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EFFECT OF PARATHYROID HORMONE ON RELEASE OF INTERLEUKIN 1 AND INTERLEUKIN 6 FROM CULTURED MOUSE OSTEOBLASTIC CELLS

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Summary This study was undertaken to examine the possibility that parathyroid hormone promotes the production of interleukin 1 and 6 in MC3T3-E1 osteoblastic cells derived from mouse calvaria. The cells were incubated in serum-free medium with or without synthetic human parathyroid hormone(1-34). The concentrations of interleukin 1 and 6 in the culture medium were determined by using specific bioassay. The cells cultured without parathyroid hormone for 24 hr released both of interleukins, and parathyroid hormone stimulated the release in a dose-dependent manner. When the cells were cultured with 10^{-6} M parathyroid hormone, the release of both interleukins from the cells remained higher than control up to 144 hr. These results suggest that interleukin 1 and 6 release stimulated by parathyroid hormone may be involved in the bone resorbing activity of the hormone. © 1991 Academic Press, Inc.

It has been well known that PTH accelerates bone resorption and osteogenic cells are believed to mediate the PTH-stimulated bone resorption (1,2). PTH has a large number of biological effects on osteoblasts or osteoblast-like cells. These include stimulation of adenylate cyclase activity (3,4), rapid activation of cyclic AMP-dependent protein kinase (5), inhibition of collagen synthesis (6), inhibition of alkaline phosphatase activity (3,7), and stimulation of calcium uptake (8,9). On the other hand, no data are available which suggest the presence of PTH receptors in osteoclasts. Recently, Löwik *et al.* reported that PTH and PTH fragment, PTH(1-34), stimulate IL-6 production in osteogenic cells and that IL-6 produced may be a mediator of PTH-induced bone resorption (10). It is possible that factors other than IL-6 are also involved in PTH-induced bone resorption. IL-1 is known as a

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Abbreviations: PTH, parathyroid hormone; hPTH, human parathyroid hormone; IL, interleukin; α-MEM, α-modified Eagle's minimum essential medium; FBS, fetal bovine serum; PBS(-), Ca^{++}, Mg^{++}-free phosphate-buffered saline; BSA, bovine serum albumin.

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potent stimulator of bone resorption (11-13). Hanazawa *et al.* have reported that IL-1 like cytokine is produced by a mouse osteoblastic cell line (14) and by cultured bone cells from mouse calvaria (15). IL-1 mRNA positive cells have also been detected in mouse humeral bone (16). Although IL-1 is known as a potent stimulator of bone resorption, the relationship between IL-1 and PTH is poorly understood. We, thus, investigated whether IL-1 is produced by osteoblastic cells, since IL-1 is a possible candidate which can mediate PTH-induced bone resorption.

Materials and Methods

Materials

Synthetic hPTH(1-34) was provided by Toyo Jyozo Co. Ltd, Shizuoka, Japan. FBS was purchased from Boeringer Mannheim Yamanouchi, Tokyo, Japan. Methyl-1',2'-[^{3}H] thymidine (107.8 Ci/mmol of specific activity) was purchased from New England Nuclear, Boston, MA. Rabbit anti-mouse IL-1α antibody was purchased from Genzyme Corporation, Boston, MA. Normal rabbit IgG was purchased from Zymed Laboratories, South San Francisco, CA. Recombinant IL-2 (17) and IL-6 (18) were kindly provided by Dr. Hajime Karasuyama of Basel Institute for Immunology, Basel, Switzerland. Recombinant mouse IL-1α and IL-1β were purchased from Collaborative Research, Bedford, MA.

Cell culture

MC3T3-E1 cells were maintained in α-MEM supplemented with 10% FBS as previously described (14). EL4-6 mouse thymoma cell line which releases IL-2 in the presence of IL-1 was cloned from parent EL4 cell line in our laboratory. EL4-6 cells were maintained in RPMI 1640 medium supplemented with 10% FBS, 5 μM 2-mercaptoethanol and 34 μg/ml gentamicin. The cells were subcultured every 3 days by diluting them 10-fold with fresh medium. IL-2 dependent murine helper T cell line HT2 (19) were maintained RPMI 1640 medium supplemented with 10% FBS, 5 μM 2-mercaptoethanol and 34 μg/ml gentamicin and 10 units/ml recombinant IL-2. HT2 cells were subcultured every 2 days by diluting the cells 10-fold with fresh medium. IL-6 dependent cell line B45-3 (18) was provided by Dr. Hajime Karasuyama. B45-3 cells were maintained in RPMI 1640 medium supplemented with 10% FBS, 5 μM 2-mercaptoethanol, 34 μg/ml gentamicin and 10 units/ml recombinant IL-6. B45-3 cells were subcultured every 3 days by diluting them 20-fold with fresh medium.

