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Tiludronate Inhibits Interleukin-6 Synthesis in Osteoblasts: Inhibition of Phospholipase D Activation in MC3T3-E1 Cells

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Abstract In previous studies, we have reported that $PGF_{2\alpha}$ stimulates phosphoinositide hydrolysis by phospholipase C and phosphatidylcholine hydrolysis by phospholipase D through heterotrimeric GTP-binding protein in osteoblast-like MC3T3-E1 cells, and that $PGF_{2\alpha}$ and PGE_1 induce interleukin-6 (IL-6) synthesis via activation of protein kinase C and protein kinase A, respectively. In the present study, we investigated the effect of tiludronate, a bisphosphonate known to inhibit bone resorption, on the $PGF_{2\alpha}$- and PGE_1-induced IL-6 synthesis in these cells. Tiludronate significantly suppressed the $PGF_{2\alpha}$-induced IL-6 secretion in a dose-dependent manner in the range between 0.1 and 30 μM. However, the IL-6 secretion induced by PGE_1 or $(Bu)_2cAMP$ was hardly affected by tiludronate. The choline formation induced by $PGF_{2\alpha}$ was reduced by tiludronate dose-dependently in the range between 0.1 and 30 μM. On the contrary, tiludronate had no effect on $PGF_{2\alpha}$-induced formation of inositol phosphates. Tiludronate suppressed the choline formation induced by NaF, known as an activator of heterotrimeric GTP-binding protein. However, tiludronate had little effect on the formation of choline induced by TPA, a protein kinase C activator. Tiludronate significantly inhibited the NaF-induced IL-6 secretion in human osteoblastic osteosarcoma Saos-2 cells. These results strongly suggest that tiludronate inhibits $PGF_{2\alpha}$-induced IL-6 synthesis via suppression of phosphatidylcholine-hydrolyzing phospholipase D activation in osteoblasts, and that the inhibitory effect is exerted at the point between heterotrimeric GTP-binding protein and phospholipase D. J. Cell. Biochem. 69:252–259, 1998. © 1998 Wiley-Liss, Inc.

Key words: bisphosphonate; prostaglandin $F_{2\alpha}$; interleukin-6; phospholipase D; osteoblast.

Bisphosphonates, analogues of pyrophosphate, are known as potent inhibitors of bone resorption [Fleisch et al., 1969]. They are widely used as therapeutic agents for the treatment of various metabolic bone diseases associated with increased osteoclastic bone resorption such as Paget's disease, tumoral bone disease, and osteoporosis [Reginster et al., 1989; Valkema et al., 1989; Storm et al., 1990; Kanis, 1991; Fleisch, 1991]. As for the mechanism of the inhibitory effect on bone resorption, it has been proposed that bisphosphonates inhibit osteoclast development and attachment to bone surface [Boonekamp et al., 1987; Löwik et al., 1988; Hughes et al., 1989] and induce metabolic injury of osteoclasts [Flanagan and Chambers, 1989]. On the other hand, current knowledge indicates that osteoblasts play important roles not only in bone formation but also in osteoclastic bone resorption [Nijweide et al., 1986]. It has been reported that the osteoclast inhibiting action of bisphosphonates is mediated in part through its action on osteoblasts [Sahni et al., 1993; Nishikawa et al., 1995]. Bisphosphonates, ibandronate and alendronate, have recently been shown to induce the synthesis of an inhibitor of osteoclastic bone resorption in osteoblastic cell line CRP 10/30 [Vitte et al., 1996]. However, the detailed mechanism of bisphosphonates in bone resorption through osteoblasts has not yet been fully clarified.

