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Mechanism of Mitogenic Action of Aluminum Ion on Bone Cells : Potential Involvement of the Insulin-Like Growth Factor Regulatory System

Aera Yoo

ABSTRACT

MECHANISM OF MITOGENIC ACTION OF ALUMINUM ION ON BONE CELLS: POTENTIAL INVOLVEMENT OF THE INSULIN-LIKE GROWTH FACTOR REGULATORY SYSTEM.

by

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Micromolar concentrations of aluminum ion (oxidation state of 3^+) consistently stimulated [^{3}H]thymidine incorporation into cell DNA and increased cellular alkaline phosphatase activity (an osteoblastic differentiation marker) in osteoblast-like cells of chicken and human origin. Although biphasic, dose-dependent stimulations were highly reproducible, the maximal stimulatory dose varied among different experiments. Mitogenic doses of aluminum ion also stimulated collagen synthesis in cultured human osteosarcoma TE85 cells. In addition, the same mitogenic concentrations of aluminum ion enhanced the 1,25 dihydroxyvitamin D_3-dependent stimulation of osteocalcin secretion. Together, these findings indicate that aluminum at micromolar concentrations, stimulates the proliferation, differentiation, and activity of human osteoblastic-line cells *in vitro*. With respect to the mechanism of its mitogenic action, the 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. Additional studies have also revealed that the mitogenic activity of aluminum ion is different from that of fluoride because (a) unlike fluoride, the mitogenic action of aluminum was unaffected by culture medium changes (*i.e.*, it was not dependent on the presence of essential factors in the cell-conditioned media);

(b) unlike fluoride, the mitogenic action of aluminum was not specific for bone cells; and (c) interaction studies with fluoride revealed that aluminum and fluoride did not share the same rate-limiting step(s) for their effects in stimulating the proliferation of osteoblastic-like cells. To test the additional hypothesis that the mitogenic action of aluminum ion is mediated through the insulin-like growth factor (IGF) regulatory system, the effects of mitogenic concentrations of aluminum on IGF-I and IGF-II production were evaluated. TE85 cells treated with aluminum ion for 48 hours showed biphasic stimulation of the synthesis/secretion of IGF-I and IGF-II into the conditioned medium (CM). The addition of IGFBP-4, an inhibitory IGF binding protein, significantly reduced the effect of aluminum to stimulate [^{3}H]thymidine incorporation, supporting the hypothesis that the mitogenic action of aluminum was mediated by an increase in IGF production. Western ligand blot analysis revealed that mitogenic concentrations of aluminum ion also inhibited the secretion of IGF binding proteins, particularly the inhibitory IGFBP-4, an action which could lead to the potentiation of the overall activity of IGFs. Based on these findings, it is concluded that aluminum ion, at micromolar concentrations, acts directly on human bone cells to stimulate their proliferation and differentiation by a mechanism that involves increase in the production and activity of IGFs in bone cells.

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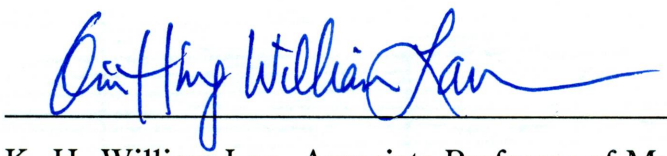
Each person whose signature appears below certifies that this thesis in their opinion is adequate, in scope and quality, as a thesis for the degree Master of Science.



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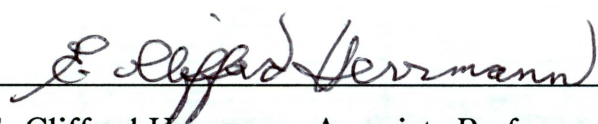

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ATP	Adenosine 5'-triphosphate
cAMP	Adenosine 3',5'-monophosphate
Ci	Curie
CM	Conditioned medium
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
FGF	Fibroblast-derived growth factor
G protein	Guanosine 5'-triphosphatase-activating protein
GDP	Guanosine 5'-diphosphate
GTP	Guanosine 5'-triphosphate
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
kD	Kilodalton
K_m	Michaelis constant
mRNA	Messenger ribonucleic acid
μM	Micromolar
PDGF	Platelet-derived growth factor
PNPP	p-Nitrophenyl phosphate
PTH	Parathyroid hormone
RIA	Radioimmunoassay
RRA	Radioreceptor assay
SDS	Sodium dodecyl sulfate
TGFß	Transforming growth factor beta
V_{max}	Maximum velocity

CHAPTER 1: BACKGROUND

I. Cellular toxicity of high concentrations of aluminum ion.

Historically, aluminum, a ubiquitous heavy metal, was considered nontoxic largely because the gastrointestinal tract was believed to act as an effective barrier to its entry, most of the metal ingested being excreted in the feces. For this reason, aluminum-containing antacids given to patients with peptic ulcers were presumed to be innocuous. However, it is now well documented that some aluminum is absorbed during therapy with this type of compounds, and a significant amount of aluminum ion can be accumulated in various tissues after prolonged administration of aluminum ion. In this regard, high levels of aluminum have been found in serum and various organs of chronic renal failure patients as a consequence of either the administration of aluminum hydroxide (1-3) or the presence of this compound in dialysis fluid (4-6).

Like other heavy metals, aluminum ion is not biologically inert, and the toxicity of high concentrations of aluminum ion in biology has been well documented (7). For example, aluminum has been implicated as the etiologic agent in the encephalopathy observed in patients with chronic renal failure who are treated by long-term hemodialysis with aluminum-rich dialysate (8). It has also been considered a contributing factor to the pathogenesis of several neurological disorders (*e.g.*, amyotrophic lateral sclerosis, presenile dementia, *etc.*) of environmental etiology (9,10). Furthermore, aluminum deposits have also been found in brains of Alzheimer's disease patients, which led to the speculation that the accumulation of aluminum ion in the brain may be associated with the development of Alzheimer's disease (11). While the proposal for an etiological role of aluminum in Alzheimer's type senile dementia is controversial (9,11-13), evidence for the neurotoxicity of the accumulation of high concentrations of aluminum in brain tissues is compelling.

In addition to its neurotoxic effects, aluminum loading has also been implicated as an important contributing factor in the development of non-iron-deficient anemia in patients with chronic renal failure (14,15). Supporting evidence for this conclusion is that hemoglobin levels in these patients fall with continued aluminum exposure (14,16) and rise when the body burden of aluminum is decreased by therapy with deferoxamine [a specific chelator for aluminum ion] (17,18) or by dialysis against aluminum-free solutions (14,15). Furthermore, it has been shown that aluminum ion inhibited erythropoiesis (19) by binding tightly to transferrin (20,21), an action which prevented the binding and transport of iron to the marrow for hemoglobin synthesis. Accordingly, aluminum intoxication is an important cause of morbidity and mortality in hemodialysis patients (22).

II. Effects of aluminum accumulation on mineralization.

Because the mineral present in bone (*i.e.*, hydroxyapatite) has a high affinity for heavy metals, including aluminum ion, bone is a major target tissue for aluminum deposition. This expectation is consistent with the findings that aluminum is selectively deposited at the mineralization front and along trabecular bone surfaces (23,24). Once deposited in bone matrix, aluminum is removed very slowly (25). Thus, prolonged treatment with aluminum should lead to accumulation of high aluminum concentrations in bones, and this is supported by findings that aluminum administration in animals and humans causes elevated aluminum content in bone matrix (26-29).

Aluminum toxicity to bone and bone cells has been widely reported. In this regard, renal osteodystrophy is a universal complication in patients with chronic renal failure. This bone disease comprises a heterogeneous group of disorders of diverse pathophysiology. Numerous studies have previously established that the vast majority of patients with uremia exhibit osteitis fibrosa, a disease characterized by evidence of increased bone resorption, peritrabecular fibrosis, and a normal or accelerated rate of

bone formation (30). However, osteomalacia and aplastic bone disease, unique bone abnormalities marked by a low rate of bone turnover, are the most prevalent complications in patients with chronic renal failure and, particularly, those who are dialysis dependent. It has been suggested that aluminum may be the etiologic agent in the pathogenesis of this osteomalacia (31-33). Histological studies have shown that aluminum-induced osteomalacia is accompanied with impaired mineralization and, in some cases, with decreased bone matrix formation (26,35). The aluminum-associated osteomalacia differs from "classical" vitamin D-deficiency osteomalacia in that patients have an increased incidence of bone fractures, are resistant to treatment with even large doses of vitamin D, and seldom have secondary hyperparathyroidism (22). Accordingly, the aluminum-associated osteomalacia has been a significant problem in the management of renal failure patients.

