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PARATHYROID HORMONE-RELATED PROTEIN IS A POSSIBLE AUTOCRINE GROWTH INHIBITOR FOR LYMPHOCYTES

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SUMMARY: Adult T-cell leukemia (ATL)-related cells have the ability to produce a newly-isolated calcium-regulating protein, parathyroid hormone-related protein (PTHrP). The present study revealed that lectin-stimulated normal lymphocytes produce immunoreactive (IR)-PTHrP. When the T-cell-enriched fraction was purified from normal lymphocytes and then treated with lectin, a similar amount of IR-PTHrP was detected, suggesting that IR-PTHrP is an actual product of T-lymphocytes. A biologically active fragment of PTHrP, PTHrP(1-34), suppressed DNA synthesis in lectin-stimulated lymphocytes at concentrations greater than 50 pg/mL; the same concentration range of IR-PTHrP detected in the cultured media of lectin-stimulated lymphocytes. Therefore, it is reasonable to postulate that PTHrP is a cytokine inhibiting the cellular growth of normal lymphocytes.

Parathyroid hormone-related protein (PTHrP) is a newly-discovered protein with calcium-elevating activity (1). Pathologically, this protein is believed to be the major causative agent of humoral hypercalcemia of malignancy (HHM) (2). Its physiological role as well as the tissues synthesizing this protein, however, are not yet fully clarified. We have recently reported that peripheral leukemic cells obtained from adult T-cell leukemia (ATL) patients as well as cultured T-cell lines infected by human T-cell leukemia virus type I (HTLV-I) produce PTHrP (3); these findings are consistent with the clinical observation that over half of acute type ATL patients develop hypercalcemia (4). Two mechanisms can be speculated for PTHrP production by ATL-related cells: the first is a trans-acting mechanism related to a viral gene product of HTLV-I (5) inducing an overexpression of the PTHrP gene; alternatively, the second is that normal T-lymphocytes, or

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subpopulations, possess the ability to produce PTHrP and this characteristic is preserved in ATL-related cells. The present study examines the possibility that T-lymphocytes have the ability to produce PTHrP. Since initial experiments revealed that lectin-stimulated T-lymphocytes produced PTHrP, a possible role of PTHrP as a cytokine was further investigated.

Materials and Methods

Cell Preparation and Cell Culture: Peripheral blood was obtained from four normal volunteers. From these specimens, mononuclear cells were isolated by a density-gradient method using Ficoll-Paque (Pharmacia Fine Chemicals AB, Uppsala, Sweden) with specific gravity of 1.077 (6). Mononuclear cells were further incubated for 2 h in tissue culture dishes (Becton Dickinson and Company, NJ) to remove adherent cells. This step was repeated twice and the resultant cells were used as the normal lymphocyte preparation. In some experiments, a T-cell-enriched fraction prepared by using a nylon wool column (7) was used.

In order to test the possibility whether normal lymphocytes have the ability to produce PTHrP, lymphocytes as well as T-cell-enriched fractions were cultured in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (GIBCO) at a concentration of $5x10^5$ cells/mL. When tested, these preparations were incubated in the absence or presence of 2 μg/mL of Phytohemagglutinin (PHA)-P (Wellcome Diagnostics, Dartford, GB) and Concanavalin A (Con A) (Seikagaku Kogyo Co., LTD, Tokyo, Japan), and the resultant conditioned media harvested after 3 day culture were used as test samples. As controls, a HTLV-I-infected T-cell line (MT-2) (8) producing a large amount of IR-PTHrP and a non-producing T-cell line (MT-1) (9) were cultured at the same cell numbers with that of lymphocytes for 3 days, and their conditioned media were investigated also.

IR-PTHrP in Conditioned Media: IR-PTHrP in the conditioned media was extracted by the method using an immune-affinity column (3). The levels of IR-PTHrP in these extracts were determined by the PTHrP radioimmunoassay (RIA) (3) with a slight modification; a newly-developed anti-human PTHrP-(1-34) antiserum, NCC-PTHrP-R-030301, was used at the final dilution of 1:60,000 (total incubation volume, 0.7 mL/tube). When assayed, the lyophilized sample was reconstituted to 1.0 mL by adding assay buffer, and 100 μL of each dilution was used for the RIA. The detection limit of the present PTHrP RIA was 2.8 pg/tube. Therefore, the minimum concentration of IR-PTHrP detected in cultured media was 5.6 pg/mL when 5.0 mL of cultured media was used as the starting material.

