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Interleukin (IL)-4 and IL-13 Inhibit the Differentiation of Murine Osteoblastic MC3T3-E1 Cells

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Abstract. Interleukin-4 (IL-4) inhibits the spontaneous and stimulated bone resorption resulting from the inhibition of osteoclast formation, as well as osteoclastic activity. Since IL-13 shares some biological properties with IL-4, it was recently reported that IL-13 inhibits bone resorption. The present study was designed to determine the effects of murine IL-4 (IL-4) and murine IL-13 (IL-13) on the murine osteoblastic cell line MC3T3-E1. IL-4 and IL-13 stimulated ^{3}H-thymidine incorporation in the MC3T3-E1 cells and its proliferation in dose dependent manners. A spontaneous increase in alkaline phosphatase (ALP) activity in the cells after plating was inhibited by IL-4 or IL-13, and both cytokines blunted an increase in ALP activity by human parathyroid hormone (PTH) (1–34). PTH-stimulated cyclic AMP (cAMP) production was inhibited by pretreatment with IL-4 and IL-13 for 48 hr in dose dependent manners. Pretreatment with IL-4 and IL-13 for 48 hr caused a decrease in PTH-induced cAMP production at any stimulatory concentration. However, the effective dose (ED_{50}) was unchanged by the pretreatment with these cytokines. Pretreatment with IL-4 and IL-13 did not modulate cAMP generation by forskolin. In contrast, cAMP generation by PGE_2 is greater in the cells treated with the cytokines compared to those without the cytokines. These results indicate that IL-4 and IL-13 act on MC3T3-E1 cells in the same manner, stimulating cell proliferation, but inhibiting cell differentiation. The inhibition of osteoblast differentiation by IL-4 and IL-13 may be associated with a decrease in PTH actions on osteoblasts.

Key words: Interleukin-13, Interleukin-4, Osteoblast differentiation, Parathyroid hormone

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INTERLEUKIN-4 (IL-4) inhibits the spontaneous and stimulated bone resorption resulting from the inhibition of osteoclast formation [1–4]. IL-4 also acts on osteoclasts, inhibiting their functional activity. Lewis *et al.* reported that bone formation is also decreased in IL-4 transgenic mice, which leads to a low turnover of bone metabolism and subsequent osteoporosis [5]. Our histomorphometrical study indicated that IL-4 inhibits not only bone resorption but also bone formation in normal and ovariectomized mice *in vivo*, resulting in a low rate of bone turnover [6]. These *in vivo* studies [5, 6] suggest that IL-4 also acts on osteoblasts, leading to inhibition of bone formation.

IL-13, a recently identified Th2 cytokine, shares some, but not all, of the *in vitro* functions of IL-4. Each cytokine inhibits the activation of monocytes and macrophages and stimulates human B cells. IL-13 does not act on T cells, whereas IL-4 does. Murine IL-13 (mIL-13) does not act on murine B cells, in contrast to mIL-4 which acts on murine B

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cells [7]. IL-4 and IL-13 may share a common signaling receptor subunit, although they have specific ligand-binding subunits [8–10]. It was recently reported that both IL-4 and IL-13 inhibit osteoclast formation and bone resorption, resulting from the suppression of cyclooxygenase (COX)-2-dependent prostaglandin synthesis in osteoblasts [11]. Taken together, these results suggest that IL-4 and IL-13 act on osteoblasts in a similar manner. In this study, we examined the effects of IL-4 and IL-13 on a murine osteoblastic cell line, MC3T3-E1 cells. The results suggest that IL-4 as well as IL-13 inhibits the differentiation of these cells, leading to the inhibition of the osteoblastic functions.

Material and Methods

The murine osteoblastic cell line MC3T3-E1 was provided by the Riken Cell Bank (Tsukuba, Japan). The MC3T3-E1 cells were maintained in alpha-minimum essential medium (α-MEM) supplemented with penicillin/streptomycin (100 U/ml, 100 μg/ml) and 10% heat-inactivated fetal calf serum (FCS) in a humidified atmosphere at 37°C in 5% CO_2 in air. Murine recombinant interleukin 4 (mIL-4) was kindly provided by Schering-Plough (Osaka, Japan), and murine interleukin 13 (mIL-13) was kindly supplied by Dr. R. de Waal Malefyt (Dnax Research Institute, Palo Alto, CA, U.S.A.). Human parathyroid hormone (hPTH) (1–34) was purchased from Peptide Institute Co. (Osaka). Forskolin was purchased from Wako Pure Chemical Co. (Osaka). PGE_2 was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