While the cause and treatment for vitamin D-resistant osteomalacia has been extensively investigated, the mechanism by which aluminum induces osteomalacia is not well understood. Because aluminum is found in the areas corresponding to the mineralization front (24,35), it has been thought that aluminum could physically and/or chemically interfere directly with the normal mineralization process (36), which in turn, would lead to severe osteomalacia (23,27,32). However, since osteoblasts are responsible for bone matrix synthesis, it has also been suggested that aluminum ion may act to affect the proliferation and/or activity of osteoblasts, which would lead to a reduction in the rate of bone matrix formation (37). In addition, aluminum accumulation is associated with reduced PTH secretion, which could also cause a reduction in osteoblastic activity (38). These observations raise the interesting possibility that an aluminum-induced reduction in serum PTH may be responsible for some of the effects of aluminum in the development of osteomalacia. On the other hand, in some uremic patients who had high bone aluminum content and also high serum PTH levels, no osteomalacia was observed, whereas they all showed increased

osteoclastic resorption (*i.e.*, a well known characteristic of hyperparathyroidism) (39,40). Thus, these findings are in support of a key role for PTH deficiency in aluminum-associated osteomalacia, but they are in contradication to the proposal of a direct inhibition of the mineralization process by aluminum. Moreover, it is now clear from histomorphometric studies that the aluminum-dependent decrease in osteoblastic activity cannot be explained by reductions in PTH levels alone (26). It has also been shown with isolated perfused tibia that bones of aluminum treated dogs are resistant to the effects of PTH, suggesting that aluminum treated bones may also have defects in the responsiveness to PTH (41). Consequently, while PTH deficiency may be important in aluminum-associated osteomalacia, the exact cause is unclear and remains controversial.

III. Osteogenic effects of low concentrations of aluminum ion.

While evidence in the literature indicates that the accumulation of high concentrations of aluminum in bone matrix of chronic renal failure patients is harmful to skeletal metabolism, there is evidence that low concentrations of aluminum may actually increase bone formation. In this regard, Quarles and Drezner have recently shown that short term (8-16 weeks) administration of aluminum to normal adult dogs induced *de novo* bone formation within the marrow cavity of the iliac crest (42) [as indicated by significant increases in histomorphometric bone formation parameters] and increased bone density (measured by Quantitative Computed Tomography) and histomorphometric bone formation parameters in thoracic and lumbar vertebrae (43,44). These investigators also demonstrated that the osteogenic effects of aluminum appeared to be dependent on PTH, since the osteogenic effects of aluminum were reduced in parathyroidectomized dogs (44). Therefore, these findings raise the interesting possibility that low concentrations of aluminum ion might have a stimulatory effect on osteoblasts, and thereby increase bone formation. In support of this

hypothesis are the previous findings that: a) administration of aluminum to normal dogs induced a transient increase in osteoblastic activity and increased osteoblast numbers (41), and b) short term treatment of beagle dogs with aluminum increased bone collagen synthesis (42).

The *in vivo* findings in intact adult dogs differ from previous findings of aluminum-induced osseous events in various animal models which indicated that toxic effects, such as abnormal mineralization and decreased bone formation, are the sole outcomes of aluminum administration (23-29). The reason for the discrepancies is not clear. However, there are two potential explanations. First, the length of aluminum administration (*i.e.*, 8-16 weeks) in these dog studies was much shorter than most previous studies (*e.g.*, 1-3 years) of aluminum-induced osteomalacia. Furthermore, the doses of aluminum used in the recent dog studies (*i.e.*, 1-1.25 mg/kg, three times per week) were also lower than those of most of the previous animal studies (*i.e.*, 5-10 mg/kg/day). Consequently, the total amount of aluminum exposure was much lower in the recent dog studies than the previous animal studies. Second, the dogs used in these studies were healthy with normal kidney functions, whereas most previous studies employed uremic animals. Accordingly, we can hypothesize that these dogs were able to excrete most of the absorbed aluminum through normal kidney function, and consistent with this hypothesis, the histomorphometric studies showed that the amount of stainable bone matrix aluminum in the dogs was at least 10-fold lower than that seen in bone matrix of patients with chronic renal failure (42). Based on these observations, we have further hypothesized that while high concentrations of aluminum are harmful to bone cell metabolism, low concentrations of aluminum could stimulate bone cell proliferation, and/or differentiation and activity. The biphasic response (*i.e.*, stimulatory at low doses and inhibitory at high doses) of bone cells to aluminum is not unique since the mitogenic actions of various bone cell effectors (*e.g.*, fluoride, IGFs, PTH, *etc.*) are also biphasic in nature (45-47).

In order to fully appreciate the effects of aluminum on bone cell metabolism, it is important that we understand both the osteogenic effects of low concentrations of aluminum and the toxic effects of high doses of aluminum. An understanding of how low concentrations of aluminum can stimulate bone cell proliferation and/or differentiation could also produce invaluable insights into how bone cell proliferation and/or differentiation is regulated. It should be noted that the *in vivo* dog studies did not demonstrate whether the anabolic effect of short term aluminum treatment was mediated through direct action of the cation on bone cells, or indirectly (*e.g.*, through effects on systemic hormones). However, it has previously been reported that low concentrations (*i.e.*, μM) of aluminum ion could stimulate the proliferation of mouse lens epithelial cells and Swiss 3T3K skin fibroblasts *in vitro* (48,49). Furthermore, it has recently been shown that micromolar concentrations of aluminum ion stimulated the proliferation of mouse MC3T3-E1 cells by inducing a transition from the G_0 to the S phase of the cell cycle (50). Accordingly, it is anticipated that aluminum ion may also be a bone cell mitogen which can be osteogenic, because it acts directly on osteoblasts to affect their proliferation, differentiation, and activity. Consequently, the central theme of this thesis project was to evaluate the osteogenic effects of low doses of aluminum on human bone cells *in vitro*.

IV. Potential mechanisms of aluminum ion on cell metabolism.

1. Biologically active species of aluminum.

In order to understand the biological activity of aluminum in cells, it is necessary to understand some aspects of the chemistry of aluminum. The chemistry of aluminum in biology is dominated by its ligation by oxygen-based functional groups, principally through aluminum substitution for competitive cations, of which magnesium is considered the most biologically relevant (51). Aluminum reacts via the hexa-aqua trivalent species Al^{3+}, and it is the concentration of this species, but not the total

aluminum concentration, that is believed to be responsible for the biological activity of aluminum (52). The pH has profound effects on the free Al^{3+} concentration (53). For example, in the absence of all other competing ligands, the solubility of amorphous aluminum hydroxide controls the free Al^{3+} concentration at pH 6.0. When the pH is raised to 7.4 the control of the concentration of free Al^{3+} switches to the aluminate species, $Al(OH)_4$, the predominant aluminum species at alkaline pH. In the physiological milieu (pH in the range 6.5-7.4), citrate (found commonly at serum concentrations of 0.1 mM) is a likely pre-eminent intracellular oxygen-based ligand and will bind aluminum to decrease the cellular free Al^{3+} concentration (54). In the absence of citrate, or towards the more acidic pH of the physiological milieu, inorganic phosphate (52) or ATP (55) [both found at intracellular concentrations of about 10 mM] similarly reduce the free aluminum concentration toward femtomolar levels. Other influencing factors include the relative concentrations of competitive anions (*e.g.*, hydroxide, fluoride, silicate, inorganic phosphate, *etc.*) and cations (*e.g.*, calcium, magnesium, manganese, zinc, iron, *etc.*), the intrinsic binding strength of any ligand for aluminum and the comparative reaction kinetics (53). The biological reactivity of aluminum cannot simply be predicted on the basis of a hierarchy of known aluminum-ligand binding constants. It is the combination of the aforementioned influences which together determine the bioavailability and hence biological effects of aluminum (53).

2. Cellular uptake of aluminum.

High electropositivity, large solvation shells and a concomitant tendency toward hydrogen bonding are characteristics of the free aluminum species thought to preclude the passive diffusion of aluminum through biological membranes. However, it is now clear that aluminum is found intracellularly, even though the mechanism for its cellular uptake is not well understood. Three potential mechanisms by which aluminum permeates biological membranes and accumulates intracellularly have been proposed.

