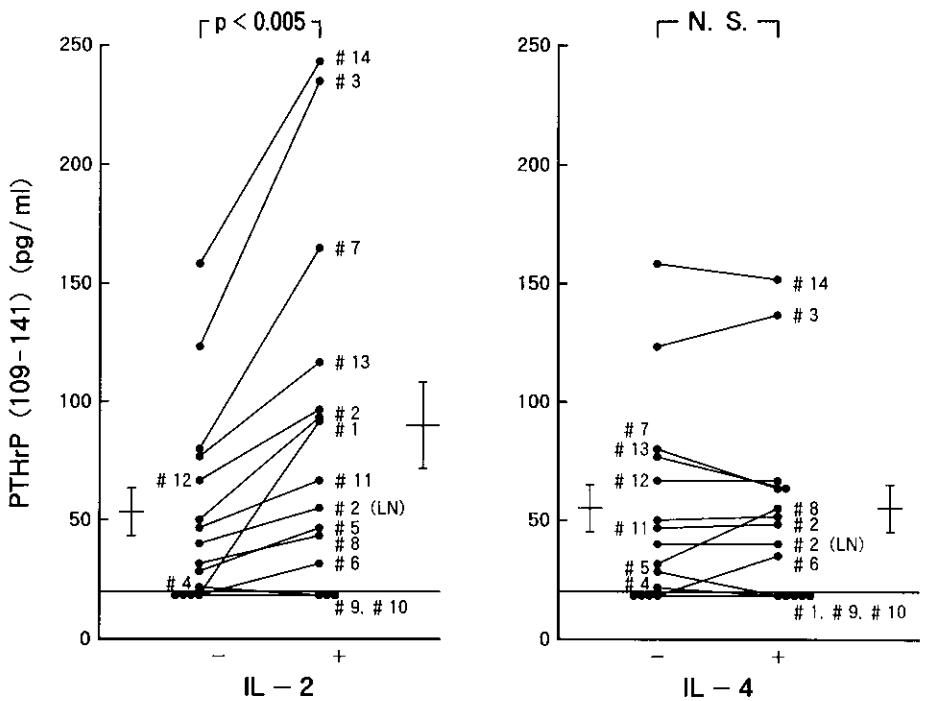

Fig. 2. Effects of IL-2 and IL-4 on PTHrP release from ATL cells. Cells were cultured for 72 h at 5×10^6 cells/ml in RPMI 1640 medium containing 10% FCS. IL-2 (10 ng/ml) and IL-4 (10 ng/ml) were added at the initiation of culture. IR-PTHrP levels were measured by a radioimmunoassay specific for the C-terminal region of PTHrP. The horizontal lines represent the detection limit of the RIA. Bars show means ± SE. #, patient No.; LN, lymph node; N.S., not significant.

from eleven of fifteen samples examined. Hypercalcemia (serum Ca > 10.5 mg/dl) was observed in 5 cases (No. 1, 3, 4, 7, and 14). However, there was no correlation between IR-PTHrP levels in the medium and the serum Ca levels (Table I). These data are in accordance with a study in which some discrepancies were found between the levels of serum Ca and the expression of PTHrP mRNA in ATL patients.[22] These data suggest that some other factors may contribute to the development of hypercalcemia along with the PTHrP. When ATL cells were cultured with IL-2, IR-PTHrP levels increased in twelve of fifteen samples ($P < 0.005$, Wilcoxon test) in a dose-dependent manner (Fig. 3). In addition, significant enhancement of PTHrP release by IL-2 was found especially in ATL cells from patients with hypercalcemia (No. 1, 3, 7, and 14). However, the difference between IR-PTHrP levels of IL-4-treated cells and nontreated cells was not statistically significant (Fig. 2). The enhancing effect of IL-2 was also observed in regard to the level of mRNA for PTHrP (Fig. 4).