^{3}H-thymidine incorporation and cell proliferation

MC3T3-E1 cells (1×10^4 cells per well) were seeded into 96-well plates in α-MEM supplemented with 1% FCS and cultured for 12 hr, and then various doses of mIL-4 or mIL-13 were added. The cells were further incubated for 24 hr in a humidified atmosphere at 37°C in 5% CO_2 in air. The cells were then pulsed with 0.5 μCi of ^{3}H-thymidine at 12 hr before harvesting. At the end of the culture period, the cells were detached with 0.25% trypsin and collected into glassfiber filters with a cell harvester (Filter-mate, Packard Instrument Co., Meriden, CT, U.S.A.). The amount of incorporated radioactivity was measured by a β-counter (Matrix 9600, Packard Instrument Co.).

Cell proliferation was determined by a cell proliferation reagent WST-1 kit (Boehringer Mannheim, Tokyo, Japan). This assay is based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenase of viable cells [12]. MC3T3-E1 cells (1×10^4 cells per well) were seeded into 96-well plates in α-MEM supplemented with 1% FCS and cultured for 12 hr, and then various doses of mIL-4 or mIL-13 were added. The cells were further incubated for 24 hr in a humidified atmosphere at 37°C in 5% CO_2 in air. After incubation, the ready-to-use WST-1 reagent was added to the cultures and incubated for 1 hr at 37°C. The absorbance at 450 nm, against a reference wavelength of 650 nm, was determined.

Cell proliferation was also assessed by direct cell counting with a hemocytometer. MC3T3-E1 cells seeded at a density of 2.5×10^5 cells/dish to 60-mm Falcon plastic dishes in α-MEM supplemented with 1% FCS and cultured for 12 hr at 37°C, and then various doses of mIL-4 or mIL-13 were added. After being cultured for 24 hr, the cell number was determined by direct counting with a hemocytometer after achieving cell detachment with trypsin.

Assay of alkaline phosphatase activity in MC3T3-E1

First, 5×10^5 MC3T3-E1 cells were plated in 60-mm Falcon plastic dishes in 4 ml of medium supplemented with 10% FCS and cultured for 3 days. The medium was then replaced with α-MEM containing 0.5% FCS plus various doses of mIL-4 or mIL-13, and the cells were cultured for an additional 24–96 hr in the absence or presence of 10 ng/ml hPTH(1–34). The cells were washed 3 times with 0.9% NaCl solution and scraped into 200 μl of 1.5 M ethylaminoethanol buffer containing 0.76 mM $MgCl_2$, pH 9.9. The cells were sonicated for 20 sec with an ultrasonic disruptor (Tomy Seiko Co., Tokyo, Japan) and then Triton-X (final concentration 0.2%) was added. The sonicates were centrifuged for 30 min at 11,000 rpm, and the supernatants were used for the ALP assay. The ALP activity was determined by measuring the release of p-nitrophenol from p-nitrophenyl phosphate using a

kit (Pure Auto ALP, Daiichi Pure Chemicals Co., Tokyo) at 30°C for 2 min by spectrophotometry at 405 nm. The enzymatic activity is expressed as unit per mg protein. The protein content in the cell lysates was measured with a protein assay kit (Bio Rad protein assay, Bio Rad, Hercules, CA).

Assay of PTH-dependent intracellular cyclic AMP levels

MC3T3-E1 cells were cultured in a 24-well plate in α-MEM containing 10% FCS (1.5×10^5 cells/well) for 3 days. The medium was then replaced with α-MEM containing 0.5% FCS plus various doses of mIL-4 or mIL-13, and the cells were cultured for 48 hr or 20 min. The cells were preincubated in 400 μl of α-MEM containing 0.2% bovine serum albumin (BSA) plus 2 mM isobutylmethylxantine (IBMX, Wako Pure Chemical, Osaka) for 30 min at 37°C. Then, 100 μl of α-MEM containing 5–800 ng/ml hPTH (PTH final 1–160 ng/ml), 50 μM forskolin (forskolin final 10 μM) or 50 nM-50 μM prostaglandin E_2 (PGE_2) (PGE_2 final 10 nM-10 μM), 0.2% BSA and 2 mM IBMX was added, and the cells were further incubated for 10 min at 37°C. After incubation, 500 μl of ice-cold 16% trichloroacetic acid (TCA) was added to the wells, and the cells were sonicated for 20 sec and then centrifuged at 3500 rpm for 20 min at 4°C. TCA in the supernatant was removed by extraction 3 times with 5 ml of water-saturated ether, and the remaining solution was used for the cAMP assay. cAMP content was measured by a cAMP radioimmunoassay kit (Yamasa cyclic AMP assay kit, Yamasa, Choshi, Japan).