Interleukin-6 (IL-6), produced and secreted in many cell types [Akira et al., 1990; Van

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Snick, 1990], is well known as a multifunctional cytokine that has important physiological effects on the immune system [Akira et al., 1990; Van Snick, 1990]. In bone metabolism, it has been reported that IL-6 stimulates bone resorption [Ishimi et al., 1990], induces osteoclast formation [Roodman, 1992], and inhibits bone formation [Hughes and Howells, 1993]. Bone resorptive agents such as parathyroid hormone, tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and platelet-derived growth factor have been reported to stimulate IL-6 synthesis in cultured osteoblasts [Helle et al., 1988; Feyen et al., 1989; Littlewood et al., 1991; Franchimont and Canalis, 1995], suggesting that IL-6 secreted from osteoblasts is an important downstream effector of bone resorptive agents. On the contrary, 17β-estradiol, known to suppress bone resorption and used as a therapeutic agent of osteoporosis, has been reported to inhibit the IL-6 production induced by IL-1 or TNF-α in osteoblasts [Girasole et al., 1992]. As for the effect of bisphosphonate on IL-6 production, it has been reported that clodronate inhibits lipopolysaccharide-stimulated IL-6 production in macrophage RAW 264 cells [Mönkkönen et al., 1994]. However, the effect of bisphosphonate on IL-6 synthesis in osteoblasts remains unclear.

Prostaglandins are well recognized to act as local modulators in osteoblasts [Nijweide et al., 1986]. Among them, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), known as a bone resorptive agent [Raisz and Martin, 1984], has been reported to stimulate the proliferation of osteoblasts and inhibit the differentiation [Raisz and Martin, 1984; Koshihara and Kawamura, 1989]. In previous studies [Miwa et al., 1990; Kozawa et al., 1994], we have shown that $PGF_{2\alpha}$ stimulates both phosphoinositide hydrolysis by phospholipase C and phosphatidylcholine hydrolysis by phospholipase D through heterotrimeric GTP-binding protein in osteoblast-like MC3T3-E1 cells. It is well known that both phosphoinositide and phosphatidylcholine hydrolysis result in the formation of diacylglycerol, known as a physiological activator of protein kinase C [Nishizuka, 1986]. We have recently reported that $PGF_{2\alpha}$ stimulates IL-6 synthesis via activation of protein kinase C in MC3T3-E1 cells [Kozawa et al., 1997]. Additionally, we have also shown that prostaglandin E_1 (PGE_1) causes IL-6 synthesis via activation of protein kinase A in these cells [Watanabe-Tomita et al., 1997]. In the present study, we examined the effect of a bisphosphonate, tiludronate (chloro-4-phenyl-thiomethylene bisphosphonate, Sanofi, Montpellier, France), on IL-6 synthesis in osteoblast-like MC3T3-E1 cells. Here, we show that tiludronate inhibits $PGF_{2\alpha}$-induced IL-6 synthesis via suppression of phosphatidylcholine-hydrolyzing phospholipase D activation in these cells, and that the inhibitory effect is exerted at the point between heterotrimeric GTP-binding protein and phospholipase D.

MATERIALS AND METHODS

Materials

$PGF_{2\alpha}$, PGE_1, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), NaF, and dibutyryl cAMP [$(Bu)_2$cAMP] were purchased from Sigma Chemical (St. Louis, MO). Tiludronate was generously provided by Meiji Seika Co. Ltd. (Kawasaki, Japan). Mouse IL-6 enzyme immunoassay (EIA) kit, [*methyl*-^{3}H]choline chloride (85 Ci/mmol) and *myo*-[^{3}H]inositol (90 Ci/mmol) were purchased from Amersham Japan (Tokyo, Japan). Human IL-6 EIA kit was purchased from Endogen, Inc. (Cambridge, MA). Other materials and chemicals were obtained from commercial sources. $PGF_{2\alpha}$ and PGE_1 were dissolved in ethanol. TPA was dissolved in dimethylsulfoxide. The maximum concentration of ethanol or dimethyl sulfoxide was 0.1%, which did not affect the assay for IL-6 nor the formation of choline and inositol phosphates.