First is passive diffusion of uncharged aluminum complexes, *e.g.*, AlF_3 (56), and aluminum-glutamate (57). Second is passive diffusion of aluminum species mediated through aluminum binding to membrane-orientated transport proteins (58) and aluminum effects on membrane fluidity (59). In this regard, it has been shown that cellular uptake of aluminum is significantly enhanced by the presence of transferrin, the principal iron-carrying protein in physiological milieu (60-62). Aluminum-transferrin is believed to follow the iron transport route into cells (62), transferrin-mediated endocytosis (60), although on internalization, aluminum-transferrin, unlike iron-transferrin, is not reduced to release the bound metal and continue the transferrin cycle. Third is adsorption-mediated endocytosis (56). Accordingly, aluminum could be taken up intracellularly into cells by any one or all of these mechanisms.

3. Intracellular actions of aluminum.

There is a large body of evidence that aluminum acts intracellularly to exert its biological effects (7,63). However, the significance of such findings at the whole cell, tissue or organism level remains largely unknown. The physico-chemistry of aluminum predicts higher binding strengths and slower reaction kinetics than, for example, the universally important divalent metal ions calcium and magnesium. It is not surprising, therefore, to find that aluminum can act as a potent inhibitor of many enzymes and/or biological processes by competing with other cations, *e.g.*, calcium and magnesium. In this regard, it has been demonstrated that aluminum inhibited several enzyme activities and cellular processes *in vitro*. For example: it has been shown that aluminum ions are required for inhibition of hepatic microsomal glucose-6-phosphatase by sodium fluoride (64), and that fluoroaluminate also inhibited the inactivation and transformation of glucocorticoid receptor (65). In addition, incubation of rat calvaria with aluminum for 24 hours significantly reduced the cellular alkaline and acid phosphatase activities (66). However, the inhibitory effects of aluminum on bone phosphatases

were probably not due to direct inhibition of the enzyme activities by aluminum since aluminum, at concentrations of up to 1 mM, did not significantly affect the activity of human osteoblastic alkaline phosphatase or acid phosphatase *in vitro* (Yoo and Lau, unpublished observations). On the other hand, fluoroaluminate has also been shown to have stimulatory effects on numerous cellular processes through its activation of G proteins (67-70).

The intracellular response to an aluminum challenge has not received a great deal of attention until recently. Even then, the types of cellular models studied are diverse and include: synaptosomes (71), hepatocytes (72), neuroblastoma cells (73), liver microsomes (74), pancreatic acinar cells (75), astrocytes (76), parotid acini (77), salt-secreting cells (78), barnacle muscle fibres (79), heart tissue (80), and osteoblasts (50,81). The comparative value of much of this research is compromised by the lack of consensus in the application of exposure systems and (in particular) culture conditions. Only rarely are the influences on aluminum binding and uptake by cells taken into account in the experimental design. Thus, the results of these studies are ambiguous for the most part. On the other hand, while the biological effects of aluminum in each of these systems appeared different, these studies have demonstrated that the intracellular effects of aluminum were consistently documented as stimulatory at low concentrations becoming inhibitory with increasing aluminum concentration. Consequently, these findings are supportive of our contention that aluminum at low concentrations can increase bone cell proliferation and bone formation.

4. Interaction of aluminum ion with G proteins.

The majority of reported intracellular responses to aluminum have been associated with second messenger systems, the central components in the control mechanisms of many (and possibly all) cells (82). For example, it has been shown that aluminum, at 10 μM, stimulated the production of calcium transients in agonist-

stimulated single rat hepatocytes (72). The mechanism by which aluminum stimulated calcium transients appeared to involve the inositol phosphate signaling pathway (72). Furthermore, a recent report (83) has demonstrated that oral aluminum consumption alters *in vitro* protein phosphorylation and protein kinase C activity in rat brain, and suggested that alteration of protein phosphorylation may be responsible for the neurotoxic effects of aluminum over exposure. Inhibitory effects of aluminum on cellular processes mediated through second messenger systems are also documented in extracellular aluminum challenges on whole cell preparations. For example, aluminum inhibits the agonist-stimulated hydrolysis of phosphoinositides and the incorporation of inositol into phospholipids (71) in rat cortical slices, the fast and slow phases of voltage-dependent Ca^{2+} uptake in synaptosomes (84), and causes the premature onset of deterioration of electrophysiological properties in differentiated neuroblastoma cells (73). Similarly, the disruption of cAMP-mediated phosphorylation of Ca^{2+} and/or K^+ channels is implicated in recent research showing an aluminum-induced decrease in maximum inward calcium currents in heart tissue of both rabbits and frogs (80).

There is evidence that aluminum affects the second messenger systems (*e.g.*, phosphoinositide pathway, cAMP pathway) that involve the G proteins (53,72). In this regard, aluminum is known to combine with fluoride to form fluoroaluminate (AlF_4^-), which has been shown to be a potent activator of G_s (85), which in turn activates adenylate cyclase to increase cellular production of cAMP (86). Similarly, fluoroaluminate has also been shown to activate G_i, which is associated with phospholipase C and D activation (87,88), and in turn leads to stimulation of phosphoinositide breakdown. The resulting products, phosphoinositides and diacylglycerol, subsequently increase intracellular calcium mobilization (82) and activation of protein kinase C (82), respectively. In this regard, Sternweis and Gilman (86) have reported that Al^{3+} was required for fluoride to activate G_s, G_i, and transducin. It has also been demonstrated that fluoroaluminate substituted for the

gamma-phosphate of GTP (85). This conclusion comes from the fact that there are structural similarities between fluoroaluminate and phosphate group. Both fluoroaluminate and phosphate are tetrahedral, the atomic sizes of phosphorus and aluminum are similar, fluorine and oxygen have the same van der Waals radii of 1.35 Å, and the bond lengths for aluminum-fluorine and phosphorus-oxygen are approximately the same. Thus, it is reasonable to conclude that fluoroaluminate can act as a potent phosphate analog in biological systems.

Perhaps the most compelling evidence to-date that points toward the involvement of G protein systems in the biological action of aluminum is the finding that aluminum treatment inhibited the activation of the signal-transducing G protein transducin (89), and that aluminum disrupted the GTP-GDP exchange activity of a G protein in the stimulation of Na^+ efflux in single barnacle muscle fibres (79). On the other hand, while the strong interaction between fluoroaluminate and the G proteins may be, in part, responsible for the observed biological effects of aluminum, there is evidence that is inconsistent with the involvement of the G proteins. For example, it has been shown that aluminum did not bind to the "model" G protein, elongation factor Tu-GDP, although those studies were complicated by the presence of millimolar concentrations of fluoride (90,91). In addition, the mitogenic effect of aluminum is abolished in cells pretreated with pertussis toxin (50), suggesting that aluminum-induced mitogenesis is probably not mediated through a G protein system. Consequently, while evidence for the involvement of the G proteins in the biological actions of aluminum is strong, that mechanism may not account for all of the biological effects of aluminum.

V. Mitogenic action of fluoride.

The facts that aluminum ion forms tight complexes with fluoride, and that the biological action of aluminum may be mediated through the formation of

fluoroaluminate, are important and could be clinically relevant as demonstrated by previous investigations that fluoride is one of the most effective single agents currently available for increasing bone density in osteoporotic spines (92-94), and that fluoride is a bone cell specific mitogen *in vitro* (47,95). Accordingly, if the hypothesis that the biological effect of aluminum is mediated through the formation of fluoroaluminate is indeed true, then the understanding of the mechanism of aluminum to stimulate bone cell proliferation and differentiation may yield important information as to how fluoride exerts its osteogenic effect. In this regard, a direct comparison between the mitogenic action of fluoride and that of aluminum on bone cells seems warranted as it would disclose information regarding whether fluoride and aluminum act through similar mechanisms. Thus a major objective of this thesis work was to compare the mitogenic action of aluminum on human bone cells to that of fluoride on the same cells *in vitro*. However, in order to have a better appreciation of the potential relationship (or the lack thereof) between aluminum and fluoride on bone cell proliferation, a brief discussion on the current knowledge of the osteogenic action of fluoride would be appropriate.