Statistical Analysis

The results are presented as the mean ± standard deviation (SD). Statistical analysis was carried out by the analysis of variance (ANOVA). The given significance levels were determined by Fisher's protected least significant difference (PLSD). P values less than 0.05 were considered to be significant.

Results

^{3}H-thymidine incorporation of MC3T3-E1 cells and cell proliferation

As shown in Fig. 1, both mIL-4 and mIL-13 increased the ^{3}H-thymidine incorporation into MC3T3-E1 cells cultured in α-MEM containing 1% FCS in dose dependent manner. This stimulatory effect was seen at concentrations of 1–100 ng/ml, and was evident in subconfluent culture. At concentrations over 100 ng/ml of mIL-4 or mIL-13, the ^{3}H-thymidine incorporation gradually decreased (data not shown), indicating that mIL-4 and mIL-13 may have a toxic effect on MC3T3-E1 cells at these con-

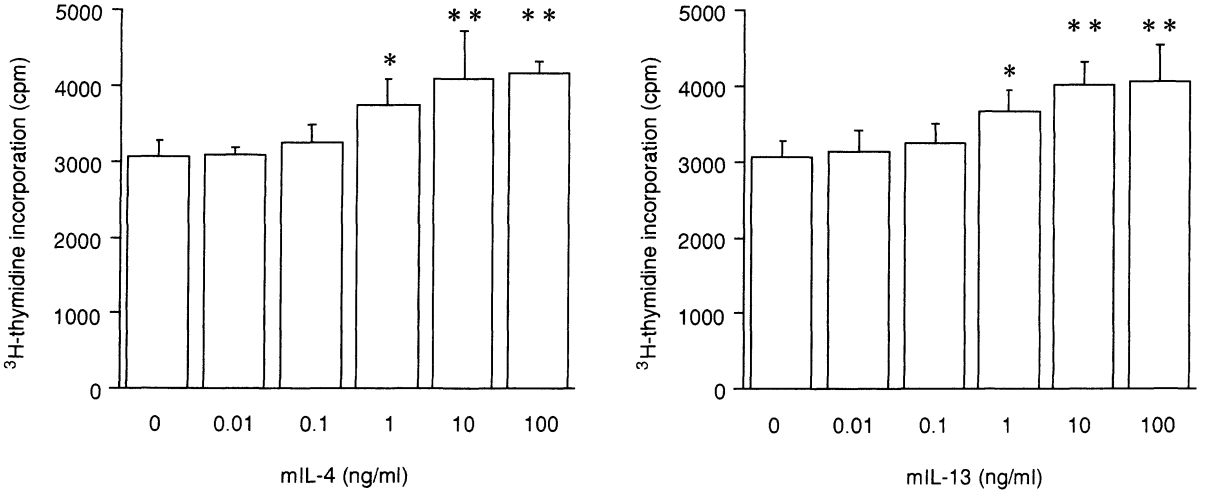

Fig. 1. Dose-response effects of mIL-4 and mIL-13 on ^{3}H-thymidine incorporation in MC3T3-E1 cells. Values are mean ± SD for six samples. *, $P<0.05$; **, $P<0.01$ compared with control.

centrations.

We also assessed the proliferation of MC3T3-E1 cells by the WST-1 test or direct cell counting. MIL-4 and mIL-13 induced an increase in the cell proliferation in dose-dependent manner (Figs. 2A and B).

Effects of mIL-4 and mIL-13 on ALP activity in MC3T3-E1 cells

ALP activity in MC3T3-E1 cells increased spontaneously during 96 hr culture and was inhibited by the presence of 10 ng/ml mIL-4 or 10 ng/ml mIL-13 (Figs. 3A and B). PTH (10 ng/ml)-stimulated ALP activity in MC3T3-E1 cells became maximum within 24 hr and a plateau was maintained 96 hr after treat-