Kinetics of PTHrP secretion We performed time-course experiments to characterize the kinetics of the effect of IL-2 on PTHrP secretion. As shown in Fig. 5, the release

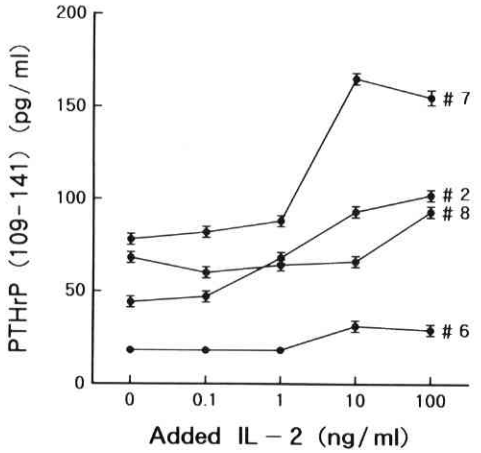

Fig. 3. Dose-response curves of the effect of IL-2 on PTHrP release from ATL cells. Cells were cultured for 72 h with various concentrations of IL-2. IR-PTHrP levels were measured by a radioimmunoassay specific for the C-terminal region of PTHrP. Data are means ± SE of triplicate cultures. #, patient No.

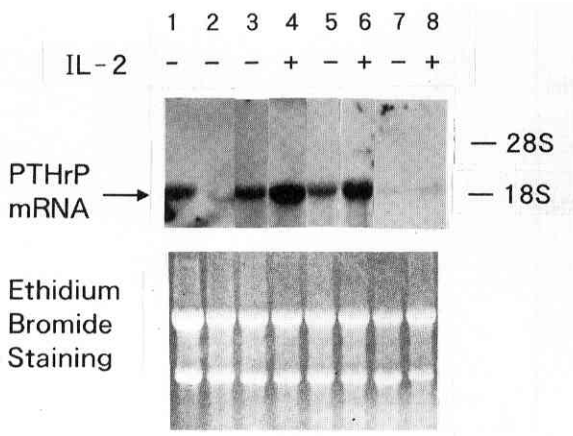

Fig. 4. Effect of IL-2 on PTHrP mRNA expression. ATL cells were cultured with medium or 10 ng/ml of IL-2 for 2 h. The upper panel shows the Northern blot analysis using a probe for PTHrP. The lower panel shows the ethidium bromide staining pattern of gel. Approximately equal amounts (20 μg) of RNA were electrophoresed in each lane. Lane 1, MT-2; lane 2, MT-1; lane 3, ATL cells from patient No. 3; lane 4, ATL cells from patient No. 3 cultured with IL-2; lane 5, ATL cells from patient No. 7; lane 6, ATL cells from patient No. 7 cultured with IL-2; lane 7, ATL cells from patient No. 9; lane 8, ATL cells from patient No. 9 cultured with IL-2.

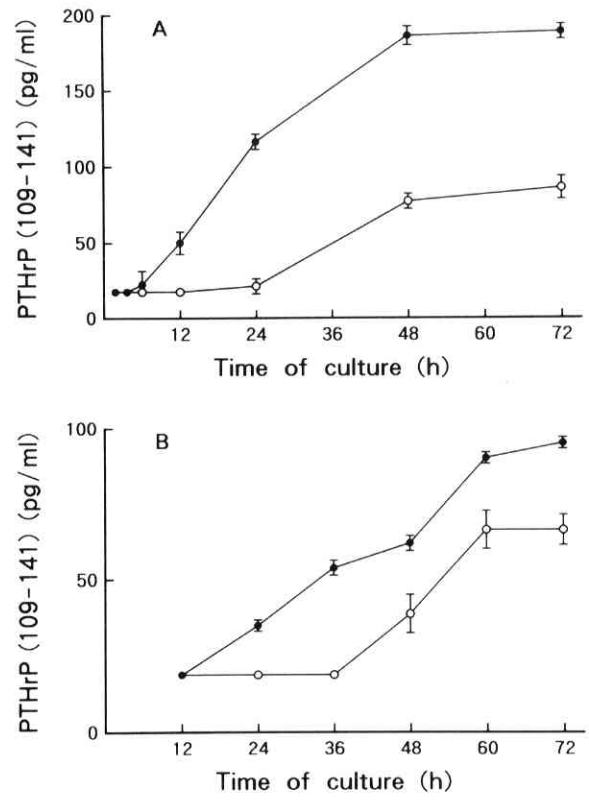

Fig. 5. Time course of PTHrP release from ATL cells cultured with or without 10 ng/ml of IL-2. IR-PTHrP levels were measured in the presence of IL-2 (●) or medium alone (○). (A) ATL cells from patient No. 3. (B) ATL cells from patient No. 2. Data are means ± SE of triplicate cultures.

of PTHrP increased after 12–24 h of culture in the presence of IL-2 compared with control cultures in medium alone.

