Aluminum stimulates the proliferation and differentiation of osteoblasts in vitro by a mechanism that is different from fluoride

Article *in* Molecular and Cellular Biochemistry · August 1991

DOI: 10.1007/BF00227749 · Source: PubMed

3 authors, including:

K-H William Lau
U.S. Department of Veterans Affairs
197 PUBLICATIONS **6,326** CITATIONS

Molecular and Cellular Biochemistry **105**: 93–105, 1991.

Aluminum stimulates the proliferation and differentiation of osteoblasts *in vitro* by a mechanism that is different from fluoride

K.-H. William Lau, Aera Yoo and Shu Ping Wang
Departments of Medicine and Biochemistry, Loma Linda University and Mineral Metabolism Unit, Jerry L. Pettis Memorial Veterans' Hospital, 11201 Benton Street, Loma Linda, California 92357, USA

Received 12 October 1990; accepted 28 January 1991

Key words: aluminum, osteoblasts, fluoride, bone formation, proliferation, differentiation

Summary

Micromolar concentrations of aluminum sulfate consistently stimulated [^{3}H]thymidine incorporation into DNA and increased cellular alkaline phosphatase activity (an osteoblastic differentiation marker) in osteoblast-line cells of chicken and human. The stimulations were highly reproducible, and were biphasic and dose-dependent with the maximal stimulatory dose varied from experiment to experiment. The mitogenic doses of aluminum ion also stimulated collagen synthesis in cultured human osteosarcoma TE-85 cells, suggesting that aluminum ion might stimulate bone formation *in vitro*. The effects of mitogenic doses of aluminum ion on basal osteocalcin secretion by normal human osteoblasts could not be determined since there was little, if any, basal secretion of osteocalcin by these cells. 1,25 Dihydroxyvitamin D_3 significantly stimulated the secretion of osteocalcin and the specific activity of cellular alkaline phosphatase in the human osteoblasts. Although mitogenic concentrations of aluminum ion potentiated the 1,25 dihydroxyvitamin D_3-dependent stimulation of osteocalcin secretion, they significantly inhibited the hormone-mediated activation of cellular alkaline phosphatase activity. Mitogenic concentrations of aluminum ion did not stimulate cAMP production in human osteosarcoma TE85 cells, indicating that the mechanism of aluminum ion does not involve cAMP. The mitogenic activity of aluminum ion is different from that of fluoride because (a) unlike fluoride, its mitogenic activity was unaffected by culture medium changes; (b) unlike fluoride, its mitogenic activity was nonspecific for bone cells; and (c) aluminum ion interacted with fluoride on the stimulation of the proliferation of osteoblastic-line cells, and did not share the same rate-limiting step(s) as that of fluoride. PTH interacted with and potentiated the bone cell mitogenic activity of aluminum ion, and thereby is consistent with the possibility that the *in vivo* osteogenic actions of aluminum ion might depend on PTH. In summary, low concentrations of aluminum ion could act directly on osteoblasts to stimulate their proliferation and differentiation by a mechanism that is different from fluoride.

Introduction

Aluminum toxicity to bone and bone cells has been widely reported. It has been suggested that aluminum may be the etiologic agent in the pathogenesis of osteomalacia in patients with chronic renal failure [1–4]. Histological studies have shown that aluminum-induced osteomalacia is accompanied with impaired mineralization and, in some cases, with decreased matrix formation [5, 6]. It has also been suggested that aluminum may act to affect the proliferation and/or activity of osteoblasts, since osteoblasts are responsible for bone matrix synthesis [7]. However, recent studies have shown that alu-

minum administration to normal adult dogs induced *de novo* bone formation [8–10], and that the osteogenic action of aluminum appeared to be dependent on parathyroid hormone (PTH) [11]. Therefore, these findings raise an interesting possibility that aluminum ion might have a stimulatory effect on osteoblasts, and thereby stimulates bone formation.

Because it has previously been reported that low concentrations (i.e., μM) of aluminum ion stimulate the proliferation of mouse lens epithelial cells and Swiss 3T3K skin fibroblasts (12, 13), we propose that although high concentrations of aluminum might have toxic effects on the bone mineralization process, low concentrations of this ion could act directly on osteoblasts to stimulate their proliferation and differentiation. In this study, we sought to determine whether aluminum ion act on cultured osteoblasts to stimulate proliferation and differentiation of these cells. Because aluminum ion is known to form tight complexes with fluoride, and because fluoride is a bone cell mitogen, we determined whether aluminum ion would interact with fluoride to stimulate bone cell proliferation. In addition, since the *in vivo* osteogenic effects of aluminum in dogs appeared to be dependent on the presence of PTH, we examined whether aluminum ion interacts with PTH to influence bone cell proliferation *in vitro*. To determine the effects of the mitogenic concentrations of aluminum on the osteoblastic functions, we have also examined the effects of aluminum ion on osteocalcin secretion by osteoblasts and on bone collagen synthesis by cultured human osteoblast-line cells.

Experimental procedures

Materials

Tissue culture supplies were from Falcon (Oxnard, CA), Dulbecco's modified Eagle's medium (DMEM), collagenase, penicillin, and streptomycin from GIBCO Laboratories (Grand Island, NY). [^{3}H]Thymidine (48 Ci/mmol) was obtained from Research Products International (Mount Prospect, IL). [^{3}H]Proline (52 Ci/mmol) was product of New England Nuclear (Wilmington, DE). The radioimmunoassay kit for cAMP was purchased from Incstar, Inc. (St. Paul, MN). p-Nitrophenyl phosphate (PNPP), Folin-Ciocalteu's Phenol reagent, and bovine serum albumin were products of Sigma Chemical Company (St. Louis, MO). Aluminum sulfate, aluminum nitrate, aluminum chloride, and sodium sulfate were purchased from Fisher Chemical Co. (Los Angles, CA). The human osteosarcoma TE-85 cells were obtained from Dr. J. Fogh of the Slone Kettering Institute (New York, NY), and were maintained in our laboratory. Normal human bone cells were provided by Dr. J. Wergedal of Loma Linda University. 1,25 Dihydroxyvitamin D_3 was kindly provided by Dr. Milan R. Uskokovic of Hoffmann-LaRoche, Inc. (Nutley, NJ). All other chemicals were of reagent grade and were obtained through Sigma Chemical Co.