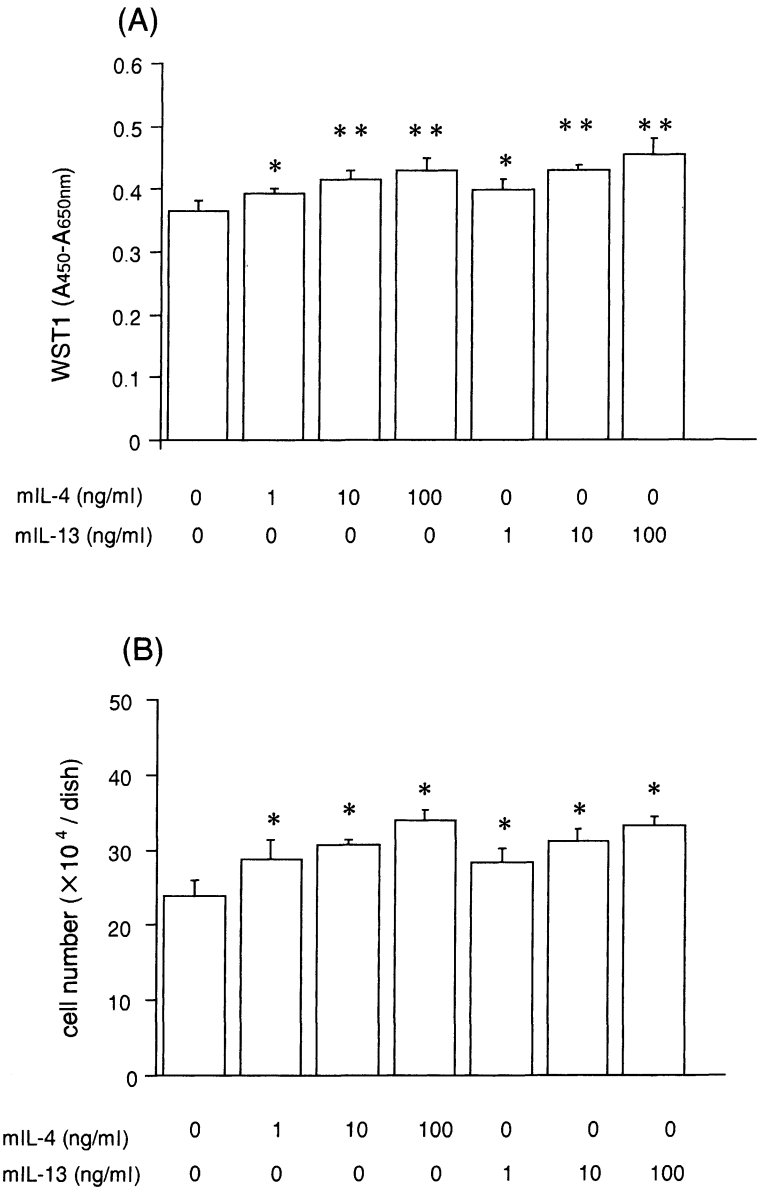

Fig. 2. Effects of mIL-4 and mIL-13 on cell proliferation. The proliferation of MC3T3-E1 cell was assesses by a cell proliferation reagent WST-1 kit (A) and direct cell counting (B). Values are mean±SD of six samples in (A) and that of four samples in (B). *, $P<0.05$; **, $P<0.01$ compared with control.