Cell Culture

Osteoblast-like MC3T3-E1 cells cloned from newborn mouse calvaria [Kodama et al., 1981; Sudo et al., 1983] were maintained as previously described [Kozawa et al., 1997]. In brief, the cells (5×10^4) were seeded into 35-mm diameter dishes in 2 ml of α-minimum essential medium (α-MEM) containing 10% fetal calf serum (FCS) at 37°C in a humidified atmosphere of 5% CO_2-95% air. After 5 days, the medium was exchanged for 2 ml of α-MEM containing 0.3% FCS. The confluent cells at resting state, which show high alkaline phosphatase activity [Sudo et al., 1983], were used for experiments after 48 hr.

The Saos-2 cells, established from an osteosarcoma of an 11-year-old Caucassian girl [Fogh and Trempe, 1975], were obtained from American Type Culture Collection (Rockville, MD). The cells were maintained in McCoy 5A medium containing 10% FCS at 37°C in a humidi-

fied atmosphere of 5% CO_2-95% air. The cells (1×10^5) were seeded into 35-mm dishes in 2 ml of McCoy 5A medium containing 10% FCS and fed every 3 days. After 7 days, the medium was exchanged for 2 ml of McCoy 5A medium containing 0.3% FCS. The confluent cells at resting state, which show high alkaline phosphatase activity [Rodan et al., 1987], were used for experiments after 48 hr.

Assay for IL-6

The cultured cells were pretreated with tiludronate for 8 hr. The MC3T3-E1 cells were subsequently stimulated by $PGF_{2\alpha}$, PGE_1, $(Bu)_2$cAMP, or NaF in 1 ml of α-MEM containing 0.3% FCS for the indicated periods. The Saos-2 cells were stimulated by NaF in 1 ml of McCoy 5A medium containing 0.3% FCS for the indicated periods. The conditioned medium was collected, and IL-6 in the medium from MC3T3-E1 cells and Saos-2 cells was measured by a mouse and a human IL-6 EIA kit, respectively.

Measurement of the Formation of Choline

To determine phosphatidylcholine-hydrolyzing phospholipase D activity in osteoblast-like MC3T3-E1 cells, the cultured cells were labeled with [*methyl*-^{3}H]choline chloride (2 μCi/dish) for 24 hr. The labeled cells were pretreated with tiludronate for 8 hr, and then stimulated by $PGF_{2\alpha}$, NaF, or TPA in 1 ml of an assay buffer [5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, 150 mM NaCl, 5 mM KCl, 5.5 mM glucose, 0.8 mM $MgSO_4$, and 1 mM $CaCl_2$] containing 0.01% bovine serum albumin (BSA) for the indicated periods. The reaction was terminated by adding 0.75 ml of ice-cold methanol. The dishes were placed on ice for 10 min. The contents were transferred to tubes to which chloroform was added and placed on ice for a further 60 min. Chloroform and water were then added for a final chloroform:methanol: water ratio of 1:1:0.9. The tubes were centrifuged at 14,000*g* for 5 min and the upper aqueous methanolic phase was taken for analysis of the water-soluble choline-containing metabolites. The methanolic phase was separated on 1 ml of Dowex 50-WH^+ column (200–400 mesh) as described by Cook and Wakelam [Cook and Wakelam, 1989] with a minor modification [Kozawa et al., 1994]. In brief, the phase was diluted to 5 ml with water and applied to the column. Glycerophosphocholine and phosphocholine were removed by 24 ml of water, and the radiolabeled choline was then eluted with 10 ml of 1 M HCl.