Cell cultures

Embryonic chicken calvarial cells were prepared by sequential crude collagenase digestions in serum-free Dulbecco's modified Eagle's medium (DMEM) at 37° C as previously described [14]. The resulting cultures were enriched in osteoblasts and osteoblast precursors [14]. Isolated cells were plated at 7,000 cells per well (in 1 ml medium) in 24-well plates in serum-free DMEM for 24 hr. Normal human bone cells, which were prepared according to Wergedal and Baylink [15], were plated at 10,000 cells (in 1 ml) per well in 24-well plate in DMEM containing 10% bovine calf serum for 24 hr. These human bone cells were shown to be of osteoblastic nature [16]. Human osteosarcoma TE-85 cells (a cell line showing osteoblastic characteristics [17]) were routinely maintained by weekly passaging in DMEM containing 10% bovine calf serum. The TE-85 cells were plated at either 5,000 cells per well (for DNA synthesis studies) or 20,000 cells per well (for alkaline phosphatase studies) in 24–well plates in serum-free DMEM for 24 hr. After plating, each bone cell culture was changed to serum-free DMEM and effectors were added half an hour later, either for 20–24 hr (for [^{3}H]thymidine incorporation assay) or 48 hr (for alkaline phosphatase specific activity measurements). Aluminum was added in DMEM supplemented with

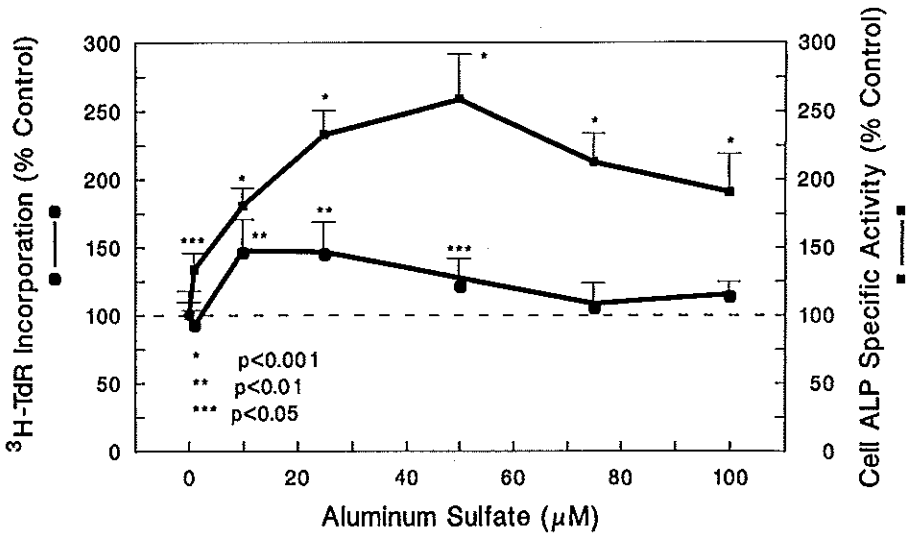

Fig. 1. Stimulation of [^{3}H]thymidine incorporation and cellular alkaline phosphatase activity in cultured embryonic chicken calvarial cells by aluminum sulfate. The data are shown as percentage of the corresponding no addition controls. The closed circles show the [^{3}H]thymidine incorporation; while the closed squares represent the cellular alkaline phosphatase activity. The dashed line indicates 100% of the corresponding control value, which was 143 cpm incorporated per well for the [^{3}H]thymidine incorporation experiment, and 0.178 mU/μg DNA for the cellular alkaline phosphatase experiment.

0.1% bovine serum albumin as carrier. Bovine serum albumin by itself had no significant effect on [^{3}H]thymidine incorporation nor on cellular alkaline phosphatase activity (data not shown).

Cells from embryonic chicken intestine, liver, muscle, and heart were also prepared (according to the method for embryonic chicken calvarial cells [14]) for determination of cell specificity for mitogenic activity of aluminum ion.

Cell DNA synthesis assay

Bone cell mitogenic activity was assayed by the stimulation of incorporation of [^{3}H]thymidine into cell DNA. The assay was adapted from the method of Gospodarowicz *et al.* [18] and has previously been described [14, 19, 20]. To confirm the assay as a measurement of cell proliferation, the number of cells in duplicate wells of TE-85 cells were counted after an incubation with or without aluminum ion for 48 hr in two separated experiments.

Cellular alkaline phosphatase activity assay

Cellular alkaline phosphatase activity was determined with 10 mM PNPP in 0.15 M sodium carbonate buffer (pH 10.3) in the presence of 1 mM $MgCl_2$ [21]. One unit of enzyme activity was the amount of enzyme that is required to hydrolyze 1 μ mol PNPP per min at room temperature (25° C). The cellular enzyme activity was normalized against DNA content measured by a fluorescent method [22], or against cellular protein determined according to Lowry *et al.* [23].

Collagen synthesis assay

The ability of aluminum ion to stimulate bone formation was assessed by the stimulation of collagen synthesis in monolayer cultures of human osteosarcoma TE-85 cells. The procedure has previously been described [24].

cAMP assay

Cellular cAMP production in confluent cultures of TE-85 cells was determined as previously described [20]. The amount of cellular cAMP was normalized against cellular protein.

Osteocalcin production assay

Normal human bone cells were plated at 15,000 cells per well in 1 ml of serum-free DMEM in 24-well plate. The cell medium was replaced with 1 ml

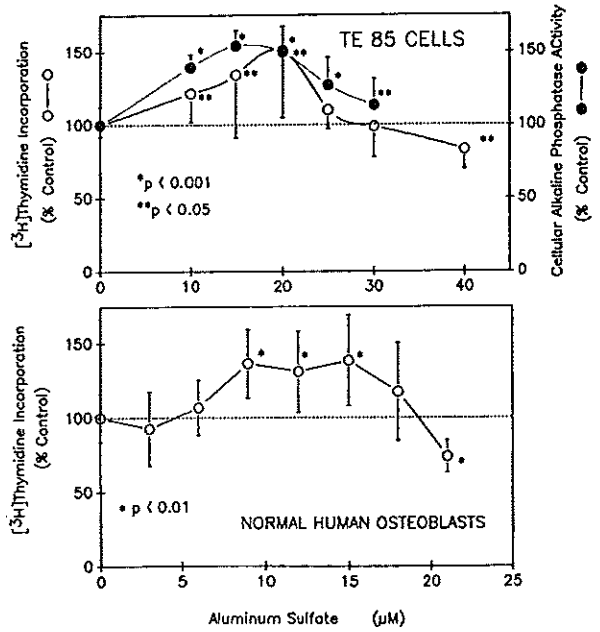

Fig. 2. Stimulation of [^{3}H]thymidine incorporation and cell alkaline phosphatase activity in monolayer cultures of human osteosarcoma TE-85 cells (upper panel) and in normal human osteoblasts (bottom panel) by aluminum sulfate. The data were presented as precentage of the corresponding controls. Closed squares represent [^{3}H]thymidine incorporation and open circles indicate the cellular alkaline phosphatase specific activity. The dashed lines indicate 100% of the corresponding control value. In the top panel, 100% of [^{3}H]thymidine incorporation was 3878 cpm per well; and that of cellular alkaline phosphatase specific activity was 0.16 mU/μg DNA. In the bottom panel, the 100% of control represents 599 cpm per well.

per well fresh DMEM supplemented with 10^{-8} M vitamin K 24 hr later. Various concentrations of aluminum sulfate were then added in the presence or absence of 10^{-8} M 1,25 dihydroxyvitamin D_3. Cells were allowed to incubate for 72 hr at 37° C under an atmosphere of 5% CO_2 in air. Conditioned medium was collected and frozen until assay. Osteocalcin concentration in the conditioned medium was assayed with an RIA specific for the mid-molecule of human osteocalcin [26]. Cells were then extracted with 0.1% Triton X-100, and cell extract alkaline phosphatase activity and protein were assayed as described above.

Statistical methods

The statistical significance of the differences was determined with two-tailed Student's t-test and analysis of variance using a MICROSTAT computer program. The differences were significant when p was < 0.05. The results in this report were presented as mean ± S.D. of six replicates.