Previous histomorphometric studies indicate that fluoride increases bone density by stimulating osteoblastic proliferation (93). The bone cell mitogenic activity of fluoride has been confirmed *in vitro* by several laboratories, including ours (47,95-98). Although the exact mechanism of the mitogenic action of fluoride is not known at the present time, it is now clear that the mitogenic action of fluoride exhibits the following characteristics: a) mitogenic effect of fluoride is specific for bone and, perhaps, kidney cells (95,99), b) the effective mitogenic concentrations of fluoride are in the micromolar range (47,95,100), c) fluoride-induced osteoblast proliferation is dependent on the presence of a growth factor whose receptor is a tyrosyl kinase (101), d) mitogenic activity of fluoride can be potentiated by inorganic phosphate (101), and e) fluoride synergizes with growth factors whose receptor is a tyrosyl kinase to increase osteoblastic proliferation (100,101). These characteristics are unique for fluoride-

induced mitogenesis. Accordingly, if the aluminum-induced osteogenesis is mediated through fluoride, the *in vitro* mitogenic activity of aluminum should show the same properties as fluoride.

With respect to the potential mechanism of action of fluoride on bone cells, Lau and coworkers have purified an acid phosphatase from osteoblasts, which displayed phosphotyrosyl protein phosphatase activity at neutral pH (102,103). These investigators have provided a large body of circumstantial evidence supporting the notion that this nonlysosomal osteoblastic acid phosphatase may function as a physiological phosphotyrosyl protein phosphatase in bone cells (102,103). A unique property of this enzyme is its inhibition by mitogenic concentrations (*i.e.*, micromolar) of fluoride. Because tyrosyl phosphorylation is closely associated with cell proliferation (104), and because increased tyrosyl phosphorylation can also be achieved by an inhibition of phosphotyrosyl protein dephosphorylation (105,106), it has been hypothesized that the mitogenic action of fluoride on bone cells may be mediated by a direct inhibition of the fluoride-sensitive phosphotyrosyl protein phosphatase activity (100). This hypothesis assumes: a) that the increase in tyrosyl protein phosphorylation is responsible for the stimulation in bone cell proliferation, b) that the binding of a growth factor (*e.g.*, IGF-I) to its membrane receptor on osteoblasts activates the intrinsic tyrosyl kinase activity of the receptor, and thereby stimulates the phosphorylation of the key mitogenic signaling proteins at the tyrosyl residues, which eventually leads to an increase in osteoblast proliferation, c) that this mitogenic signal should be terminated by the dephosphorylation reaction catalyzed by the fluoride-sensitive phosphotyrosyl protein phosphatases, and d) that the action of fluoride is to inhibit the activity of this phosphotyrosyl protein phosphatase and prolongs the mitogenic signal, resulting in a stimulation of osteoblast proliferation.

The following is a brief enumeration of the evidence that supports the concept that the mitogenic action of F is mediated by inhibition of fluoride-sensitive

phosphotyrosyl protein phosphatase: First, the mitogenic dose of fluoride is very low compared to fluoride doses that affect other biological systems, and yet it is very similar to the dose that is required for inhibition of this phosphatase activity and for increased tyrosyl phosphorylation in human bone cells (100,107). Second, other inhibitors of this phosphotyrosyl protein phosphatase, such as vanadate and molybdate are, like fluoride, mitogens for bone cells at concentrations that inhibit the F-sensitive phosphotyrosyl protein phosphatase activity (100,108). Moreover, the mitogenic effect of vanadate is identical to the effect of fluoride in that it requires the presence of a skeletal growth factor (108). Third, fluoride treatment of normal bone cells leads to a net increase in protein phosphorylation (100). Fourth, fluoride stimulates enzymes in the bone cell membrane to increase the overall tyrosyl phosphorylation of an artificial substrate *in vitro* (100). Fifth, thiophosphorylation studies suggest that fluoride acts to increase tyrosyl phosphorylation through an inhibition of phosphotyrosyl protein phosphatase rather than a stimulation of tyrosyl kinase (100). Sixth, fluoride causes an increase in the tyrosyl phosphorylation of, at least, ten cellular proteins (Thomas and Lau, unpublished observations). Seventh, the time course for the effect of fluoride on both mitogenesis and on tyrosyl phosphorylation exhibits a lag period consistent with an inhibition of phosphotyrosyl protein phosphatase and inconsistent with an effect on tyrosyl kinase (Thomas and Lau, unpublished observation). Finally, bone cells (and perhaps also kidney cells) have been shown to contain this fluoride-sensitive phosphotyrosyl protein phosphatase activity (100), a finding that is consistent with the observation that the mitogenic action of fluoride is specific for bone cells (and perhaps also kidney cells - 95,99).

It should be noted that there are some observations that are inconsistent with the hypothesis that the aluminum ion acts through a mechanism which is similar to that of fluoride to exert its mitogenic actions. First, while the mitogenic action of fluoride is specific for bone cells (95,100), the mitogenic effect of aluminum does not appear to be

tissue-specific as it stimulates the proliferation of many cell types, *e.g.*, mouse lens epithelial cells (48) and Swiss 3T3K skin fibroblasts (49). Second, the mitogenic action of aluminum on bone cell proliferation is highly reproducible, whereas the bone cell mitogenic effect of fluoride is inconsistent.

VI. Potential involvement of local bone cell production of growth factors in the osteogenic action of aluminum.

Recent investigations have revealed that many bone cell effectors exert their osteogenic effects by modulating the production and/or the activity of bone cell growth factors. It has been reported that a) progestins stimulate human bone cell proliferation by increasing the bone cell production of IGF-II (109); b) estrogen affects bone formation by altering the production of TGFß (110); c) 1,25 dihydroxyvitamin D_3 inhibits bone cell proliferation by altering the production of IGFs and their binding proteins (111); d) androgens stimulate bone cell proliferation and differentiation by modulating IGF production (112); and e) low-amplitude, low frequency electric field-stimulated bone cell proliferation is mediated, in part, by increased production of IGF-II (113). Consequently, these findings suggest the interesting possibility that some bone cell mitogens may induce cell proliferation indirectly through stimulation of production of bone cell growth factors, and we have, therefore, hypothesized that aluminum may be acting as a bone cell mitogen by altering the effective concentration or activity of bone cell-derived growth factors. Accordingly, one of the goals of this thesis work was to determine whether the mitogenic action of aluminum is mediated by the production of growth factors by human bone cells *in vitro* (or by synthesizing products, such as IGFBPs, that modulate the activity of the growth factors).

Bone cells are known to produce several growth factors, *i.e.*, IGF-I, IGF-II, TGFß, PDGF, and FGF (114), and it has been hypothesized that these growth factors, upon secretion from osteoblasts, will be deposited in bone matrix (114), and released

during resorption (115). In human bone cells, the most abundant and apparently most important growth factors are IGF-II and TGFß (116,117). TGFß is synthesized by bone cells as latent holoenzyme form, which needs to be activated by proteolysis (118). Because our laboratory has the expertise and reagents to measure IGFs accurately, whereas measurements of active TGFß is based on an insensitive and less reliable bioassay (117), this thesis work had been focused on investigation of the potential effects of aluminum on the IGF regulatory system.

VII. Insulin-like growth factor regulatory system and bone cell proliferation.

It is now clear that the IGF regulatory system is complex and highly regulated. Human bone cells produce two IGFs (*i.e.*, IGF-I and IGF-II) and at least six binding proteins (*i.e.*, IGFBP-1, -2, -3, -4, -5, and -6). These two classes of molecules, the IGFs and their binding proteins, represent two major components of the IGF regulatory systems. My thesis work has been focused primarily on these two components of the system.