Measurement of the Formation of Inositol Phosphates

To determine phosphoinositide-hydrolyzing phospholipase C activity in MC3T3-E1 cells, the cultured cells were labeled with *myo*-[^{3}H]inositol (3 μCi/dish) for 48 hr. The labeled cells were pretreated with tiludronate for 8 hr, and preincubated with 10 mM LiCl for 10 min in 1 ml of the assay buffer containing 0.01% BSA. The cells were then stimulated by $PGF_{2\alpha}$ at 37°C. The reaction was terminated by adding 1 ml of 30% trichloroacetic acid and the acidic supernatant was treated with diethyl ether to remove the acid. After being neutralized with 0.1 M NaOH, the supernatant was applied to 1 ml of Dowex AG1-X8 column (100–200 mesh, formate form) as described by [Berridge et al., 1983, 1984] with a minor modification [Miwa et al., 1990]. The radioactive inositol phosphates were eluted from the column with 8 ml of 0.1 M formic acid containing 1 M ammonium formate.

Determination

The radioactivity of ^{3}H-labeled samples was determined with a Beckman (Palo Alto, CA) LS-6500IC liquid scintillation spectrometer.

Statistical Analysis

The data were analyzed by Student's *t*-test and a $P < 0.05$ was considered significant. All data are presented as the mean ± SE of triplicate independent cell preparations. Each experiments was repeated three times with similar results.

RESULTS

Effects of Tiludronate on $PGF_{2\alpha}$- or PGE_1-Induced IL-6 Secretion in MC3T3-E1 Cells

We have recently reported that $PGF_{2\alpha}$ stimulates IL-6 synthesis via protein kinase C activation, and that PGE_1 induces IL-6 synthesis via protein kinase A activation in osteoblast-like MC3T3-E1 cells [Kozawa et al., 1997; Watanabe-Tomita et al., 1997]. Thus, we first examined the effect of tiludronate on the secretion of IL-6 induced by $PGF_{2\alpha}$ in these cells. Tiludronate, which by itself had little effect on IL-6 secretion, significantly reduced the $PGF_{2\alpha}$-induced

IL-6 secretion in a dose-dependent manner in the range between 0.1 and 30 μM. The maximum effect of tiludronate was observed at 30 μM, and the IL-6 secretion decreased to about 60% of control (Fig. 1). At the end of the incubation, the cell density was confluent and similar to that at the initiation of the incubation (data not shown). We next examined the effect of tiludronate on IL-6 secretion induced by PGE_1 or $(Bu)_2cAMP$, a permeable analogue of cAMP. However, tiludronate hardly affected IL-6 secretion induced by these agents (Table I).

Effects of Tiludronate on the Formation of Choline and Inositol Phosphates Induced by $PGF_{2\alpha}$ in MC3T3-E1 Cells

In previous studies [Miwa et al., 1990; Kozawa et al., 1994], we have shown that $PGF_{2\alpha}$ activates both phosphatidylinositol-hydrolyzing phospholipase C and phosphatidylcholine-hydrolyzing phospholipase D via independent pathways in MC3T3-E1 cells. We next examined the effects of tiludronate on these two