Results

Effects of aluminum ion on bone cell DNA synthesis and alkaline phosphatase activity

Micromolar concentrations of aluminum sulfate significantly stimulated the [^{3}H]thymidine incorporation into DNA of the chicken calvarial cells in a biphasic, dose-dependent manner (Fig. 1). The maximal stimulation was seen between 10–25 μM of aluminum sulfate. Aluminum sulfate also significantly increased the specific activity of cellular alkaline phosphatase in the monolayer chicken calvarial cells (Fig. 1). The stimulation was also biphasic with the maximal stimulation seen at doses between 15 and 50 μM of aluminum sulfate (Fig. 1). Other forms of aluminum salt, i.e., aluminum nitrate and aluminum chloride, also stimulated cell DNA synthesis and cellular alkaline phosphatase specific activity in chicken calvarial cells, whereas sodium sulfate at the same concentrations was ineffective (data not shown). Thus, the mitogenic activities were attributed to the aluminum ion and not to its conjugated salt.

The mitogenic effects of aluminum ion were not unique to chicken bone cells, since aluminum caused a similar dose dependent stimulation of [^{3}H]thymidine incorporation and cellular alkaline phosphatase activity in a human osteosarcoma TE-85 cell line (Fig. 2, top panel), and in normal human osteoblasts (Fig. 2, bottom panel). In addition, μM concentrations of aluminum ion were also mitogenic to chicken intestinal cells, but not to chicken liver, heart, or muscle cells (Fig. 3). Thus, our results suggest that the mitogenic actions of aluminum are not specific to chicken bone cells.

It should be noted that the increases in [^{3}H]thymidine incorporation appeared to reflect the stimulation of cell proliferation since the increases in [^{3}H]thymidine incorporation after 24 hr incubation with aluminum sulfate corresponded to the increas-

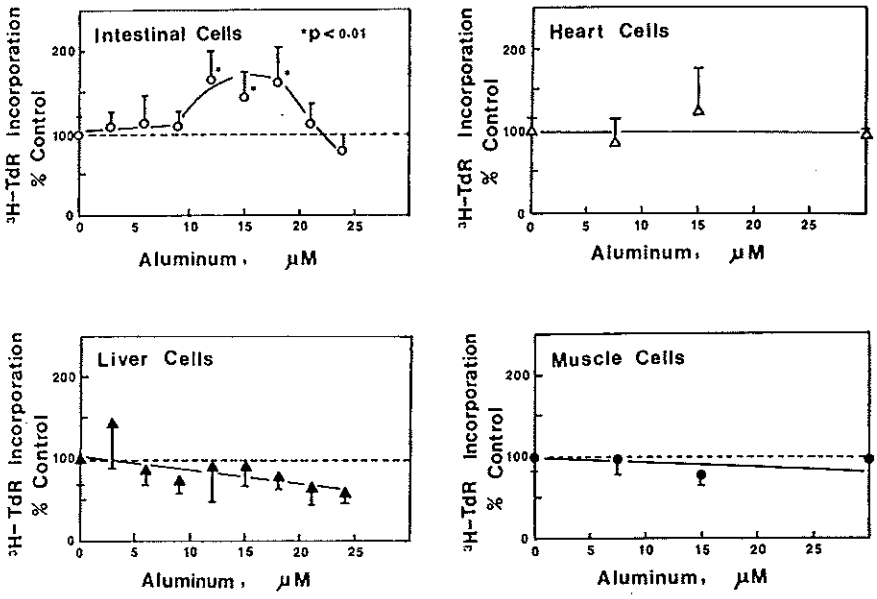

Fig. 3. Cell specificity of the mitogenic activity of aluminum sulfate. Embryonic chicken intestinal, liver, heart and muscle cells were prepared from 16-days-old embryos. The mitogenic activity of aluminum sulfate was measured by the stimulation of [^{3}H]thymidine incorporation into cell DNA and is shown as percentage of the corresponding no addition controls. The dashed lines are the correponding 100% controls, which represented 1346 cpm per well for the intestinal cells, 1455 cpm per well for the liver cells, 792 cpm per well for heart cells, and 613 cpm per well for the muscle cells. As positive control, 1% bovine calf serum was added to each cell monolayer cell cultrues, and each gave significant stimulations of [^{3}H]thymidine incorporation ranging from 406% of control for intestinal cells to 200% of the control for liver cells.

es in the number of TE-85 cells after an 48-hr incubation (Fig. 4).

Stimulation of collagen synthesis in monolayer human osteosarcoma TE-85 cells by the mitogenic concentrations of aluminum

The effect of the mitogenic concentrations of aluminum on the stimulation of bone formation *in vitro* was assessed by the ability of the cation to stimulate incorporation of [^{3}H]proline into collagenase digestable proteins. Table 1 shows that aluminum ion significantly stimulated the incorporation of [^{3}H]proline into collagenase-digestable and total proteins, suggesting that aluminum ion stimulated both the *de novo* synthesis of total and collagen proteins. That the mitogenic concentrations of aluminum (i.e., 10–25 μM) significantly increased the relative proportion of collagen indicated that the observed stimulation in collagen synthesis was not a result of a nonspecific overall stimulation of protein synthesis. As positive controls, the mitogenic doses of fluoride (i.e., 100 μM) and insulin (0.1 μM) [26] were both shown to stimulate collagen synthesis in these cells.

Effects of mitogenic doses of aluminum on osteocalcin production

The actions of mitogenic concentrations of aluminum sulfate on the secretion of osteocalcin by normal human osteoblasts were examined. The human osteoblasts under basal conditions secreted little, if any, osteocalcin into their conditioned medium (i.e., undetectable by our assay), and mitogenic concentrations of aluminum did not stimulate its secretion, or the effect was masked by the insensitivity of the assay (Fig. 5, top panel). However, μM aluminum ion significantly increased cellular alkaline phosphatase specific activity in the same cells (Fig. 5, bottom panel). High concentrations of aluminum appeared to be toxic to these cells, since

200 μM aluminum sulfate reduced the specific activity of alkaline phosphatase to the undetectable level, and since the cells also looked very unhealthy.

Because it has been suggested that 1,25 dihydroxyvitamin D_3 is required for the activation of osteocalcin synthesis by osteoblasts [27], the effect of aluminum ion on the 1,25 dihydroxyvitamin D_3-stimulated osteocalcin release by these cells was also evaluated. 1,25 dihydroxyvitamin D_3 (10^{-8} M) stimulated osteocalcin secretion, and mitogenic doses of aluminum ion significantly potentiated the release of osteocalcin by human osteoblasts. However, it is not clear as to why there were two apparent 'peaks' of stimulation. Nonetheless, the stimulation by aluminum was confirmed in two separate experiments. In contrast, aluminum ion inhibited the 1,25 dihydroxyvitamin D_3-dependent stimulation of alkaline phosphatase specific activity in a dose-dependent manner. This finding indicates that the effects of aluminum on these two 1,25 dihydroxyvitamin D_3-dependent events were different; and raises the possibility that they might act through different mechanisms.