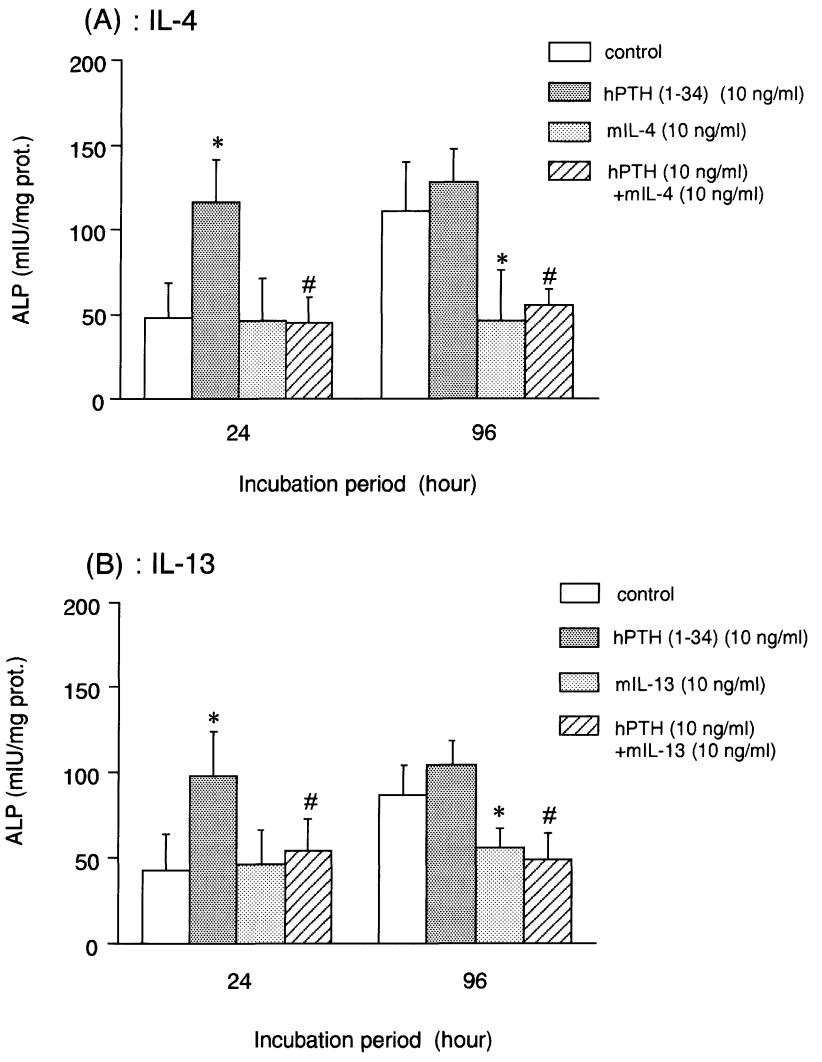

Fig. 3. Alkaline phosphatase activity (ALP) in spontaneous and hPTH-stimulated MC3T3-E1 ells after treatment with mIL-4 (A) and mIL-13 (B) for 1 and 4 days. Values are mean ± SD for quadruplicate cultures. *, $P < 0.01$ compared with control; #, $P < 0.01$ compared with hPTH(1–34).

ment. PTH-stimulated ALP activity was inhibited by simultaneous addition of 10 ng/ml mIL-4 or 10 ng/ml mIL-13 to the level of control cells cultured for 24 hr (Figs. 3A and B).

Effects of mIL-4 and mIL-13 on hPTH(1-34) and PGE_2-stimulated cAMP generation in MC3T3-E1 cells

PTH induces an increase in cAMP levels in MC3T3-E1 cells [13, 14]. In the present study, the hPTH(1–34) (10 ng/ml)-stimulated cAMP generation in MC3T3-E1 cells was inhibited by pretreatment with mIL-4 or mIL-13 for 48 hr in a dose-dependent manner (Fig. 4). The dose dependent increase in cAMP generation by PTH was not inhibited when the cells were pretreated with mIL-4 (10 ng/ml) or mIL-13 (10 ng/ml) for 20 min (Fig. 5A). However, pretreatment with mIL-4 or mIL-13 for 48 hr caused a decrease in hPTH(1–34)-induced cAMP production at all stimulatory concentrations of PTH (Fig. 5B). However, the half-maximal effective dose (ED_{50}) was essentially unchanged by pretreatment with mIL-4 or mIL-13 (hPTH(1–34) alone, 23.0 ng/ml; mIL-4, 27.6 ng/ml; mIL-13, 27.0 ng/ml). To examine the effect of mIL-4 or mIL-13 on cAMP generation by