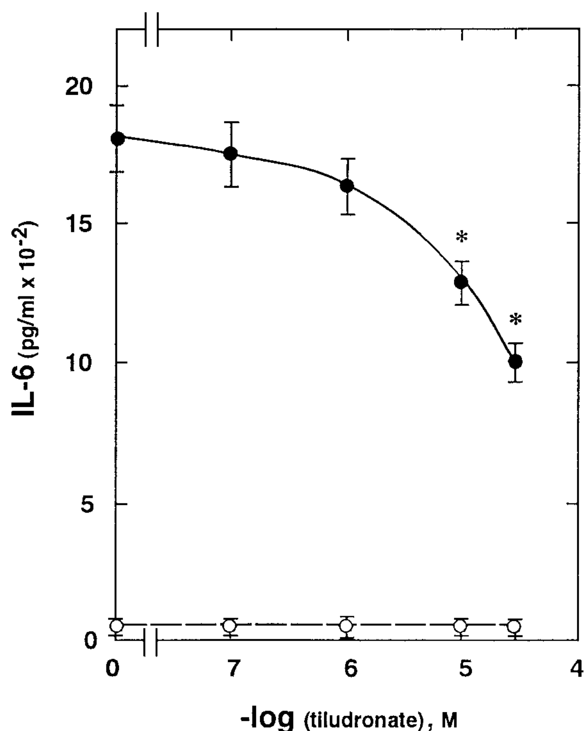

Fig. 1. Effect of tiludronate on $PGF_{2\alpha}$-induced IL-6 secretion in MC3T3-E1 cells. The cultured cells were pretreated with various doses of tiludronate for 8 hr, and then stimulated by 10 μM $PGF_{2\alpha}$ (●) or vehicle (○) for 48 hr. Each value represents the mean ± SE of triplicate independent cell preparations. Similar results were obtained with two additional and different cell preparations. $^{*}P < 0.05$, compared to the value of $PGF_{2\alpha}$ alone.

TABLE I. Effects of Tiludronate on PGE_1- or $(Bu)_2cAMP$-Induced IL-6 Secretion in MC3T3-E1 Cells*

Tiludronate (30 μM)	Stimulator	IL-6 (pg/ml)
−	−	<50
−	PGE_1 (10 μM)	925 ± 85
−	$(Bu)_2cAMP$ (3 mM)	512 ± 42
+	−	<50
+	PGE_1 (10 μM)	906 ± 79
+	$(Bu)_2cAMP$ (3 mM)	491 ± 38

*Cultured cells were pretreated with 30 μM tiludronate or vehicle for 8 hr, and then stimulated by 10 μM PGE_1, 3 mM $(Bu)_2cAMP$, or vehicle for 48 hr. Each value represents the mean ± SE of triplicate independent cell preparations. Similar results were obtained with two additional and different cell preparations.

activations induced by $PGF_{2\alpha}$. Tiludronate, which by itself hardly affected the formation of choline, significantly inhibited the $PGF_{2\alpha}$-induced choline formation in a dose-dependent manner in the range between 0.1 and 30 μM (Fig. 2A). The maximum effect of tiludronate was observed at 30 μM, and the formation decreased to 20% of control. On the contrary, tiludronate, which alone had no effect on inositol phosphates formation, hardly affected the formation of inositol phosphates induced by $PGF_{2\alpha}$ (Fig. 2B).

Effects of Tiludronate on NaF- or TPA-Induced Choline Formation in MC3T3-E1 Cells

We have previously reported that heterotrimeric GTP-binding protein is involved in the $PGF_{2\alpha}$-induced activation of phospholipase D in MC3T3-E1 cells [Kozawa et al., 1994]. To clarify the point exerted by tiludronate in the inhibitory effect on the $PGF_{2\alpha}$-induced choline formation in these cells, we examined the effect of tiludronate on the formation of choline induced by NaF, known as an activator of heterotrimeric GTP-binding protein [Gilman, 1987]. As previously reported [Kozawa et al., 1994], NaF (40 mM) stimulated choline formation. Tiludronate significantly inhibited the NaF-induced choline formation (Table II). The inhibitory effect was dose-dependent in the range between 0.1 and 30 μM (data not shown). The maximum effect was observed at 30 μM and the choline formation induced by NaF was reduced to about 60% of the control. In addition, we previously showed that phospholipase D is also protein kinase C–dependently activated in MC3T3-E1 cells [Kozawa et al., 1994]. Thus, we next exam-