Effect of medium change on the mitogenic activity of aluminum ion

Aluminum is known to form tight complexes with fluoride [28], which was shown to be a bone cell mitogen [29, 30]. Because no attempt to remove contaminating fluoride from reagents and glassware was made in this study, one might argue that the observed mitogenic actions of aluminum could be due to fluoride. To rule out this possibility, the mitogenic activity of aluminum was compared to that of fluoride. We previously showed that the osteogenic actions of fluoride were abolished by medium changes, and that the mitogenic activity of fluoride depended on the coexistence of growth factors in conditioned medium [26, 31, 32]. In this regard, we found that removal of endogenous growth factors by medium change immediately before the addition of aluminum, unlike fluoride, did not abolish the stimulatory effects of aluminum ion on the [^{3}H]thymidine incorporation into cell DNA (Fig. 6, top panel) or on cellular alkaline phosphatase activity (Fig. 6, bottom panel). Analyses of variances indicate that the stimulations of both [^{3}H]thymidine incorporation and of cellular alkaline phosphatase activity were significantly higher in the group without medium change than that with medium change ($p < 0.00001$ for [^{3}H]thymidine incorporation; and $p < 0.0027$ for cell alkaline

Table 1. Aluminum ion stimulates collagen synthesis in human osteosarcoma TE-85 cells *in vitro*. TE-85 cells were plated at 50,000 per well of 24-well plates in 1 ml of DMEM containing 10% bovine calf serum for 24 hr. Cells were then changed to 0.5 ml serum-free DMEM containing 50 μg/ml ascorbate. The cells were then incubated for 48 hr. After the incubation, a solution of 2 μ Ci [^{3}H]proline (52 Ci/mmol) and 25 μg/ml β-aminoproprionitrile were added and the cells were further incubated for 4 hr. The amount of collagen and total protein synthesis were determined as previously described [25]. The results are reported in mean ± S.D., n = 6. Statistical significance was determined by comparing the effector-treated cells to the 'no addition control' cells using a two-tailed Student's t-test.

Effector	Total protein		Collagen			
	(cpm/well)	p	(cpm/well)	p	% Collagen	p
Control	3845.2 ± 692.0	–	140.2 ± 45.23	–	3.59 ± 0.67	–
10 μM $Al_2(SO_4)_3$	12708.3 ± 3718.5	<0.001	812.0 ± 180.8	<0.001	6.53 ± 0.77	<0.001
25 μM $Al_2(SO_4)_3$	11695.9 ± 1776.1	<0.001	546.8 ± 75.9	<0.001	4.82 ± 1.32	<0.050
50 μM $Al_2(SO_4)_3$	10190.9 ± 2379.9	<0.001	480.0 ± 117.7	<0.001	4.63 ± 1.10	n.s.
75 μM $Al_2(SO_4)_3$	10293.8 ± 3561.9	<0.001	368.9 ± 147.8	<0.001	3.55 ± 0.62	n.s.
100 μM $Al_2(SO_4)_3$	10357.3 ± 1980.0	<0.001	465.0 ± 89.4	<0.001	4.50 ± 0.43	<0.020
100 μM F	13062.3 ± 2574.5	<0.001	648.5 ± 76.4	<0.001	5.05 ± 0.62	<0.010
0.1 μM Insulin	13234.0 ± 2484.2	<0.001	689.4 ± 160.5	<0.001	5.25 ± 0.90	<0.010

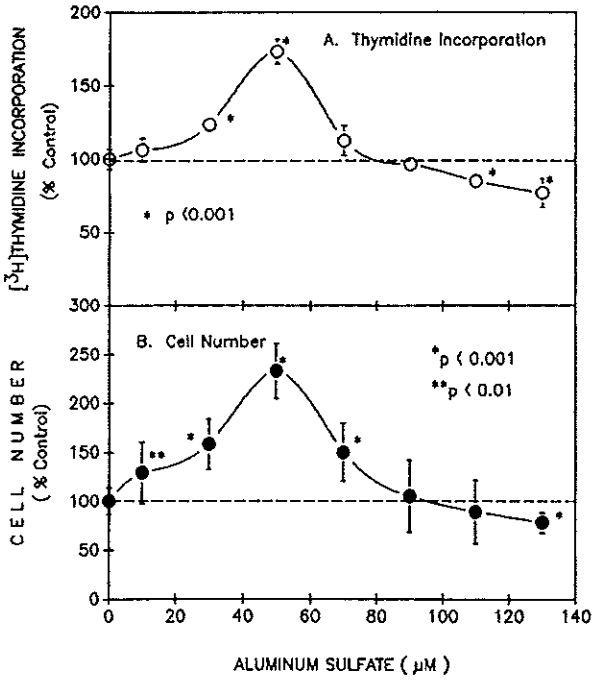

Fig. 4. Stimulation of the proliferation of human osteoblastic TE-85 cells by aluminum ion. TE-85 cells were plated at 5,000 cells per well in 1 ml serum-free DMEM for 24 hr. After plating, the cells were changed with fresh serum-free DMEM, and effectors were added half an hour later. In one set of the experiment, the thymidine incorporation was determined after an 24-hr incubation (panel A); and in a duplicate set of the experiment, the number of cells per well was counted after an 48-hr incubation using a hemocytometer. Results were presented as percentage of the no effector controls. The dashed lines are the corresponding 100% controls. The error bars indicate the standard deviation of the mean of 6 replicates. The 100% of control in panel A was the incorporation of 4576 cpm per well; and that in panel B was 4,055 cells per well.

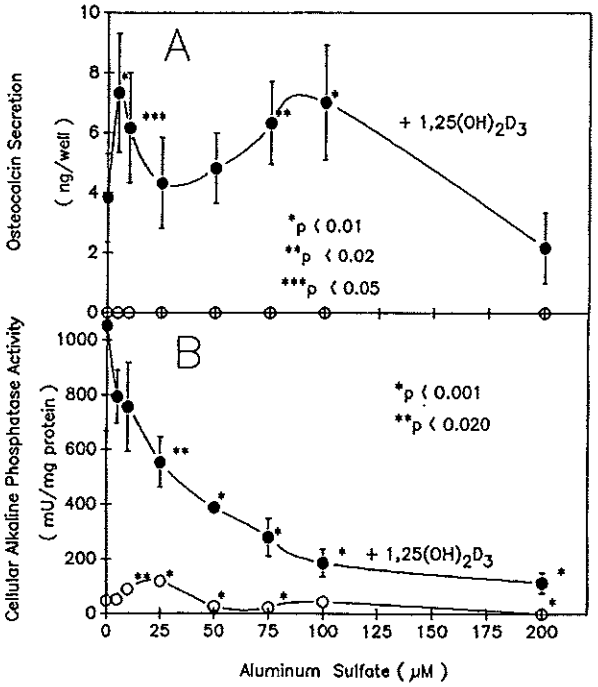

Fig. 5. Effects of aluminum ion on basal and 1,25 dihydroxyvitamin D_3-stimulated osteocalcin secretion and cellular alkaline phosphatase specific activity in normal human osteoblasts. The top panel shows the effects of aluminum ion on the secretion of osteocalcin by normal human bone cells. The open symbols represent the amount of osteocalcin in the conditioned medium after an 24-hr incubation in the absence of $1,25(OH)_2D_3$ (but in the presence of the ethanol vehicle control). The closed symbols indicate those in the presence of 10^{-8} M $1,25(OH)_2D_3$. The detection limit of the osteocalcin RIA was 30 pg/well. The bottom panel shows the effects of aluminum ion on the specific activity of the cellular alkaline phosphatase in the same experiment. The open symbols are the cells incubated for 24 hr without $1,25(OH)_2D_3$; and the closed symbols are those in the presence of $1,25(OH)_2D_3$.

phosphatase activity), suggesting that there were interactions between aluminum and the endogenous growth factors in the stimulations of these two parameters, even though the effects of aluminum ion did not require the presence of growth factors.