Effects of PTHrP(1-34) on Cellular Growth: These experiments were performed in triplicate using 96-well round-bottomed plates (Nunc, Roskilde, DK). PHA-P or Con A-stimulated cells ($1x10^5$/well) cultured in RPMI 1640 medium supplemented with 10% FBS were incubated in the presence or absence of exogenously added PTHrP(1-34) (Peninsula Laboratories, Belmont, CA). The final incubation volume was 200 μL, and each 24 h, 150 μL of medium was gently aspirated and the same volume of fresh medium containing PTHrP(1-34), at various concentrations, was added. Fourty-eight hours later, [^{3}H]Thymidine (37 KBq/well, specific activity 248 GBq/mmol; New England Nuclear, Boston, MA) was added to each well and incubated for an additional 24 h. Incorporation of [^{3}H]Thymidine into the cells was determined by liquid scintillation counter.

Statistics: All samples were assayed in triplicate. The data are expressed as the mean±SD. Statistical analyses were performed using the paired Student's t-test. A probability value of 0.05 was considered to be significant.

Results

IR-PTHrP Produced by Lectin-stimulated Lymphocytes: IR-PTHrP was undetectable in the cultured media conditioned by unstimulated normal lymphocytes, whereas with PHA-P-stimulated lymphocytes appreciable amounts of IR-PTHrP were detected (Table 1). In the case of Con A-stimulated lymphocytes, IR-PTHrP was also detected (data not shown). When two preparations of T-cell- enriched fractions were examined, similar IR-PTHrP amounts with those of lectin-stimulated normal lymphocytes in the respective individual were detected (Table 1). IR-PTHrP levels in the cultured media conditioned by MT-2 were 400 pg/mL; in contrast, MT-1 did not produce detectable IR-PTHrP levels (less than 5.6 pg/mL).

Suppression of Lymphocytes Proliferation by PTHrP(1-34): PTHrP(1-34) was not cytotoxic for lymphocytes when examined by the trypan blue dye exclusion test. PTHrP suppressed the DNA synthesis of lectin-stimulated lymphocytes in a dose dependent manner (Fig. 1). Statistically significant suppressive effects were observed at the concentrations of PTHrP(1-34) ranging from 50-250 pg/mL. This effect reached its maximum at concentrations of 250 pg/mL or greater by suppressing DNA synthesis to 65% of the basal level. PTHrP(1-34) did not modulate DNA synthesis in unstimulated lymphocytes (data not shown).

Table 1. Concentrations of IR-PTHrP in the cultured media conditioned by normal lymphocytes ae well as T-lymphocyte-enriched fraction obtained from 4 volunteers

Subjects	Normal lymphocytes		T-lymphocytes	
	PHA(-)	PHA(+)[a]	PHA(-)	PHA(+)
A	<5.6[b]	20	<5.6	17
B	<5.6	21	<5.6	30
C	<5.6	57	NT[c]	NT
D	<5.6	47	NT	NT

a) PHA-P (2 μg/mL) was used as a stimulant.
b) expressed as equivalents to pg of PTHrP(1-34) per mL of cultured media.
c) not tested.

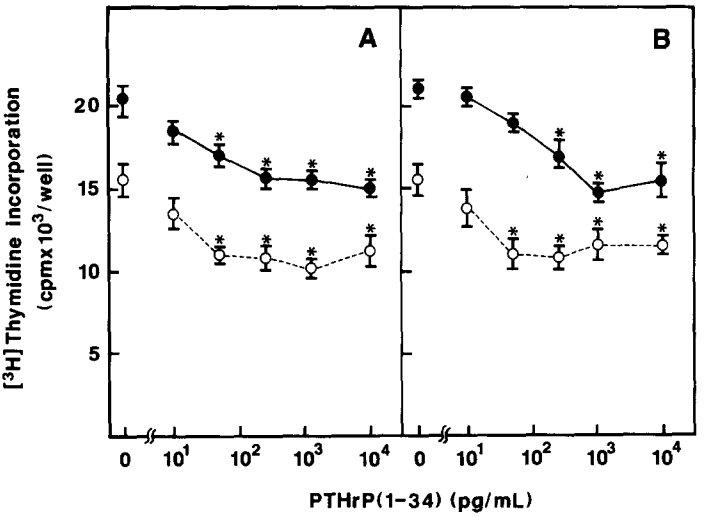

Fig. 1. Effect of PTHrP(1-34) on DNA synthesis of lectin-stimulated lymphocytes. Concentrations of PTHrP(1-34) added were plotted on a log scale. Each points represents the mean±SD of triplicated experiments. (A) lymphocytes stimulated with Con A (●—● , 2 μg/mL; o---o , 1 μg/mL). (B) lymphocytes stimulated with PHA-P (●—● , 2 μg/mL; o---o , 1 μg/mL). *, $p<0.05$.