IGF-II is the most abundant growth factor in human bone matrix (114), and is produced by bone cells from a number of species, including human, mouse, and rat (116). IGF-II is synthesized as a prepropolypeptide with an approximate molecular mass of 20 kD that includes a 24 amino acid signal peptide and an 89 amino acid carboxyterminal peptide (119). IGF-II stimulates cell proliferation in a dose-dependent manner in serum-free cultures of bone cells isolated from a number of species, including chick, mouse, and human (120). It has been shown that IGF-II is one of the few growth factors that is mitogenic for untransformed normal human bone cells isolated from the trabecular bone of femoral head samples (121). In addition to stimulating bone cell proliferation, IGF-II also stimulates the differentiated function of bone cells. In this regard, IGF-II stimulated synthesis of type I collagen in mouse calvarial MC3T3-E1 cells (122). The production rate of IGF-II by human bone cells is

regulated by a variety of bone cell regulators. PTH has been shown to stimulate IGF-II release in newborn mouse calvaria (123); and 1,25 dihydroxyvitamin D_3 stimulated production of IGF-II in serum-free organ cultures of newborn mouse calvaria (111). Also 17-ß estradiol, was found to increase the secretion of IGF-II from the rat osteosarcoma UMR106 cell line (124). Thus, the bone cell production of IGF-II is regulated, and agents that stimulate bone formation may act, at least in part, by altering the production of IGF-II by bone cells.

IGF-I is produced by human bone cells at concentrations of 50- to 100-fold less than those of IGF-II (125). IGF-I stimulates human bone cell proliferation in a dose-dependent manner (120). The mitogenic efficiencies of IGF-I and IGF-II *in vitro* were almost identical, since identical dose response curves for IGF-I and -II were obtained in untransformed normal human bone cells and embryonic chick calvarial cells under serum-free conditions (120). Studies on the regulation of IGF-I production by bone cells have shown that 17ß-estradiol stimulated the production of IGF-I in UMR106 cells (124). PTH has also been shown to increase the production of IGF-I by monolayer cultures of rat osteoblasts (*i.e.*, 1.5- to 2-fold after 24 hour treatment). These findings are consistent with the notion that the bone cell production of IGF-I, like that of IGF-II, is regulated and may play a role in mediating the mitogenic effects of bone cell effectors.

IGF-I and II are bound to specific binding proteins in the serum, tissue extracts, and conditioned media (126,127). Six different IGFBPs have been identified thus far. IGFBP-1 has 234 amino acids with a predicted molecular mass of 25.3 kD, somewhat smaller than the apparent molecular size (30-35 kD) estimated by SDS polyacrylamide gel (128). IGFBP-1 binds to both IGF-I and -II with similar affinity (128). Human IGFBP-2 contains a 39-residue signal peptide followed by a mature protein of 289 residues with a predicted molecular mass of 31 kD. While IGFBP-2 binds both IGF-I and -II, it has a much higher affinity for IGF-II than IGF-I (129). IGFBP-3, the

major IGFBP in serum, has a 27 amino acid signal sequence followed by a mature protein of 264 amino acid residues, and it binds both IGF-I and -II with similar affinity (130).

The exact functions of these IGFBPs is not fully understood. IGFBP-1 has been shown to induce IGF-I-stimulated cell proliferation in muscle cells (133). On the other hand, high concentrations of IGFBP-1 inhibited the actions of IGF-I in cultured choriocarcinoma cells (134). The function of IGFBP-2 is unknown. Purified IGFBP-3 either inhibits or stimulates cell proliferation in neonatal skin fibroblasts depending on culture conditions (135).

Perhaps the most important IGFBPs in human bones are IGFBP-4 and IGFBP-5. IGFBP-4, molecular size of approximately 25 kD as determined by SDS polyacrylamide gel electrophoresis, is a potent inhibitor of IGF activity on bone cells (131) that binds IGF-I and -II but not insulin. IGFBP-4 is the most potent inhibitory binding protein identified thus far. IGFBP-5 is a newly discovered binding protein (132). IGFBP-5, upon binding to IGFs, enhances the mitogenic activity of the bound IGF; thus, IGFBP-5 has been described as a stimulatory IGFBP (132). The most unique feature of IGFBP-5 is that it has high binding affinity for the mineral phase of bone, and may play a role in fixing IGFs in mineralizing human bone matrix (132). IGFBP-6 is a newly discovered binding protein, whose function and properties are not yet well understood.

VIII. Human TE85 osteosarcoma cells as a model system for human osteoblasts.

In order to evaluate the mechanism of the mitogenic action of aluminum on human bone cells, an appropriate cell model is necessary. The normal human bone cell population is generally heterogeneous consisting of many cell types with various degrees of differentiation. Human osteosarcoma cells are transformed tumor cells, which are more homogeneous than normal human bone cell populations. Moreover,

some osteosarcoma cells appear to have retained some of the normal regulatory mechanisms for cell proliferation. Hence, useful information can be obtained from appropriate osteosarcoma cells.

In these studies I have used human TE85 osteosarcoma cells. These cells were chosen because a) they grow much faster than the normal untransformed human osteoblasts, and thus would provide sufficient cell numbers for my experiments in a reasonable time frame; b) unlike normal human bone cells, they are relatively homogeneous; c) they exhibit several characteristics which are similar to those of untransformed human osteoblasts (81); and d) previous studies have used these osteosarcoma cells to investigate the mitogenic action of fluoride on human osteoblast-line cells. Consequently, we have been able to compare the results of our studies with previous work on fluoride in the same cells.

CHAPTER 2: MATERIALS and METHODS

I. MATERIALS.

Tissue culture supplies were from Falcon (Oxnard, CA). Dulbecco's modified Eagle's medium (DMEM), collagenase, bovine calf serum, penicillin, and streptomycin were 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 a product of New England Nuclear (Wilmington, DE). [^{125}I]NaI (2,125 Ci/mmol) and [^{32}P]ATP (10-25 Ci/mmol) were products of ICN Biochemicals (Irvine, CA). 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). Chloroform, formaldehyde, and 2-propanol were obtained from Fisher Scientific (Irvine, CA). Molecular biology grade phenol was purchased from USB Corp. (Cleveland, OH). Guanidine thiocyanate was a product of Fluka Chemicals Corp. (Ronkonoma, NY). All other chemicals were molecular biology grade (or reagent grade, as appropriate) and were obtained from Sigma Chemical Co. (St. Louis, MO). Recombinant human IGF-I and IGF-II were purchased from Bachem Chemicals (Torrance, CA). The human osteosarcoma TE85 cells were obtained from Dr. J. Fogh of the Sloan 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. M. R. Uskokovic of Hoffmann-LaRoche, Inc. (Nutley, NJ).

II. METHODS.

1. Cell cultures.

Embryonic chicken calvarial cells were prepared by sequential crude collagenase digestion in serum-free DMEM at 37°C as previously described which resulted in enriched cultures of osteoblasts and their precursors (136). Cells isolated by trysin digestion were plated at 7,000 cells per well (in 1 ml medium) in 24-well plates in serum-free DMEM and cultured for 24 hours. Normal human bone cells, which were prepared according to Wergedal and Baylink (137), were plated at 10,000 cells (in 1 ml) per well in 24-well plates in DMEM containing 10% bovine calf serum and cultured for 24 hours. These human bone cells were shown to be of osteoblastic nature (121). Human osteosarcoma TE85 cells, a cell line showing osteoblastic characteristics (138), were routinely maintained by weekly passaging in DMEM containing 10% bovine calf serum. The TE85 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 and cultured for 24 hours. 24 hours after plating, each bone cell culture was changed to serum-free DMEM and effectors were added half an hour later, for incubations of either 20-24 hours for [^{3}H]thymidine incorporation assay or 48 hours for alkaline phosphatase specific activity measurements. Aluminum salts were added in DMEM supplemented with 0.1% bovine serum albumin as carrier. Bovine serum albumin by itself had no significant effect on [^{3}H]thymidine incorporation or 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 cell preparation (136), for determination of cell specificity for mitogenic activity of aluminum ion.

2. Cell DNA synthesis assay.

Bone cell mitogenic activity was assayed by measuring the stimulation of incorporation of [^{3}H]thymidine into cellular DNA. The assay was adapted from the method of Gospodarowicz *et al.* (139) and has previously been described (136,140,141). Briefly, the bone cells were plated at 2,500 cells per cm^2 in 24-well plates in DMEM containing 10% bovine calf serum for 24 hours. After plating, the bone cells were changed to serum-free DMEM and incubated for 24 hours. The medium was then changed to fresh serum-free DMEM and effectors (*e.g.*, aluminum) were added half an hour later, and the cultures were incubated for an additional 24 hours. Aluminum ion (*i.e.*, aluminum sulfate) was added in DMEM with 0.1% bovine serum albumin as carrier. Addition of aluminum ion did not alter the pH of the culture medium according to the pH indicator in the culture medium. [^{3}H]thymidine was added to each culture (20 μl, containing 0.75 μCi) 2 hours before the end of the incubation. Bone cell mitogenic activity was assayed by the stimulation of incorporation of [^{3}H]thymidine into cellular DNA during the last 2 hours of the exposure to effectors. To confirm the assay as a measurement of cell proliferation, the number of cells in parallel replicate cultures of TE85 cells were also counted after incubation with or without aluminum ion for 48 hours (*i.e.*, in two separate experiments). A minimum of six replicate culture wells were used to measure [^{3}H]thymidine incorporation for each tested dose of effector.