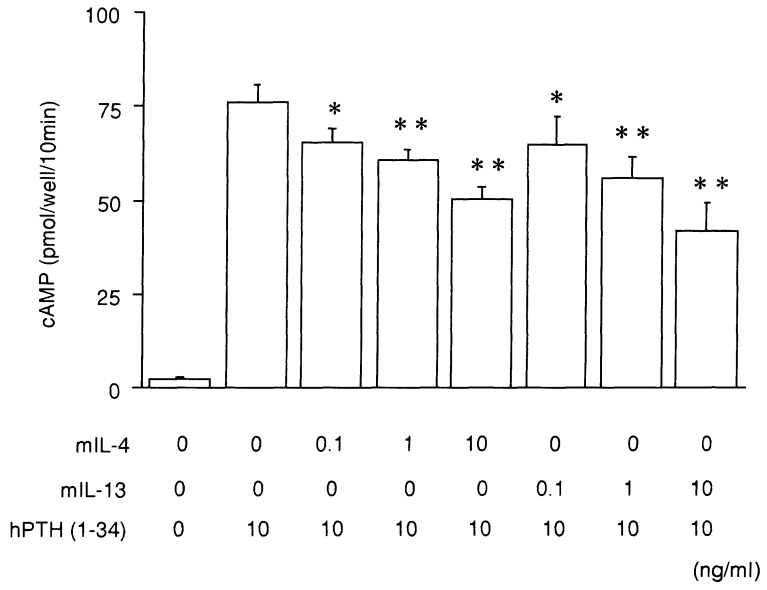

Fig. 4. Effects of pretreatment with mIL-4 or mIL-13 for 48 hr on PTH-stimulated cAMP generation in MC3T3-E1 cells. The cells preincubated with vehicle, mIL-4 or mIL-13 for 48 hr were incubated for 10 min with hPTH(1–34) in α-MEM containing 0.2% BSA with 2 mM IBMX. Values are mean±SD for quadruplicate cultures. *, $P<0.05$; **, $P<0.01$ compared with hPTH only.

other stimulatory factors, PGE_2 was employed under the same experimental condition. Unlike the PTH responses, PGE_2-induced cAMP production was greater in the cells treated with 10 ng/ml mIL-4 or 10 ng/ml mIL-13 for 48 hr compared to those without cytokines (Fig. 6). The effect of mIL-4 or mIL-13 on non-PTH receptor mediated activation of cAMP was evaluated. cAMP generation by 10 μM forskolin, which has a direct stimulatory effect on catalytic component of adenylate cyclase, was not modulated by pretreatment with 10 ng/ml mIL-4 or 10 ng/ml mIL-13 for 48 hr (Table 1).

Discussion

IL-4 inhibits bone resorption stimulated by systemic and local bone resorption agents such as PTH(1–34), PTHrP(1–34), 1α, 25$(OH)_2D_3$, IL-1α, IL-1β and PGE_2 *in vitro* and *in vivo* [4, 15], as a result of the inhibition of osteoclast formation and activation [1–3, 11, 15]. IL-13 has been shown to share a series of structural and functional properties with IL-4 [7]. These include inhibiting the production of inflammatory cytokines by monocytes, modulating the growth and phenotype of B lymphocytes, and stimulating the proliferation of hematopoietic progenitor cells. Recently it was reported that IL-13 acts on bone metabolism. Onoe *et al.* reported that IL-4 and IL-13 inhibits IL-1α-induced bone resorption in mouse long bone cultures and osteoclast formation in a coculture system in similar manners [11].

As murine osteoblast-like cells, MC3T3-E1 cells progress through a temporal sequence beginning with an initial proliferation phase followed by the expression of osteoblast differentiation markers, ALP and osteocalcin, similar to that of murine primary osteoblast cells [16]. In the present study, IL-4 and IL-13 caused an increase in ^{3}H-thymidine incorporation into MC3T3-E1 cells. Cell proliferation assays showed that both cytokines stimulated the proliferation in dose dependent manners. Riancho *et al.* have reported that mIL-4 did not have any significant effect on the proliferation of MC3T3-E1 cells [17]. The discrepancy between their results and our results may derive from the methodological difference. Both cytokines inhibited the spontaneous increase in ALP activity, a marker of osteoblast differentiation. These results indicate that mIL-4 and mIL-13 act in similar manners on MC3T3-E1 osteoblastic cells, that is, stimulating the proliferation and inhibiting the