DISCUSSION

Since leukemic cells from ATL patients and HTLV-I-infected cell lines express IL-2 receptor α and/or β chain,[21, 23] and IL-2 and IL-4 are known to enhance the proliferation of peripheral leukemic cells in some ATL patients,[20, 24] we thought it worthwhile to assess the effects of IL-2 and IL-4 on PTHrP release into the conditioned medium. IL-4 did not affect PTHrP release by ATL cells. In addition, IL-1α, IL-6, interferon-γ, tumor necrosis factor-α, and $1,25(OH)_2D_3$ had no effect (data not shown). However, PTHrP levels in the medium increased when ATL cells were cultured with IL-2. IL-2 and IL-4 could augment the DNA synthesis of ATL cells and subsequently increase the number of these cells, but no significant increase in cell number was observed after at least 72 h of culture in ATL cells from most patients. Furthermore, in response to IL-2, PTHrP levels in cell lysates also increased, whereas IL-4 had no effect (data not shown). Therefore, it was concluded that the high levels of PTHrP observed with IL-2 were the result of increased PTHrP synthesis.

As shown in Fig. 1 and Fig. 2, some discrepancies exist between the proliferative responses to IL-2 and IL-4, and the effects of these cytokines on PTHrP release. In addition, our preliminary studies indicated that IL-4 inhibits the production of IL-1α, IL-1β, IL-6 and TNF-α by ATL cells, whereas IL-2 enhances the production of these cytokines. These findings suggest that there is no correlation between the production of cytokines and response to IL-2 and IL-4.

How IL-2 influences the production of PTHrP is not clear yet. Our studies indicate that PTHrP steady-state mRNA levels are enhanced in ATL cells cultured in the presence of IL-2, but further analyses are required to assess the relative contribution of changing rates of transcription and/or mRNA stability to determine the influence of IL-2 on steady-state mRNA levels in ATL cells. The pX gene of the HTLV-I genome encodes a transcriptional activator known as *tax* which *in vitro* activates transcription of a variety of host cellular genes including IL-2, IL-2R α chain, and PTHrP genes.[11, 12, 14] However, it has been reported that in the leukemic cells from ATL patients, viral genes are not expressed or are expressed at a very low level.[25] Therefore, some factors other than the HTLV-I *tax* gene may also be involved in the production of PTHrP. Interestingly, we showed that PTHrP release from ATL cells cultured with IL-2 was significantly enhanced especially in patients with hypercalcemia. We suggest that the production of PTHrP is induced by HTLV-I infection through transactivation of the *tax* gene and that further overproduction of PTHrP induced by some other factors associated with malignant transformation, for example, IL-2, may accelerate the development of hypercalcemia.

Kodaka *et al.* have reported that IL-2 mRNA expression was undetectable in leukemic cells from ATL patients.[26] However, it is possible that ATL cells are acted upon by IL-2 which normal activated T cells produce in lymphoid tissue *in vivo*. In fact, although IL-2 was not detected in cultures of peripheral blood mononuclear cells from one ATL patient (No. 2) by immunoassay, we detected IL-2 in cultures of mononuclear cells from a lymph node of the same patient.

We have demonstrated that PTHrP is regulated in freshly isolated leukemic cells from ATL patients by a positive modulator, IL-2. It is possible, therefore, that T cells activated either by HTLV-I infection or unknown stimuli are responsible for producing IL-2, which contributes to the hypercalcemia through overproduction of PTHrP. We believe that IL-2 plays a significant role in the regulation of PTHrP production in ATL.

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