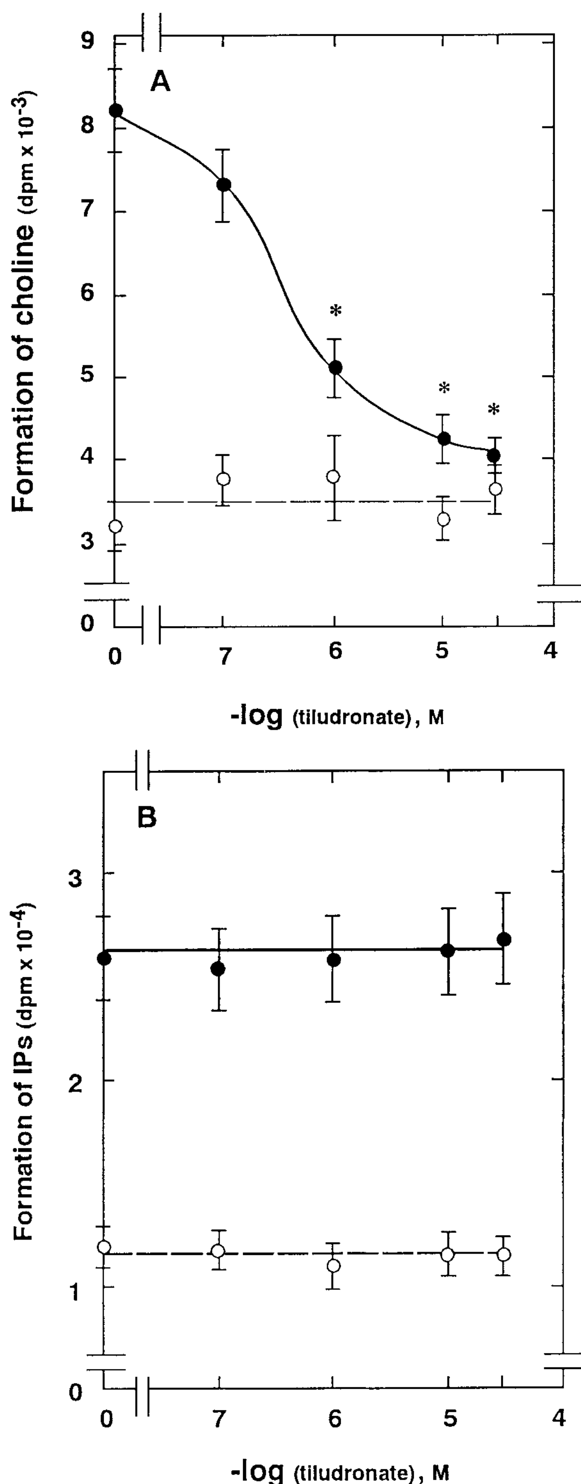

Fig. 2. Effects of tiludronate on the formation of choline and inositol phosphates induced by $PGF_{2\alpha}$ in MC3T3-E1 cells. The cultured cells labeled with [^{3}H]choline chloride (**A**) or [^{3}H]inositol (**B**) were pretreated with various doses of tiludronate for 8 hr, and then stimulated by 10 μM $PGF_{2\alpha}$ (●) or vehicle (○) for 15 min. Each value represents the mean ± SE of triplicate independent cell preparations. Similar results were obtained with two additional and different cell preparations. *$P < 0.05$, compared to the value of $PGF_{2\alpha}$ alone.

TABLE II. Effects of Tiludronate on NaF- or TPA-Induced Choline Formation in MC3T3-E1 Cells[1]

Tiludronate (30 μM)	Stimulator	Formation of choline (dpm)
−	−	3,275 ± 305
−	NaF (40 mM)	7,558 ± 483
−	TPA (0.1 μM)	8,520 ± 515
+	−	3,332 ± 391
+	NaF (40 mM)	5,863 ± 347*
+	TPA (0.1 μM)	8,435 ± 722

[1]Cultured cells labeled with [^{3}H]choline were pretreated with 30 μM tiludronate or vehicle for 8 hr, and then stimulated by 40 mM NaF, 0.1 μM TPA, or vehicle for 15 min. Each value represents the mean ± SE of triplicate independent cell preparations. Similar results were obtained with two additional and different cell preparations.
*$P < 0.05$, compared to the value of NaF alone.

ined the effect of tiludronate on the formation of choline induced by TPA, a protein kinase C activator [Nishizuka, 1986]. Tiludronate (30 μM) had little effect on TPA-induced choline formation in these cells (Table II).