Interaction of fluoride and aluminum on bone cell proliferation

If aluminum acts via the formation of aluminum fluoride, then addition of fluoride should shift the bone cell mitogenic dose-dependent curve of aluminum to the left (i.e., lower optimal doses) without altering its maximal stimulation. Furthermore, if fluoride and aluminum act through the same biochemical mechanism (i.e., through the formation of aluminum fluoride), there should be no interaction between the two mitogenic activities. Accordingly, we tested the effects of several doses of fluoride on the stimulation of TE-85 cell proliferation by aluminum sulfate (Fig. 7). There were two noteworthy observations: (a) fluoride did not significantly shift or alter the dose-dependent cruves of aluminum; and (b) fluoride potentiated the stim-

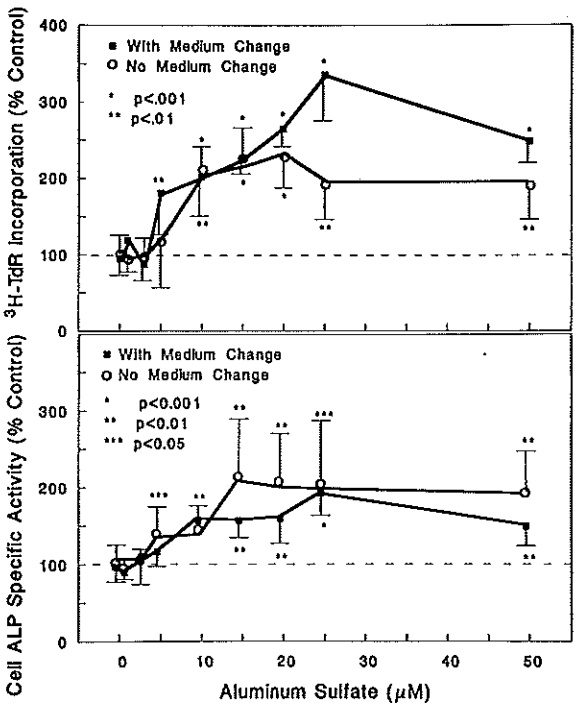

Fig. 6. Evidence that the stimulation of [^{3}H]thymidine incorporation (upper panel) and of cellular alkaline phosphatase specific activity (lower panel) of aluminum on human osteosarcoma TE-85 cells is not dependent on a factor(s) in conditioned medium. The osteogenic effects of aluminum sulfate were determined in human osteosarcoma cells in conditioned medium, i.e., no medium change, and in fresh medium, i.e., with medium change. Conditioned medium had been conditioned by overnight incubation with TE-85 cells. Fresh medium indicates replacement of conditioned medium with fresh unconditioned DMEM half an hour before the addition of aluminum sulfate. The results are presented as the percentage of the untreated control value. Values for untreated controls (indicated by the dashed lines) reflected [^{3}H]thymidine incorporation or the cellular alkaline phosphatase specific activity in the absence of aluminum. The 100% control value of [^{3}H]thymidine incorporation for the conditioned medium group and the fresh medium group was 276 cpm and 359 cpm incorporated per well, respectively. The 100% control value of cellular alkaline phosphatase specific activity for the conditioned medium group and the fresh medium group was 0.468 U/μg DNA and 0.218 U/μg DNA, respectively.

ulation of [^{3}H]thymidine incorporation by aluminum, i.e., the maximal stimulation was higher when both effectors were present than each alone. To further determine whether fluoride interacted with aluminum, the data of Fig. 7 was replotted in a manner analogous to the Lineweaver-Burk kinetic plot, i.e., 1/(stimulation of [^{3}H]thymidine incorporation) vs 1/[aluminum sulfate] (Fig. 8). This plot indicates that the maximal stimulation (i.e., analogous to 'V_{max}') by aluminum in the presence of fluoride was greater than that by aluminum alone, and that the presence of fluoride had no effect on the dose of aluminum that was required to produce half maximal stimulation (i.e., analogous to 'K_m'). This non-competitive type of interaction, which indicates that fluoride indeed interacted with aluminum, is consistent with the interpretation that the rate-limiting steps for the mitogenic actions of fluoride and aluminum are different.

Effects of aluminum on cAMP production

Aluminum fluoride is a potent stimulator of adenylate cyclase (through its action on the G-proteins) [33, 34], and cAMP has been shown to be an important cellular regulator. Consequently, the effect of aluminum ion on the cAMP production in TE-85 cells was also measured. Table 2 shows that mitogenic concentrations of aluminum did not significantly increase the cellular cAMP production in these cells. PTH and 10 mM fluoride both significantly stimulated the cellular cAMP production in these cells, indicating that the adenylate cyclase system in these cells was reponsive to stimulations.

Interaction of the mitogenic action of aluminum ion with PTH

Because the *in vivo* osteogenic effects of aluminum in dogs appeared to require PTH [11], and because physiological doses of PTH stimulated osteoblast proliferation *in vivo* [35] and *in vitro* [36, 37], we investigated whether there was an interaction between aluminum and PTH to stimulate osteoblast proliferation. Table 3 indicates that 1 ng/ml PTH alone significantly stimulated the [^{3}H]thymidine incorporation in TE-85 cells. When this dose of PTH was added together with the mitogenic concentrations of aluminum, the stimulation of [^{3}H]thymidine incorporation was more than additive, suggesting a synergistic interaction between PTH and

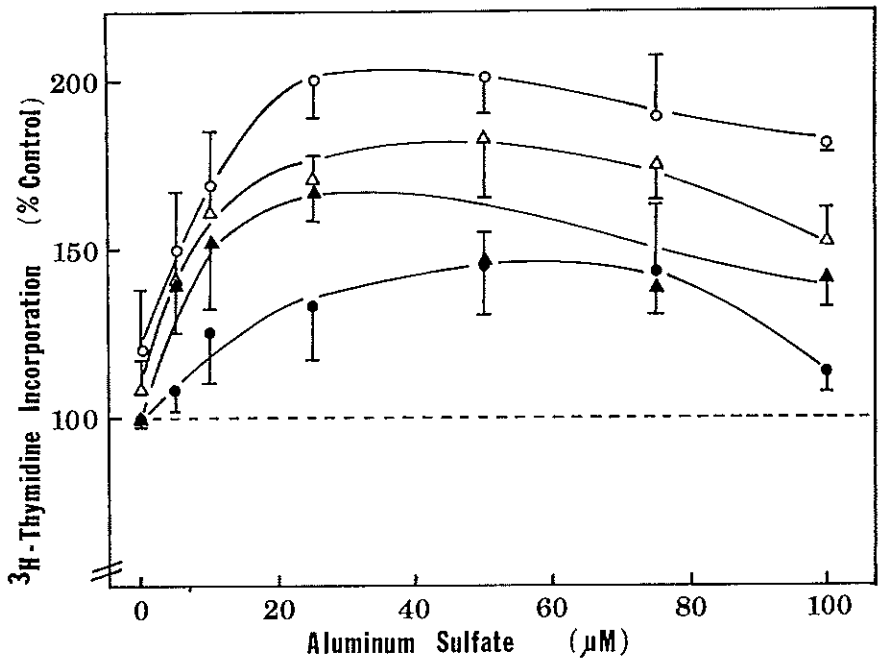

Fig. 7. Interaction of the mitogenic activity of aluminum and fluoride on human osteosarcoma TE-85 cells. Various concentration of sodium fluoride [i.e., 0 (closed circles), 50 (closed triangles), 100 (open triangles), and 200 μM (open circles)] potentiated the bone cell mitogenic activity of aluminum sulfate. The results were presented as percentage of the no addition control (i.e., no aluminum nor fluoride). The 100% of the control value (indicated by the dashed line) was 4891 cpm incorporated per well.

aluminum in the stimulation of the proliferation of human osteoblasts.