3. Cellular alkaline phosphatase activity assay.

Cell layer protein was extracted in a buffered solution of 0.01% triton X-100, and cellular alkaline phosphatase activity was determined in an aliquot of this extract with 10 mM PNPP in 0.15 M sodium carbonate buffer (pH 10.3) in the presence of 1 mM MgCl (142). One unit of enzyme activity was the amount of enzyme that hydrolyzed 1 μmol PNPP per min at room temperature (25°C). The cellular enzyme

activity was normalized against DNA content measured by a fluorescent method (143), or against cellular protein determined according to Lowry *et al.* (144). Alkaline phosphatase specific activity was determined in six replicate cultures for each tested effector concentration.

4. Collagen synthesis assay.

The ability of aluminum ion to stimulate bone formation was assessed by the measurement of collagen synthesis in six replicate monolayer cultures of human osteosarcoma TE85 cells. The procedure has previously been described (145). Briefly, sub-confluent cultures of TE85 osteosarcoma cells were plated in 24-well culture plates in serum-free DMEM, and 24 hours later effectors were added for an additional 48 hours incubation. Sodium fluoride (100 μM) and insulin (0.1 μM) were included as positive controls. During the final 6 hours, [^{3}H]proline (8 μCi/ml) and ß-aminopropionitrile (50 μg/ml) were added to each well. Radioactivity in collagenase-digestible and -nondigestible proteins was determined according to Beresford *et al.* (145). Percent collagen synthesized was calculated after multiplying collagenase-nondigestible proteins by 5.4 to correct for the relative abundance of proline in collagenase-digestible and -nondigestible proteins (146).

5. cAMP assay.

Cellular cAMP production in confluent cultures of TE85 cells was determined as previously described (141). The cells were plated in 24-well plates in 1 ml DMEM with 10% fetal bovine serum, and allowed to grow for 24 hours. The cells were then washed three times with serum-free DMEM, and placed in 1 ml fresh serum-free DMEM containing 1 mM isobutylmethylxanthine to inhibit phosphodiesterase activity. After incubation with effectors at room temperature for 15 min, incubation medium was decanted rapidly. The cells were washed with ice-cold phosphate-buffered saline

(0.15 M NaCl/5.6 mM Na_2HPO_4, pH 7.4) and 1 ml of 10% trichloroacetic acid (4°C) was added to each cell layer. The cell layer was then scraped from the plate with a rubber policeman and transferred into a test tube. The cell extracts were then centrifuged for 5 min at 2,500 x g, and the acid was removed by extracting four times with 2 ml of diethyl ether. The aqueous extract was dried by Speed-Vac vacuum centrifugation, and stored at -20°C until assay.

Shortly before assay, each dried extract residue was redissolved in 0.4 ml sodium acetate (50 mM, pH 6.2). cAMP was measured by a radioimmunoassay (RIA). The overall recovery of cAMP was estimated by adding [^{3}H]cAMP (20,000 cpm) to replicate cell cultures during initial trichloroacetic acid extraction. Recovery was found to be 85-90%. The cellular protein concentration in the trichloroacetic acid-insoluble fraction was measured by the protein assay of Lowry *et al.* (144). The amount of cellular cAMP was normalized against cellular protein.

6. 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 per well fresh DMEM supplemented with 10^{-8} M vitamin K 24 hours 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 hours at 37°C under an atmosphere of 5% CO_2 in air. CM was collected and frozen until assay. Osteocalcin concentration in the CM was assayed with an RIA specific for the mid-molecule of human osteocalcin (147). Cells were then extracted with 0.1% Triton X-100, and cell extract alkaline phosphatase activity and protein were assayed as described above.

7. IGF assays.

IGF-I was determined in CM by an RIA, using rabbit polyclonal anti-human IGF-I antiserum UB3-189 from the National Institutes of Health. Recombinant human IGF-I was used as standard and tracer. IGF-I was labeled with [^{125}I]NaI by chloramine-T iodination as described previously (125) and separated from free ^{125}I using a Sep-Pak (Waters, Milford, MA). The range of the assay was 0.05-3 ng/ml, the cross-reactivity with IGF-II and insulin was $< 0.5\%$ (125), and there was no cross-reactivity with other growth factors. IGF-II was assayed with a radioreceptor assay (RRA) using competitive displacement of ^{125}I-labeled human IGF-II binding to monolayer cultures of H-35 rat hepatoma cells. These cells have no type 1 IGF receptors. Human recombinant IGF-II was used for standard and tracer. [^{125}I]IGF-II was prepared as described for IGF-I. The RRA, described previously (125), had a range of 6-80 ng/ml, a cross-reactivity with IGF-I and insulin of $< 2\%$, and no cross-reactivity with other growth factors. Both IGF-I and IGF-II assays have been standardized and validated (125). IGFBP artifacts in the CM samples have been eliminated by pre-incubating the samples with 50 ng/ml IGF-II in the IGF-I RIA or with 400 ng/ml IGF-I in the IGF-II RRA (125).

8. Western ligand blotting of IGFBPs.

Human TE85 cells were plated at a density of 17,500 cells per cm^2 in 2 ml of DMEM containing 10% calf serum in 60-mm culture plates and cultured overnight. The culture media were then changed to serum-free DMEM and incubated for 24 hours. After the incubation, the culture media were changed to fresh DMEM and effector was added. CM was collected after 6, 24, or 48 hours incubation, and dried by SpeedVac centrifugation. Each sample was then resuspended in 0.1 ml of deionized water and 0.1 ml SDS-polyacrylamide gel electrophoresis treatment buffer without ß-mercaptoethanol. A 0.05 ml volume of each CM sample was electrophoresed on a

10-20% gradient SDS-polyacrylamide gel under non-reducing conditions. The size-fractionated proteins were electroblotted onto a nitrocellulose membrane for 24 hours at 50 volts. The immobilized proteins on nitrocellulose membrane were incubated overnight with 2 x 10^6 cpm each of [^{125}I]IGF-I and [^{125}I]IGF-II at 4°C. IGFBPs were visualized by autoradiography. The relative concentrations of each IGFBP was determined by measuring the relative intensity of the bands with laser densitometry (Biomed Instruments, Fullerton, CA).

9. Statistical Methods.

The statistical significance of the differences was determined by 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 thesis are presented as mean $\pm$ S.D. of six replicates. Each experiment has been repeated at least twice.

CHAPTER 3: RESULTS

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

Micromolar 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 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 doses was ineffective (data not shown). Thus, the mitogenic activities were attributed to 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 TE85 cell line (Fig. 2, top panel), and in normal human osteoblasts (Fig. 2, bottom panel). In addition, micromolar 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 action of aluminum is 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 hours incubation with aluminum sulfate corresponded to the increases in the number of TE85 cells after an 48-hour incubation (Fig. 4).

Figure 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.