Effects of Tiludronate on NaF-Induced IL-6 Secretion in Saos-2 and MC3T3-E1 Cells

To clarify whether the inhibitory effect of tiludronate on IL-6 secretion is exerted in other osteoblast-like cells, we examined the effect of tiludronate on the IL-6 secretion in human osteosarcoma Saos-2 cells, which has been reported to possess osteoblastic characteristics [Boland et al., 1986; Weiss et al., 1986; Rodan et al., 1987]. NaF stimulated IL-6 secretion in a dose-dependent manner in the range between 1 and 3 mM (data not shown). Tiludronate, which alone hardly affected IL-6 secretion, significantly reduced the IL-6 secretion induced by NaF (3 mM) in a dose-dependent manner in the range between 1 and 30 μM (Fig. 3). The maximum effect of tiludronate was observed at 30 μM, and the IL-6 secretion reduced to about 70% of the control. At the end of the incubation, the cell density was confluent and similar to that at the initiation of the incubation (data not shown). Tiludronate inhibited the NaF-induced IL-6 secretion also in MC3T3-E1 cells (data not shown).

DISCUSSION

In the present study, we showed that tiludronate suppressed $PGF_{2\alpha}$-induced IL-6 synthesis in osteoblast-like MC3T3-E1 cells. We mea-

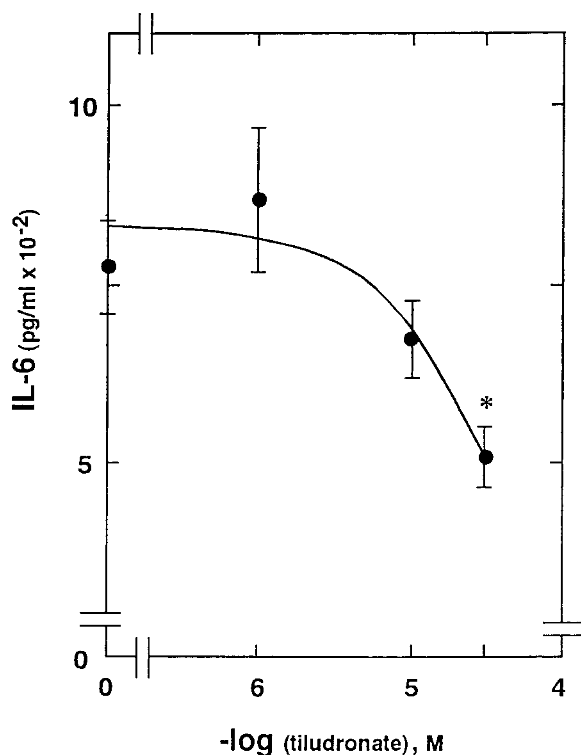

Fig. 3. Effect of tiludronate on NaF-induced IL-6 secretion in osteosarcoma Saos-2 cells. The cultured cells were pretreated with various doses of tiludronate for 8 hr, and then stimulated by 3 mM NaF for 48 hr. Values for unstimulated cells have been subtracted from each data point. Each value represents the mean ± SE of triplicate independent cell preparations. Similar results were obtained with two additional and different cell preparations. *$P < 0.05$, compared to the value of NaF alone.