Discussion

The present study demonstrates that low (i.e., μM) concentration of aluminum stimulated proliferation (e.g., the incorporation of [^{3}H]thymidine into cell DNA) and differentiation (e.g., cellular alkaline phosphatase activity, a recognized marker of osteoblastic differentiation [38]) of osteoblast-line cells of chicken and human. The stimulations were highly reproducible (i.e., observed in every experiment). However, the extent of stimulation and the optimal dose varied from experiment to experiment. For example: The stimulation of [^{3}H]thymidine incorporation by aluminum in some experiments was as high as 300% of control, but in others was as low as 150% of controls. The optimal doses of aluminum also varied (i.e., from 10 to 75 μM). The cause for this variation is unknown. However, it should be noted that aluminum is one of the most abundant elements in the Earth, and is a major contaminant found in reagent chemicals, culture media, and laboratory glasswares. Our experiments did not take into account the amount of contaminating aluminum. Thus, the amount of aluminum added exogenously did not necessarily represent the actual concentration of aluminum in each incubation, which was likely to be varied from experiment to experiment, depending on the amount of the contaminating aluminum in the reagents and media.

This and previous studies [12, 13] show that the mitogenic action of aluminum, unlike fluoride [29, 30, 32], was not specific for bone cells, suggesting that it, unlike fluoride is probably not an osteoblast-specific mitogen. That the mitogenic concentrations of aluminum also influenced the 1,25 dihydroxyvitamin D_3-dependent stimulation of osteocalcin secretion (an index of osteoblastic function [39, 40]) by human osteoblasts and that these concentrations of aluminum stimulated collagen

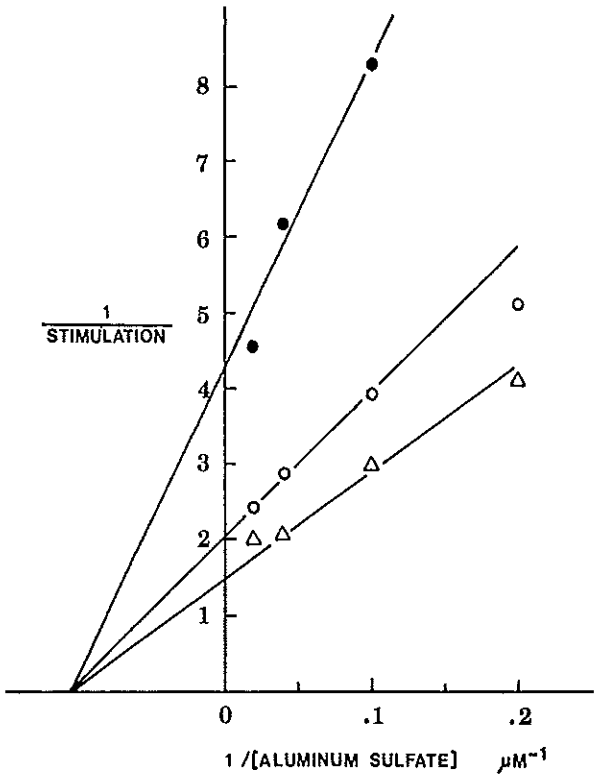

Fig. 8. Analysis of interaction between fluoride and aluminum with a plot analogous to the Lineweaver-Burk kinetic plot. The results of Fig. 7 are plotted as 1/stimulation of [^{3}H]thymidine incorporation (i.e., difference in the cpm incorporated/well of the treated group and that of the no addition control) vs 1/ aluminum sulfate concentration. Closed circles are for the no fluoride; open circles are for 100 μM fluoride; and the open triangles are for the 200 μM fluoride.

synthesis in human osteoblast-line cells provided strong evidence that aluminum affects the activity of human osteoblast-line cells directly. Consequently, this study can be considered as the first *in vitro* evidence that aluminum ion acts directly on human osteoblasts to stimulate their proliferation and differentiation.

An important observation with respect to the *in vivo* osteogenic action of aluminum in dog is the apparent dependency of PTH [11]. The reason for this apparent dependency is unclear. However, this study shows that aluminum (a relatively weak bone cell mitogen compared to growth factors) became a much more potent bone cell mitogen in the presence of PTH. Hence, it is possible that the apparent synergistic interaction between PTH and aluminum could explain, at least in part, the apparent requirement of PTH for the osteogenic effects of aluminum *in vivo*.

The mechanism(s) by which aluminum stimulates osteoblastic proliferation and differentiation is unknown. It has been suggested that aluminum might act through the activation of G proteins [33, 34, 41]. (G proteins have been implicated to be key regulators of cellular signal transduction mechanism [42], an important initial step in the cell proliferation and differentiation processes). However, our results indicate that mitogenic concentrations of aluminum did not stimulate cAMP production in TE-85 cells (G proteins are known to influence the activity of adenylate cyclase). Furthermore, a recent study showed that agents which affected the adenylate cyclase activity through the interaction with G proteins would significantly inhibit the 1,25 dihydroxyvitamin D_3-dependent stimulation of osteocalcin production [43]. We, on the other hand, found that aluminum, not only not inhibited, but significantly enhanced the 1,25 dihydroxyvitamin D_3-dependent stimulation of osteocalcin production by normal human osteoblasts. Hence, these findings provide indirect arguments against the possible involvement of G proteins in the mitogenic action of aluminum. Nevertheless, we should emphasized that G proteins have other function, e.g., activation of phospholipase C and the phosphoinositide pathway [44], and that aluminum has been shown to stimulate phosphoinositide breakdowns in fibroblasts [45]. Thus, the possibility that G proteins may involved in the osteogenic actions of aluminum cannot be discarded entirely.

Fluoride has been shown to be a bone cell mitogen [29, 30]. We recently presented evidence supporting the possibility that the osteogenic actions of fluoride are mediated through the inhibition of an osteoblastic acid phosphatase-like phosphotyrosyl protein phosphatase [32]. Because aluminum is known to form tight complexes with fluoride [28], and because aluminum has previously been shown to be a potent inhibitor of bone acid and alkaline phosphatases [46], we reasoned that it is possible that aluminum and fluoride could act through the

Table 2. Effect of the mitogenic concentrations of aluminum on cAMP production in TE-85 cells. Confluent TE-85 cell cultures were treated with effectors in fresh serum-free DMEM containing 1 mM isobutylmethylxanthine at room temperature for 15 min. cAMP content in the cell extract after extraction with ether to remove trichloroacetic acid was measured with a commercial RIA kit. The results are presented in mean ± S.D. of 6 replicates

Effector	Cellular cAMP Concentration (pmol/mg cell protein)	p*
No addition control	46.12 ± 15.06	–
10 μM $Al_2(SO_4)_3$	76.98 ± 30.37	n.s.
30 μM $Al_2(SO_4)_3$	56.85 ± 17.83	n.s.
50 μM $Al(SO_4)_3$	86.30 ± 43.27	n.s.
100 μM $Al_2(SO_4)_3$	58.80 ± 17.68	n.s.
150 μM $Al(SO_4)_3$	70.58 ± 39.09	n.s.
10 mM fluoride**	82.15 ± 23.93	<0.020
100 nM PTH	208.82 ± 135.31	<0.050

* Statistical significance was determined by comparing the effector-treated cells to the 'no addition control' cells. One way of Analysis of Variance evaluation indicates that there was no significant dose-dependent stimulation of cAMP by aluminum ion ($p = 0.2563$).