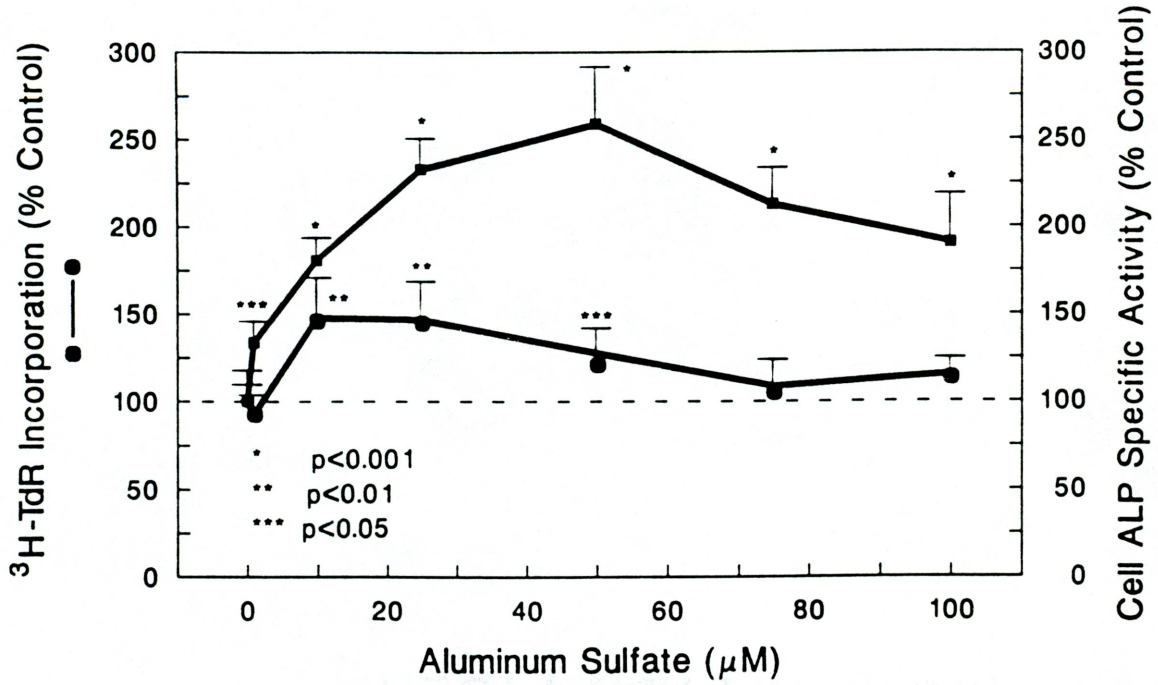

^{3}H-TdR Incorporation (% Control)
Cell ALP Specific Activity (% Control)
Aluminum Sulfate (μM)
300
250
200
150
100
50
0
0
20
40
60
80
100
* p<0.001
** p<0.01
*** p<0.05

Figure 2. Stimulation of [^{3}H]thymidine incorporation and cell alkaline phosphatase activity in monolayer cultures of human TE85 osteosarcoma cells (upper panel) and in normal human osteoblasts (bottom panel) by aluminum sulfate. The data were presented as percentage of the corresponding controls. Open circles represent [^{3}H]thymidine incorporation and closed 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.

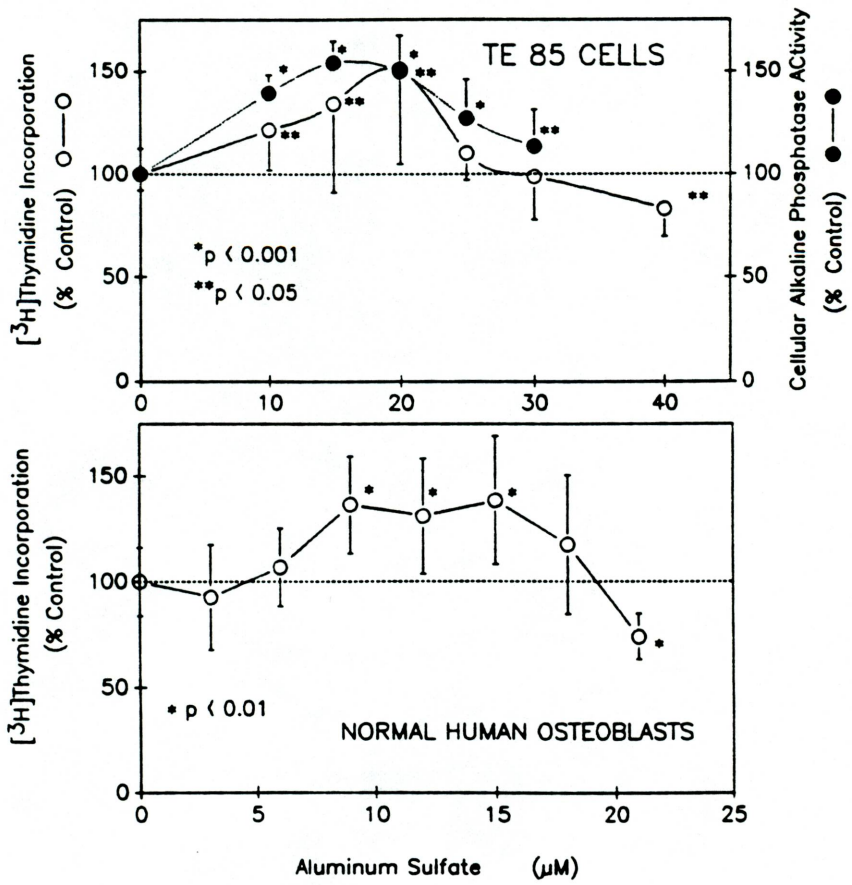
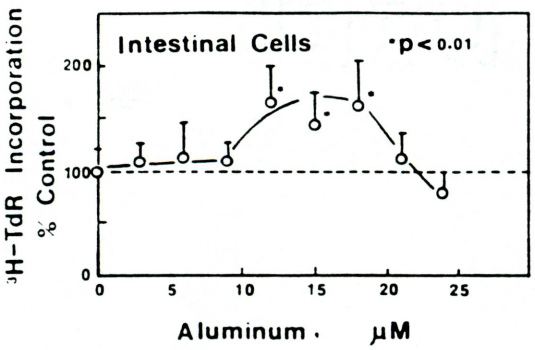
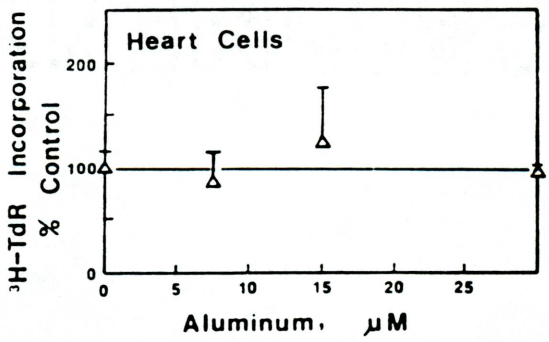
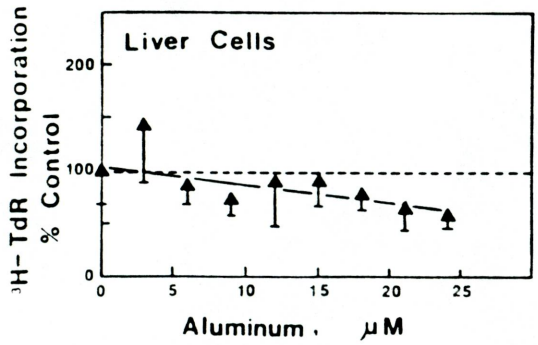

TE 85 CELLS
[3H]Thymidine Incorporation (% Control) O—O
Cellular Alkaline Phosphatase ACtivity (% Control) ●—●
*p < 0.001
**p < 0.05
150
100
50
0
0
10
20
30
40
[3H]Thymidine Incorporation (% Control)
* p < 0.01
NORMAL HUMAN OSTEOBLASTS
5
15
25
Aluminum Sulfate (μM)

Figure 3. Cell specificity of the mitogenic activity of aluminum sulfate. Embryonic chicken intestinal, liver, heart, and muscle cells were prepared from 16-day-old embryos. The mitogenic activity of aluminum sulfate was measured by the stimulation of [^{3}H]thymidine incorporation into cellular DNA and is shown as percentage of the corresponding no addition controls. The dashed lines are the corresponding 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.