sured IL-6, which is secreted from these cells, in the conditioned medium. However, it is most likely that the changes in long-term secretion represent the changes in synthesis rather than those in secretory process. We have recently reported that $PGF_{2\alpha}$ stimulates IL-6 synthesis via activation of protein kinase C in MC3T3-E1 cells [Kozawa et al., 1997]. In previous studies [Miwa et al., 1990; Kozawa et al., 1994], we have shown that $PGF_{2\alpha}$ induces phosphoinositide hydrolysis by phospholipase C and phosphatidylcholine hydrolysis by phospholipase D, and that phospholipase D is activated independently from phospholipase C. They are major pathways of protein kinase C activation [Nishizuka, 1986; Billah and Anthes, 1990; Exton, 1990]. Therefore, we examined the effect of tiludronate on these two pathways. We found that tiludronate inhibited $PGF_{2\alpha}$-induced choline formation without affecting the formation of inositol phosphates. It is recognized that phosphatidylcholine hydrolysis by phospholipase D is necessary for the long-term activation of protein kinase C [Billah and Anthes, 1990; Exton, 1990]. Thus, our results suggest that tiludronate reduces the activity of phosphatidylcholine-hydrolyzing phospholipase D induced by $PGF_{2\alpha}$, resulting in the inhibition of protein kinase C activation in osteoblast-like MC3T3-E1 cells. We showed that tiludronate had little effect on PGE_1- or $(Bu)_2$cAMP-induced IL-6 secretion. In the recent study [Watanabe-Tomita et al., 1997], we have demonstrated that PGE_1 causes IL-6 synthesis via activation of protein kinase A in these cells. Thus, it is unlikely that tiludronate affects IL-6 synthesis induced by the activation of protein kinase A in osteoblast-like cells.

We next showed that tiludronate inhibited the choline formation induced by NaF. Heterotrimeric GTP-binding proteins are involved in many kinds of transmembrane signaling [Gilman, 1987]. We previously reported that $PGF_{2\alpha}$ stimulates phosphatidylcholine hydrolysis by phospholipase D in a heterotrimeric GTP-binding protein-dependent manner [Kozawa et al., 1994]. Thus, our present results suggest that tiludronate affects $PGF_{2\alpha}$-induced phosphatidylcholine hydrolysis by phospholipase D at a point downstream from the heterotrimeric GTP-binding protein in MC3T3-E1 cells. In addition, we showed that tiludronate had little effect on TPA-induced choline formation. We have reported that TPA stimulates phosphatidylcholine-hydrolyzing phospholipase D through protein kinase C activation in these cells [Kozawa et al., 1994]. Therefore, these results strongly suggest that tiludronate inhibits $PGF_{2\alpha}$-induced phosphatidylcholine hydrolysis at the point between heterotrimeric GTP-binding protein and phospholipase D in osteoblast-like MC3T3-E1 cells.

Furthermore, we showed here that tiludronate reduced NaF-induced IL-6 secretion in human osteoblastic osteosarcoma Saos-2 cells. Thus, this finding suggests that tiludronate affects IL-6 synthesis at a point downstream of heterotrimeric GTP-binding protein in these cells. We also found the inhibitory effect of tiludronate on NaF-induced IL-6 secretion in MC3T3-E1 cells. Nowadays, it is well known that IL-6 induces osteoclastic bone resorption [Ishimi et al., 1990]. Therefore, it is probable that tiludronate reduces the synthesis of IL-6 commonly in osteoblasts, resulting in the reduction of bone resorption. However, primary cultures of osteoblasts and/or osteogenic stem cells

may elicit different responses to tiludronate. It is currently well recognized that increased production of IL-6 by osteoblastic lineage plays an important role in the development of estrogen-withdrawal osteoporosis [Manolagas and Jilka, 1995]. Taking them into account, it is most likely that the inhibitory mechanism of tiludronate on IL-6 synthesis as shown here, in addition to the direct effects of bisphosphonate on osteoclast such as the promotion of osteoclast apoptosis [Parfitt et al., 1996], might contribute to the effectiveness of bisphosphonates at least in postmenopausal osteoporosis.

In conclusion, our results strongly suggest that tiludronate inhibits $PGF_{2\alpha}$-induced IL-6 synthesis via suppression of phosphatidylcholine-hydrolyzing phospholipase D activation in osteoblasts, and that the inhibitory effect is exerted at the point between heterotrimeric GTP-binding protein and phospholipase D.

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