** Fluoride, at millimolar concentrations, is known to stimulate adenylate cyclase. Thus, 10 mM fluoride was included in this study as a positive control.

same mechanism to stimulate the proliferation and differentiation of osteoblasts.

We have previously demonstrated that the mitogenic actions of fluoride and another phosphate transition state analog, vanadate, require the presence of growth factors whose receptor is a tyrosyl protein kinase [26, 31, 32]. The requirement of the co-presence of growth factors for the mitogenic action of fluoride was established by the findings: (a) the removal of endogenous growth factors by replacing the conditioned medium with fresh unconditioned medium completely abolished the mitogenic effects of fluoride [26, 31]; and (b) the addition of a growth factor that would stimulate tyrosyl kinase activity, e.g., insulin or IGF-1, in the fresh medium restored the mitogenic activity of fluoride [26, 31]. Our findings that the osteogenic effects of aluminum ion could not be abolished by medium change, indicating that the actions of aluminum, unlike that of fluoride, were independent of bone cell growth factors. Our conclusion that the mechanism of action of aluminum is different from that of fluoride was further supported by the following observations: (a) unlike fluoride, aluminum is not a bone cell specific mitogen; (b) addition of

Table 3. Interaction between PTH and aluminum sulfate on [^{3}H]thymidine incorporation in human TE-85 osteosarcoma cells. Thymidine incorporation was measured during the last two hr of the 24-hr incubation with the effectors by adding 1.5 μ Ci carrier-free [^{3}H]thymidine (48 Ci/mmol) into each well of the 24-well plates. (Mean ± S.D., n = 6)

Effector	[^{3}H]Thymidine incorporation				
	cpm		(% Control)		p
	Mean	S.D.	Mean	S.D.	
No addition control	329	21	100	6	–
PTH*	554	27	168	8	<0.001
10 μM $Al_2(SO_4)_3$	284	18	86	6	<0.001
30 μM $Al_2(SO_4)_3$	468	46	142	14	<0.001
50 μM $Al_2(SO_4)_3$	1013	174	307	53	<0.001
100 μM $Al_2(SO_4)_3$	455	36	138	11	<0.001
PTH + 10 μM $Al_2(SO_4)_3$	360	59	109	18	n.s.**
PTH + 30 μM $Al_2(SO_4)_3$	1609	124	488	38	<0.001**
PTH + 50 μM $Al_2(SO_4)_3$	1979	74	601	22	<0.001**
PTH + 100 μM $Al_2(SO_4)_3$	549	164	167	50	<0.010***

* The concentration of 1–34 PTH was 1 ng/ml.

** $p < 0.001$ when compared to the corresponding doses of aluminum sulfate without PTH.

*** p = n.s. when compared to the corresponding doses of aluminum sulfate without PTH.

Two way Analysis of Variance analysis reveals that PTH significantly ($p = 0.0$) potentiate the mitogenic action of aluminum ion.

fluoride did not appreciably alter the dose dependent curve of aluminum in the stimulation of human osteoblast proliferation; (c) addition of fluoride potentiated the maximal mitogenic actions of aluminum; and (d) the interaction between fluoride and aluminum appeared to be noncompetitive, indicating that the two osteogenic agents each have different rate-limiting step(s).

We have not yet determined the mechanism of the osteogenic actions of aluminum ion. A recent preliminary report using mouse MC3T3-E1 cells suggested that the mitogenic action of aluminum might involve protein kinase C [47]. This finding needs to be further examined and comfirmed. Nevertheless, regardless of what mechanism by which aluminum stimulates bone cell proliferation and differentiation, this study clearly demonstrates that low (μM) concentrations of aluminum could directly act on human osteoblast-line cells to stimulate their proliferation and differentiation by a mechanism that is different from fluoride.

Acknowledgements

This work was supported in part by research grants from the Arthritis Foundation, the Southern California Chapter, and the National Institute of Dental Research (RO1 DE08681). The authors wish to thank Mr. Noah Awoniyi for his technical assistance, and the Medical Media staff of the Jerry L. Pettis Memorial Veterans' Hospital for their assistance in the preparation of the manuscript.

References

1. Ward MK, Feest TG, Ellis HA, Parkinson IS, Kerr DNS: Osteomalacic dialysis osteodystrophy: evidence for a water-borne etiological agent, probably aluminum. Lancet 1: 841–845, 1978
2. Boyce BF, Fell GS, Elder HY, Junor BJ, Elliot HL, Beastall G, Fogelman I, Boyle IT: Hypercalcaemic osteomalacia due to aluminum toxicity. Lancet 2: 1009–1013, 1982
3. Platts MM, Goode GC, Hislop LS: Composition of the domestic water supply and the incidence of fractures and encephalopathy in patients on home dialysis. Br Med J 2: 657–660, 1977
4. Blumenthal NC, Posner AS: *In vitro* model of aluminum-induced osteomalacia: inhibition of hydroxyapatite formation and growth. Calcif Tissue Int 36: 439–441, 1984
5. Maloney NA, Ott SM, Alfrey AC, Miller NL, Coburn JW, Sherrard DJ: Histological quantitation of aluminum in iliac bone from patients with renal failure. J Lab Clin Med 99: 206–216, 1982
6. Hodsman AB, Sherrard DJ, Alfrey AC, Ott S, Brickman AS, Miller NL, Maloney NA, Coburn JW: Bone aluminum and histomorphometric features of renal osteodystropy. J Clin Endocrinol Metab 54: 539–546, 1982
7. Dunstan CR, Evans RA, Hills E, Wong SVP, Alfrey AC: Effect of aluminum and parathyroid hormone on osteoblasts and bone mineralization in chronic renal failure. Calcif Tissue Int 36: 133–138, 1984
8. Galceran T, Finch J, Bergfeld M, Coburn J, Martin K, Teitelbaum S, Slatopolsky E: Biological effects of aluminum on normal dogs: studies on the isolated perfused bone. Endocrinology 121: 406–413, 1987
9. Quarles LD, Gitelman HJ, Drezner MK: Induction of the *de novo* bone formation in the beagle. A novel effect of aluminum. J Clin Invest 81: 1056–1066, 1988
10. Quarles LD, Murphy G, Vogler JB, Drezner MK: Aluminum-induce neo-osteogenesis: a generalized process affecting trabecular network in the axial skeleton. J Bone Min Res 5: 625–635, 1990
11. Quarles LD, Gitelman HJ, Drezner MK: Aluminum-induced de novo bone formation in the Beagle. A parathyroid hormone-dependent event. J Clin Invest 83: 1644–1650, 1989
12. Jones TR, Antonetti DL, Reid TW: Aluminum ions stimulate mitosis in murine cells in tissue culture. J Cell Biochem 30: 31–39, 1986
13. Smith JB: Aluminum ions stimulate DNA synthesis in quiescent cultures of swiss 3T3 cells. J Cell Physiol 118: 298–304, 1984
14. Puzas JE, Drivdahl RH, Howard GA, Baylink DJ: Endogenous inhibitor of bone cell proliferation. Proc Soc Exp Biol Med 166: 113–122, 1981
15. Wergedal JE, Baylink DJ: Characterization of cells isolated and cultured from human bone. Proc Soc Expl Biol Med 176: 27–31, 1984
16. Wergedal JE, Mohan S, Lundy M, Baylink DJ: Skeletal growth factor and other growth factors known to be present in bone matrix stimulate proliferation and protein synthesis in human bone cells. J Bone Min Res 5: 179–186, 1990
17. Rhim JS, Cho HY, Huebner RJ: Non-producer human cells induced by murine sarcoma virus. Int J Cancer 15: 23–29, 1975
18. Gospodarowicz D, Bialecki H, Greenburg G: Purification of the fibroblast growth factor activity from bovine brain. J Biol Chem 253: 3736–3741, 1978
19. Lau KHW, Lee MY, Linkhart TA, Mohan S, Vermeiden J, Liu CC, Baylink DJ: A mouse tumor-derived osteolytic factor stimulates bone resorption by a mechanism involving local prostaglandins production in bone. Biochim Biophys Acta 840: 56–68, 1985