Treatment of MC3T3-E1 cells with hPTH(1-34)

MC3T3-E1 cells ($4x10^5$) suspended in 4 ml of the medium were seeded onto plastic dish (60 mm in diameter; Falcon, Oxnard, CA). At day 3 after seeding, the medium was removed and the cells were washed twice with PBS(-). Four ml of α-MEM supplemented with 0.2% bovine serum albumin (BSA) and hPTH(1-34) was then added to the dish to give desired concentration. The cells were further cultured with or without hPTH(1-34) for 6 to 144 hr, and the culture medium was collected at 6, 12, 24, 48, 96 and 144 hr, respectively. After removal of culture medium, the cells attached to the plastic dish were washed twice with PBS(-) and were lysed in 1.8 ml of 1N NaOH by incubating at 37°C for 1 hr. An aliquot of cell lysate was titrated with 1N HCl, and protein concentration in the sample was determined by using Bio-Rad Protein Assay Kit (Bio-Rad Lab., Richmond, CA). The culture medium was dialyzed at 4°C against PBS(-) 4 times, and subsequently dialyzed against serum-free RPMI 1640 medium. The dialyzed medium was filtered through 0.22 μm Millipore filters and stored at -20°C until the time of assay. The medium was thawed immediately before the bioassay of IL-1 and IL-6 activity. The possible effect of IL-1 on

hPTH(1-34)-stimulated release of IL-6 from MC3T3-E1 cells was examined by using anti-IL-1α antibody. The cells were cultured in 4 ml of α-MEM supplemented with 0.2% BSA and 10^{-6} M hPTH(1-34) was added to the dishes after washing the cells with PBS(-). The cells were further cultured with anti-IL-1α antibody or normal rabbit IgG for 48 hr.

IL-1 assay

IL-1 activity in the culture medium was determined by measuring its ability to stimulate EL4-6 cells to produce IL-2. IL-2 produced was determined by measuring the activity to stimulate DNA synthesis of HT2 cells (19). EL4-6 cells were washed 3 times with MEM supplemented with 2 % FBS. The cells ($1x10^5$) were suspended in 100 μl of RPMI 1640 supplemented with 10 % FBS and seeded onto each well of 96-well plastic plate (Falcon, Oxnard, CA). Then, 100 μl of the culture medium (sample) or RPMI 1640 medium containing standard IL-1α was added to the cell suspension, and the cells were cultured for 20-24 hr. At the end of culture, the supernatants were collected. HT2 cells were washed 3 times with MEM supplemented with 2 % FBS. The cells ($4x10^3$) suspended in 50 μl of RPMI 1640 supplemented with 10 % FBS were mixed with 100 μl of the culture medium of EL4-6 cells, and also mixed with 50 μl of the medium containing rat anti-IL-4 monoclonal antibody, 11B11 (20), in each well of 96-well plastic plate. The anti-IL-4 antibody was added to eliminate the possible stimulatory effect of IL-4 on HT2 cells (21). HT2 cells in the mixture were cultured for 20-24 hr, followed by a 6 hr pulse of 0.5 μCi of [^{3}H]thymidine. After the culture, HT2 cells were harvested on GF/C filter (Whatman, Maidstone, Kent, UK) with cell harvester (Labo Science Co. Ltd, Tokyo, Japan) and filters were counted with a liquid scintillation counter (LSC-700, Aloka Co. Ltd, Tokyo, Japan). IL-1 activity in the samples was measured in triplicate from the standard curve obtained by recombinant IL-1α.

IL-6 assay

IL-6 was measured by using IL-6-dependent B45-3 cells (18). The cells ($4x10^3$) suspended in 100 μl of RPMI 1640 medium supplemented with 10 % FBS were seeded onto each well of 96-well plastic plate. Then, 100 μl of samples was added to the cell suspension. After cultured for 48 hr, 0.5 μCi of [^{3}H]thymidine was added to the well and the cells were further incubated for 4 hr. The cells were harvested, and radioactivity incorporated into the cells was determined by the same method as in IL-1 assay. IL-6 activity in the samples were measured in triplicate from the standard curve obtained by recombinant IL-6.

Statistics

Data were analyzed by one-factor analysis of variance. If statistically significant effects were found, a Newman-Keuls test was performed to isolate the differences between groups. Student's t test was performed when appropriate. A p value of less than 0.05 was considered to be significant. All data are presented in the text, table and figures as the means±SEM.