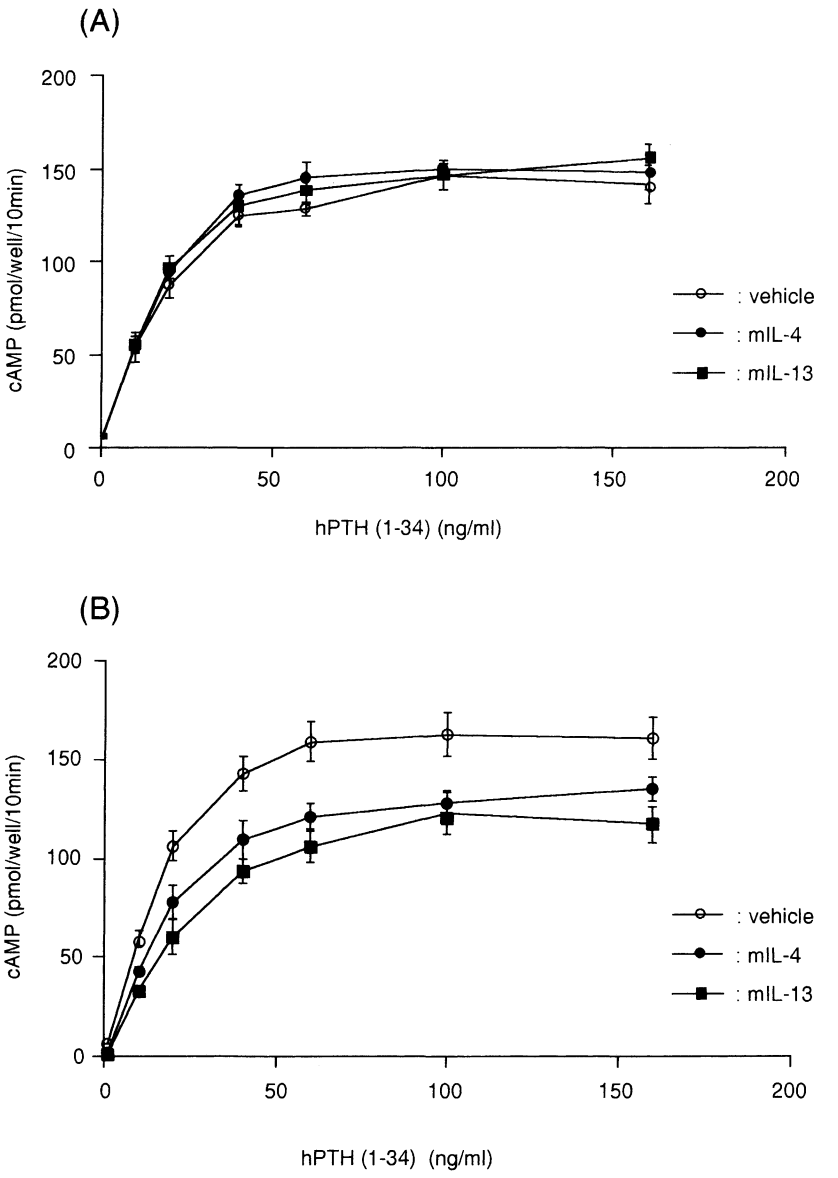

Fig. 5. Effects of pretreatment with mIL-4 or mIL-13 for 20 min and 48 hr hPTH-stimulated cAMP production in MC3T3-E1 cells. The cells pretreated with vehicle alone, 10 ng/ml mIL-4 or 10 ng/ml mIL-13 for 20 min (A) and 48 hr (B) were incubated for 10 min with various concentrations of hPTH(1–34) in α-MEM containing 0.2% BSA with 2 mM IBMX. Values are mean $\pm$ SD for quadruplicate cultures.

differentiation. Osteoblasts exhibit low levels of ALP activity before reaching a confluent state and PTH induces an increase in ALP activity in the osteoblasts, resulting from the induction of osteoblast differentiation [18]. In the present study, PTH stimulated the ALP activity in MC3T3-E1 cells within 24 hr and this stimulatory effect was inhibited by the simultaneous addition of mIL-4 or mIL-13.

PTH stimulates multiple intracellular signals including protein kinase A (PKA), protein kinase C and inositol phosphate and calcium. The activation of PKA acts as a main pathway in the regulation of ALP activity in MC3T3-E1 and other osteoblasts [19–21]. The inhibition of PTH-stimulated ALP activity by mIL-4 or mIL-13 in the MC3T3-E1 cells may be associated with the inhibition of cAMP generation. In this study, pretreatment with mIL-4 or mIL-13 for 48 hr inhibited PTH-induced cAMP