20. Farley JR, Fitzsimmons R, Talyor AK, Jorch UM, Lau KHW: Direct effects of ethanol on bone resorption and formation *in vitro*. Arch Biochem Biophys 238: 305–314, 1985
21. Farley JR, Jorch UM: Differential effects of phospholipids on skeletal alkaline phosphatase activity in extracts, in situ and in circulation. Arch Biochem Biophys 221: 477–488, 1983
22. Thomas PS, Farquhar MN: Specific measurement of DNA in nuclei and nucleic acids using diaminobenzoic acid. Anal Biochem 89: 35–44, 1978
23. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275, 1951
24. Beresford JN, Gallagher JA, Russell RGG: 1,25-Dihydroxyvitamin D_3 and human bone-derived cells *in vitro:* effects on alkaline phosphatase, type I collagen and proliferation. Endocrinology 119: 1776–1785, 1986
25. Taylor AK, Linkhart SG, Mohan S, Baylink DJ: Development of a new radioimmunoassay for human osteocalcin: Evidence for a midmolecule epitope. Metabolism 37: 872–877, 1988
26. Lau KHW, Tanimoto H, Baylink DJ: Vandate stimulates bone cell proliferation and bone collagen synthesis *in vitro*. Endocrinology 123: 2858–2867, 1988
27. Price PA, Baukol SA: 1,25-dihydroxyvitamin D_3 increases synthesis of the vitamin K-dependent bone protein by osteosarcoma cells. J Biol Chem 255: 11660–11663, 1980
28. Martin RB: Ternary hydroxide complexes in neutral solutions of Al^{+3} and F^-. Biochem Biophys Res Commun 155: 1194–1200, 1988
29. Farley JR, Wergedal JE, Baylink DJ: Fluoride directly stimulates proliferation and alkaline phosphatase activity of bone forming cells. Science 222: 330–332, 1983
30. Wergedal JE, Lau KHW, Baylink DJ: Fluoride and bovine bone extract influence cell proliferation and phosphatase activities in human bone cell cultures. Clin Orthopaed Rel Res 233: 274–282, 1988
31. Farley JR, Tarbaux N, Hall S, Baylink DJ: Evidence that fluoride-stimulated ^{3}[H]-thymidine incorporation in embryonic chick calvarial cell cultures is dependent on the presence of a bone cell mitogen, sensitive to changes in the phosphate concentration, and modulated by systemic skeletal effectors. Metabolism 37: 988–995, 1988
32. Lau KHW, Farley JR, Freeman TK, Baylink DJ: A proposed mechanism of the mitogenic action of fluoride on bone cells: inhibition of the activity of an osteoblastic acid phosphatase. Metabolism 38: 858–868, 1989
33. Marc S, Leiber D, Harbon S: Fluoroaluminates mimic muscarinic- and oxytocin-receptor-mediated generation of inositol phosphates and contraction in the intact guinea-pig myometrium. Role for a pertussis/cholera-toxin-insensitive G protein. Biochem J 255: 705–713, 1988
34. Woods NM, Dixon CJ, Cuthbertson KSR, Cobbold PH: Fluoroaluminate mimics agonist application in single rat hepatocytes. Biochem J 265: 613–615, 1990
35. Malluche HH, Sherman D, Meyer W, Ritz E, Norman AW, Massry SG: Effects of long-term infusion of physiologic doses of 1–34 PTH on bone. Am J Physiol 242: F197–F201, 1982
36. Howard GA, Bottemiller BL, Turner RT, Rader JI, Baylink DJ: Parathyroid hormone stimulates bone formation and resorption in organ culture: Evidence for a coupling mechanism. Proc Natl Acad Sci USA 78: 3204–3208, 1981
37. Wong GL: Skeletal effects of parathyroid hormone. In: Bone and Mineral Research. Peck WA (ed.) 4th ed. Elsevier Sci Publishing Co. Inc., New York. 1986, pp 103–129
38. Farley JR, Baylink DJ: Skeletal alkaline phosphatase activity as a bone formation index *in vitro*. Metabolism 35: 563–571, 1986
39. Price PA, Parthemore JG, Deftos LJ: New biochemical marker for bone metabolism. Measurement by radioimmunoassay of bone GLA protein in the plasma of normal subjects and patients with bone disease. J Clin Invest 66: 878–838, 1980
40. Garcia-Carrasco M, Gruson M, de Vernejoul MC, Denne MA, Miravet L: Osteocalcin and bone morphometric parameters in adults without bone disease. Calcif Tissue Int 42: 13–17, 1988
41. Pun KK, Ho PWM, Lau P: Effects of aluminum on the parathyroid hormone receptors of bone. Kidney Int 37: 72–78, 1990
42. Litosch I: Regulatory GTP-binding proteins: emerging concepts on their role in cell function. Life Sci 41: 251–258, 1987
43. Evans DB, Russell RGG, Brown BL, Dobson PRM: Agents affecting adenylate cyclase activity modulate the stimulatory action of 1,25-dihydroxyvitamin D_3 on the production of osteocalcin by human bone cells. Biochem Biophys Res Commun 164: 1076–1085, 1989
44. Boyer JL, Waldo GL, Evans T, Northup JK, Downes CP, Harden TK: Modification of AlF_4^-- and receptor-stimulated phospholipase C activity by G-protein $\beta\gamma$ subunits. J Biol Chem 264: 13917–13922, 1989
45. Paris S, Chambard J-C, Pouyssegur J: Tyrosine kinase-activating growth factor potentiate thrombin- and AlF^-_4-induced phosphoinositide breakdown in hamster fibroblasts. Evidence for positive cross-talk between the two mitogenic signaling pathways. J Biol Chem 263: 12893–12900, 1988
46. Lieberherr M, Grosse B, Cournot-Witmer G, Thil CL, Balsan S: *In vitro* effects of aluminum on bone phosphatases: a possible interaction with bPTH and vitamin D_3 metabolites. Calcif Tissue Int 34: 280–284, 1982
47. Quarles LD, Castillo SA, Drezner MK: Aluminum-induced replication of osteoblast precursors: A potential mechanism underlying neo-osteogenesis. J Bone Min Res 4 (Suppl 1): abs 54, 1989

Address for offprints: K.-H. William Lau, Mineral Metabolism (151), Jerry L. Pettis Memorial Veterans' Hospital, 11201 Benton Street, Loma Linda, CA 92357, USA