Results

Effect of hPTH(1-34) on the release of IL-1 and IL-6 from MC3T3-E1 cells

Confluent MC3T3-E1 cells were cultured in the presence or absence of hPTH(1-34) (10^{-9} -10^{-6} M) for 24 hr, and IL-1 and IL-6 activities released from cells were measured. As shown in Table 1, both IL-1 and IL-6 were released at a detectable level from the osteoblastic cells cultured without hPTH(1-34). hPTH(1-34) stimulated the release of IL-1 and IL-6 in a dose-dependent manner.

Table 1. Effect of hPTH(1-34) on the release of IL-1 and IL-6 from MC3T3-E1 osteoblastic cells. MC3T3-E1 cells were incubated with various concentrations of human parathyroid hormone(1-34), and the activities of IL-1 and IL-6 in the culture media were measured with specific bioassay. **p<0.01 vs. control. Number of experiments are six for all data. N.D. = not determined.

hPTH(1-34) (M)	[^{3}H]TdR incorporation (cpm/μg protein)	
	IL-1	IL-6
0 (control)	467±143	728±144
$1x10^{-9}$	481±208	1207±327
$3x10^{-8}$	723± 71	N.D.
$1x10^{-6}$	1248±154**	1636±480

Time course of hPTH(1-34)-stimulated release of IL-1 from MC3T3-E1 cells

Figure 1 shows the time course of hPTH(1-34)(10^{-6} M)-stimulated release of IL-1 from MC3T3-E1 cells. The cells cultured without hPTH(1-34) released the detectable level of IL-1 during 6 to 144 hr. Significantly higher IL-1 activity was detectable in the culture media of hPTH(1-34)-treated osteoblastic cells as compared with that in the control culture. The release of IL-1 activity from hPTH(1-34)-treated cells was higher than that from control culture at as early as 6 hr, and remained higher up to 144 hr. The IL-1 activity tended to decrease thereafter.

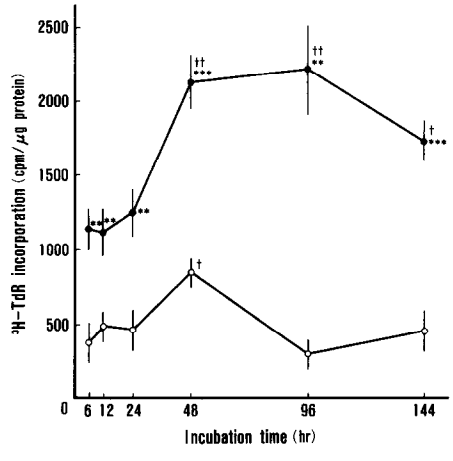

Fig. 1. Effect of incubation time on release of IL-1 from MC3T3-E1 cells stimulated by 10^{-6} M hPTH(1-34). MC3T3-E1 cells were cultured in the presence (●) or absence (○) of 10^{-6} M hPTH(1-34), and IL-1 concentration in the culture medium was measured by specific bioassay. Results are shown as means±SEM of [^{3}H]thymidine incorporation (cpm/μg protein) into cells from 6 experiments. **p<0.01, ***p<0.001 vs. control; †p<0.05, ††p<0.01 vs. 6 hr.

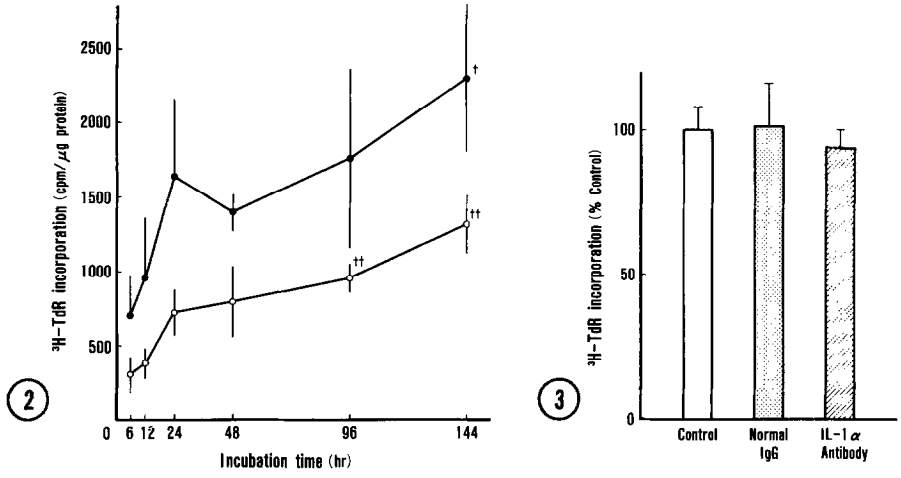

Fig. 2. Effect of incubation time on release of IL-6 from MC3T3-E1 cells stimulated by 10^{-6} M hPTH(1-34). MC3T3-E1 cells were cultured in the presence (●) or absence (○) of 10^{-6} M hPTH(1-34), and IL-6 concentration in the culture medium was measured by a specific bioassay. Results are shown as means±SEM of [^{3}H]thymidine incorporation (cpm/μg protein) into cells from 6 experiments. †$p<0.05$, ††$p<0.01$ vs. 6 hr.