Discussion

IR-PTHrP was not detectable in fresh media and in cultured media conditioned by unstimulated lymphocytes obtained from peripheral blood. However, when lymphocytes were stimulated by two different lectins, Con A and PHA-P, it was detectable. Since IR-PTHrP was detected in all extracts prepared from cultured media conditioned by lectin-stimulated lymphocytes, it is likely that lectin-stimulated lymphocytes commonly produce IR-PTHrP. When the same experiment was undertaken by using T-cell-enriched fraction, similar results were obtained. These observations indicate that IR-PTHrP is a product of stimulated T-lymphocytes.

The present finding may add a new informations regarding with the mechanism responsible for PTHrP production by ATL cells. It is well recognized that the introduction of the HTLV-I gene to lymphocytes produces a factor with trans-acting activity to the promotor or enhancer regions of other cellular genes (5); this factor is now believed to participate for development of ATL cells by activating cellular genes related to cellular proliferation (5). Accordingly, it is possible to assume that the trans-acting factor activates the PTHrP gene in the same manner, which in turn stimulates the production of PTHrP by tumor cells. However, the present finding that lectin-stimulated lymphocytes produce PTHrP indicates that the production of PTHrP by lymphocytes is not always associated with HTLV-I infection. In this context, it is worth mention here that cultured T-cell

lines infected with HTLV-I do not always produce PTHrP, as reported previously (3). Accordingly, it is more likely that ATL cells seem to maintain the ability of lectin-stimulated lymphocytes to produce IR-PTHrP. It is important to note that PTHrP was not detected in the cultured media of unstimulated lymphocytes. We believe that in the unstimulated state the amount of PTHrP produced by each cell is too low to be detected by the present RIA; stimulation by lectins increases the number of lymphocytes producing PTHrP which, in turn, increases the amount of PTHrP in the cultured media.

The biological implications of PTHrP produced by lymphocytes is another interest. Since it is now established that PTHrP has an activity to stimulate bone resorption, lymphocyte-derived PTHrP may participate in the regulation of calcium ion concentration in bone marrow in a paracrine fashion by resorbing bone. In the present study, however, we examined another potential role of PTHrP as a growth modulator of lymphocytes, according to the several reasons described below. First, it has been reported that normal lymphocytes possess parathyroid hormone (PTH) receptors (10), and that PTH inhibited lectin-induced lymphocyte proliferation (11); PTHrP is now believed to share the same receptor with PTH (12). Second, purified PTHrP as well as $[Tyr^{36}]$-PTHrP(1-36)NH_2 was reported to stimulate colony formation of NRK49F cells (13). And third, the structure of the PTHrP gene possesses some similarity with the c-myc oncogene (14). These observations led us to examine whether PTHrP has the ability to regulate the cellular growth of lectin-stimulated lymphocytes. We found that PTHrP-(1-34), a biologically active fragment of PTHrP, had the ability to suppress DNA synthesis in lectin-stimulated lymphocytes at concentrations greater than 50 pg/mL. Previous studies focused mainly on growth-stimulating factors for lymphocytes, but it is also known that there are several factors that inhibit cellular growth of lymphocytes. Examples are PTH (11), transforming growth factor (TGF)-ß (15), calcitonin gene-related peptide (16), VIP (17), somatostatin (17) and ß-endorphin (18). However, none of these factors except for TGF-ß and ß-endorphin was shown to be produced by lymphocytes. Moreover, it is worth noting that the concentration of PTHrP required for inhibition of DNA synthesis is in picogram range; this concentration is in the same range as that of cultured media conditioned by lectin-stimulated lymphocytes. These findings raise the possibility that PTHrP is an autocrine growth inhibitor for lymphocytes. Since PTHrP stimulates colony formation of NRK49F cells (13), the effect of PTHrP on cellular growth could be considered to be bifunctional, as are the cases in several peptide growth factors (19).

From the clinical standpoint, a previous report described increased susceptibility to infection in ATL patients, presumably due to a defect in

cellular immunity (20). The present study demonstrated that PTHrP possessed the activity to suppress DNA synthesis in lectin-stimulated lymphocytes. When compared to the IR-PTHrP level in the cultured media conditioned by lectin-stimulated normal lymphocytes, the level was approximately 10 times greater in the case of a HTLV-I-infected T-cell line, MT-2. Taken together these observations, it is possible to speculate that PTHrP produced by ATL cells plays an important role for the development of immunosuppressive conditions observed in ATL patients.

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