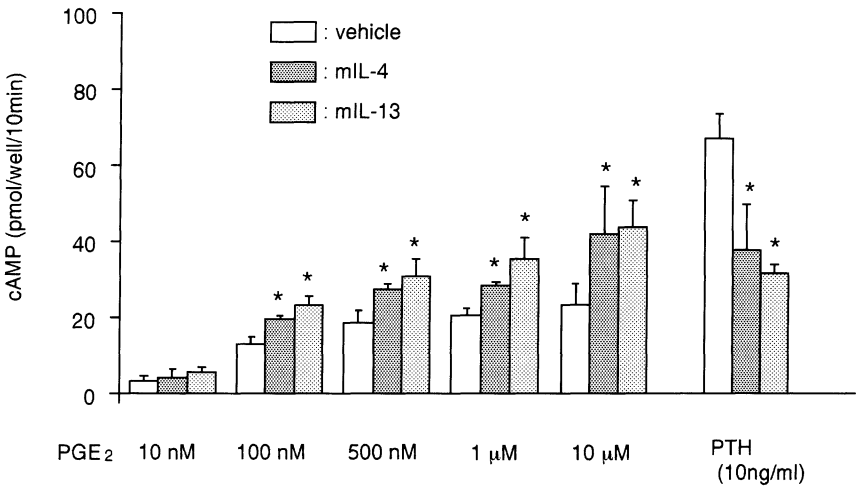

Fig. 6. Effects of pretreatment with mIL-4 or mIL-13 for 48 hr on PGE_2-stimulated cAMP production into MC3T3-E1 cells. The cells pretreated with vehicle alone, mIL-4 or mIL-13 for 48 hr were incubated for 10 min with various concentrations of PGE_2 in α-MEM containing 0.2% BSA with 2 mM IBMX. Values are mean±SD for quadruplicate cultures. *, $P<0.05$ compared with vehicle.

Table 1. Effects of pretreatment with mIL-4 and mIL-13 for 48 hr on PTH and forskolin-induced cAMP production into MC3T3-E1 cells

Stimulatory agonist	Pretreatment	cAMP content (pmol/well)
PTH (10 ng/ml)	Vehicle	60.4± 7.2
	mIL-4	30.1± 2.1*
	mIL-13	28.1± 3.6*
Forskolin (10 μM)	Vehicle	613.7±108.7
	mIL-4	637.3± 90.3
	mIL-13	622.5±111.4

Cells pretreated with vehicle, 10 ng/ml mIL-4 and 10 ng/ml mIL-13 for 48 hr were incubated for 10 min with 10 ng/ml PTH or 10 μM forskolin in α-MEM containing 0.2% BSA with 2 mM IBMX. Values are mean±SD for quadruplicate cultures. *, $P<0.05$ compared with vehicle.

generation in MC3T3-E1 in a dose dependent manner. However, the pretreatment with the cytokines for 20 min did not influence the PTH-induced cAMP generation in the cells. Neither mIL-4 nor mIL-13 inhibited forskolin or PGE_2-stimulated cAMP production in the cells. The dose dependent increase in cAMP production by PTH was inhibited when the cells were preincubated with the cytokines for 48 hr. Since the ED_{50} was no different between the cells pretreated with and without these cytokines, it is suggested that PTH binding affinity to the receptor is not affected by pretreatment with the cytokines. In MC3T3-E1 cells, the type I PTH/PTHrP receptor mRNA levels are low during cell proliferation and increase once the cells begin to differentiate [16]. Taken together, the inhibitory effects of mIL-4 and mIL-13 on PTH-stimulated cAMP generation may be associated with the receptor expression in MC3T3-E1 cells, resulting from the inhibition of differentiation of the cells.

Unlike the PTH responses, PGE_2-induced cAMP production is greater in MC3T3-E1 cells treated with the cytokines compared to those without the cytokines. There is no data whether PGE_2 action in

osteoblasts is modulated by cell differentiation, though expression of PGE_2 receptor subtype is affected at the stage of osteoblastic differentiation [22]. In vivo treatment of rat with PGE_2 analogue down-regulates PGE receptor concentration, which is associated with decreased PGE_2-stimulated cAMP production, in isolated liver plasma membrane [23] and adipocytes [24]. Since IL-4 and IL-13 suppressed IL-1-induced cyclooxygenase (COX)-2 gene expression, resulting in decreased PGE_2 production in murine osteoblastic cells [11, 25], the reduction of PGE_2 production in IL-4 and IL-13-treated MC3T3-E1 cell may up-regulate PGE_2 receptor concentration and PGE_2-dependent cAMP response. However, PGE_2 levels in the culture medium of MC3T3-E1 cell were not modulated by mIL-4 or mIL-13 (data not shown). There is a possibility that the cytokine-induced cell proliferation may be in part associated with an increase in PGE_2-induced cAMP production. Further study is required to clarify these phenomena.

In conclusion, the present study demonstrated that mIL-4 and mIL-13 stimulate murine osteoblastic MC3T3-E1 cell proliferation but inhibit their differentiation. Pretreatment with IL-4 and IL-13 inhibits PTH-stimulated cAMP generation and ALP activity. This inhibitory effect is probably associated with the inhibition of cell differentiation by both cytokines.

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