Fig. 3. Effect of anti-IL-1α antibody on hPTH(1-34)-stimulated release of IL-6 from MC3T3-E1 cells. MC3T3-E1 cells were cultured with 10^{-6} M hPTH(1-34) in the presence of rabbit anti-mouse IL-1α antibody or normal rabbit IgG as described in Materials and Methods. Results are shown as means±SEM of percent of [^{3}H]thymidine incorporation into B45-3 cells from 8 experiments.

Time course of hPTH(1-34)-stimulated release of IL-6 from MC3T3-E1 cells

As shown in Figure 2, IL-6 activity released from the cells cultured without hPTH(1-34) was relatively low during the first 12 hr and increased thereafter. IL-6 activity was found to be higher in the culture media of hPTH(1-34)-treated cells than that of control cells. IL-6 activity in the culture medium of hPTH(1-34)-treated cells remained higher during 144 hr.

Effect of anti-IL-1 antibody on hPTH(1-34)-stimulated release of IL-6 from MC3T3-E1 cells

MC3T3-E1 cells were cultured with 10^{-6} M hPTH(1-34) for 48 hr in the presence or absence of anti-mouse IL-1α antibody and IL-6 activity in the culture supernatant was determined. According to preliminary experiments, the amount of the antibody used in this study completely suppressed the activity of standard IL-1 (data not shown). As shown in Figure 3, the antibody itself had no effect on IL-6 activity.

Discussion

In the present study, we found that IL-1 and IL-6 are released from MC3T3-E1 osteoblastic cells in culture. We also found that hPTH(1-34) stimulates both IL-1 and IL-6

release from these cells. As far as we know, this is the first report showing that PTH stimulates IL-1 release from the osteogenic cells. As for IL-6, Löwik *et al.* have already shown that PTH and PTH-fragment stimulate the production of this cytokine in rat osteoblast-like cells and UMR-106 osteogenic sarcoma cells (10). We have confirmed this finding using MC3T3-E1 cells.

Hanazawa *et al.* reported that IL-1-like cytokine is released spontaneously from MC3T3-E1 cells (14). This finding is consistent with the results in the present study. It must be emphasized that the method used for IL-1 assay in the present study is entirely specific for IL-1, differing from the method using thymocyte. The two cell lines, EL4-6 and HT2, were used for the bioassay of IL-1 in the present study. IL-2 was produced specifically by EL4-6 cells in response to IL-1, and HT2 cells proliferates by stimulatory effect of produced IL-2.

PTH-activated osteogenic cells can stimulate osteoclastic bone resorption by increasing the activity of existing mature osteoclasts and by increasing osteoclast number through enhanced recruitment from hemopoietic precursors (22). The factors involved in the PTH-stimulated processes are still unknown. Since both IL-1 and IL-6 are known as a stimulator of bone resorption, we examined the possibility that IL-1 besides IL-6 may function as a mediator in PTH-stimulated bone resorption.

Recently, interaction between IL-1 and IL-6 in their production in human blood mononuclear cells has been reported (23). Production of IL-6 in the mononuclear cells is reported to be stimulated by IL-1α and, in turn, IL-6 is reported to suppress the production of IL-1β (23). To examine the possibility that IL-6 production in MC3T3-E1 cells may be stimulated by IL-1α, effect of anti-IL-1α antibody on the release of IL-6 from the hPTH(1-34)-treated osteoblastic cells was investigated. The results showed that the antibody had no effect on the release of IL-6. Thus, the increased release of IL-6 from hPTH(1-34)-treated osteoblastic cells may be independent of IL-1 production. Further studies, however, will be required to clarify this point.

In conclusion, the present study showed that hPTH(1-34) stimulates the release of IL-1 and IL-6 from MC3T3-E1 osteoblastic cells. Since IL-1 is a potent stimulator of bone resorption, it is possible that IL-1 besides IL-6 may function as a mediator of PTH-induced bone resorption.

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