Intestinal Cells
*p< 0.01
³H-TdR Incorporation
% Control
200
100
0
0
5
10
15
20
25
Aluminum, μM


Heart Cells
³H-TdR Incorporation
% Control
200
100
0
0
5
10
15
20
25
Aluminum, μM


Liver Cells
³H-TdR Incorporation
% Control
200
100
0
0
5
10
15
20
25
Aluminum, μM

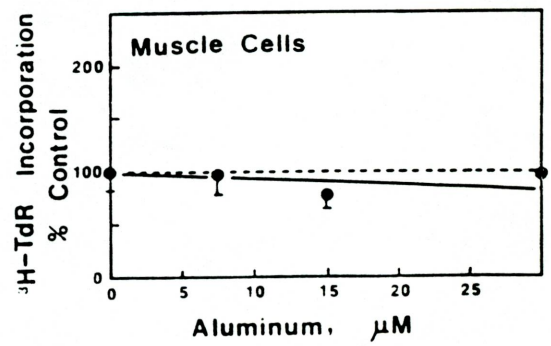
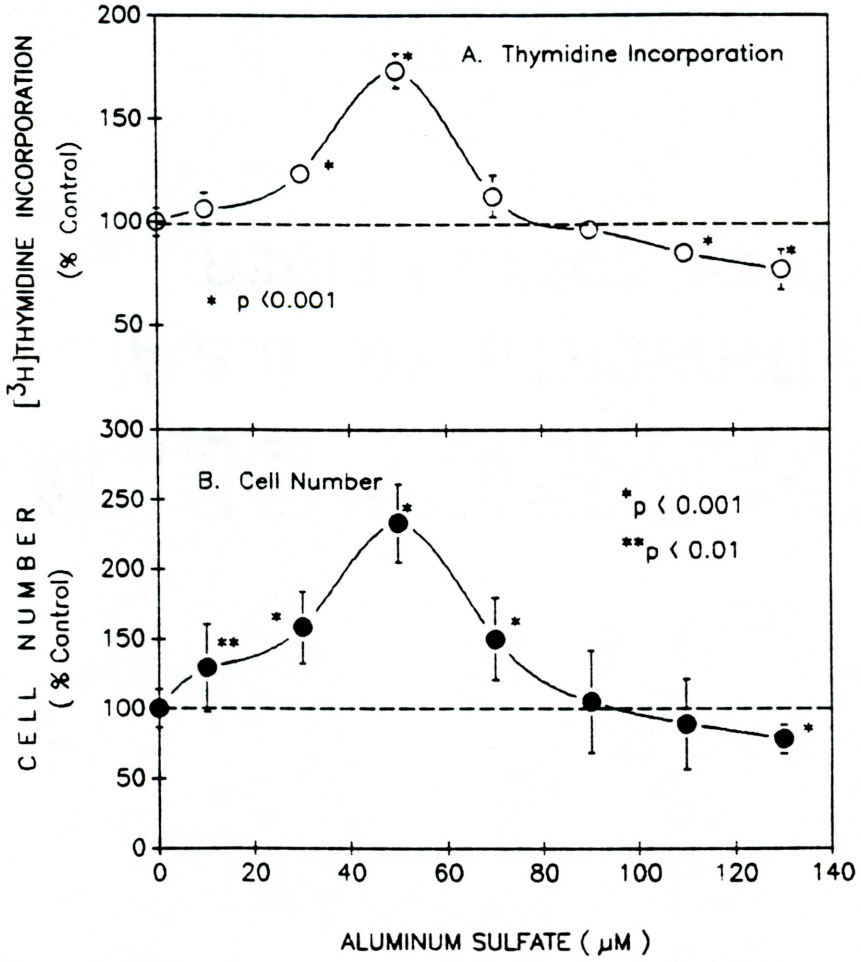

Muscle Cells
³H-TdR Incorporation
% Control
200
100
0
0
5
10
15
20
25
Aluminum, μM

Figure 4. Stimulation of the proliferation of human TE85 osteosarcoma cells by aluminum ion. TE85 cells were plated at 5,000 cells per well in 1 ml serum-free DMEM and cultured for 24 hours. The cells were then changed to 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-hour incubation (panel A); and in a duplicate set of the experiment, the number of cells per well was counted after an 48-hour 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.


A. Thymidine Incorporation
* p <0.001
[3H]THYMIDINE INCORPORATION (% Control)
B. Cell Number
*p < 0.001
**p < 0.01
CELL NUMBER (% Control)
ALUMINUM SULFATE (μM)

II. Stimulation of collagen synthesis by mitogenic doses 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 digestible proteins. Table 1 shows that aluminum ion significantly stimulated the incorporation of [^{3}H]proline into collagenase-digestible and total proteins, suggesting that aluminum ion stimulated both the *de novo* synthesis of total and collagen proteins. That the mitogenic concentrations of aluminum ion (*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) (108) were both shown to stimulate collagen synthesis in these cells.

III. Effects of mitogenic doses of aluminum ion on osteocalcin production.

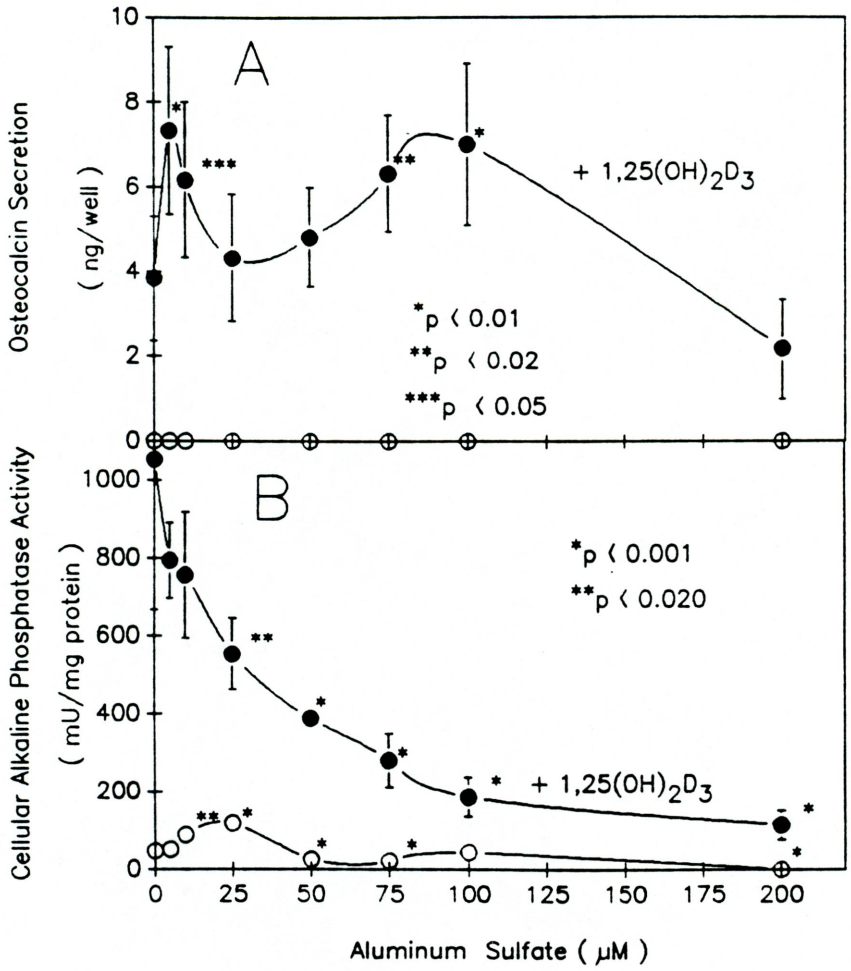
The action of mitogenic concentrations of aluminum sulfate on the secretion of osteocalcin by normal human osteoblasts was examined. The human osteoblasts under basal conditions secreted little, if any, osteocalcin into their conditioned medium (*i.e.*, undetectable by our assay), and mitogenic concentrations of aluminum did not stimulate its secretion, or the effect was masked by the insensitivity of the assay (Fig. 5, top panel). However, micromolar 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 unhealthy, since a significant amount of cell debris was seen in these cultures.

Because it has been suggested that 1,25 dihydroxyvitamin D_3 is required for the activation of osteocalcin synthesis by osteoblasts (148), the effect of aluminum ion on

Table 1. Aluminum ion stimulates collagen synthesis in human osteosarcoma TE85 cells *in vitro*. TE85 cells were plated at 50,000 per well of 24-well plates in 1 ml of DMEM containing 10% bovine calf serum and cultured for 24 hours. Cells were then changed to 0.5 ml serum-free DMEM containing 50μg/ml ascorbate, for an additional 48 hour incubation. At the end of that incubation, a solution of 2 μCi [^{3}H]proline (52 Ci/mmol) and 25 μg/ml ß-aminopropionitrile were added and the cells were further incubated for 4 hours. 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 (cpm/well)	p	Collagen (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 Fluoride	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

Figure 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-hour incubation in the absence of $1,25(OH)_2D_3$ (but in the presence of the ethanol vehicle control). The closed symbols indicate osteocalcin production 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 hours without $1,25(OH)_2D_3$; and the closed symbols are those in the presence of $1,25(OH)_2D_3$.

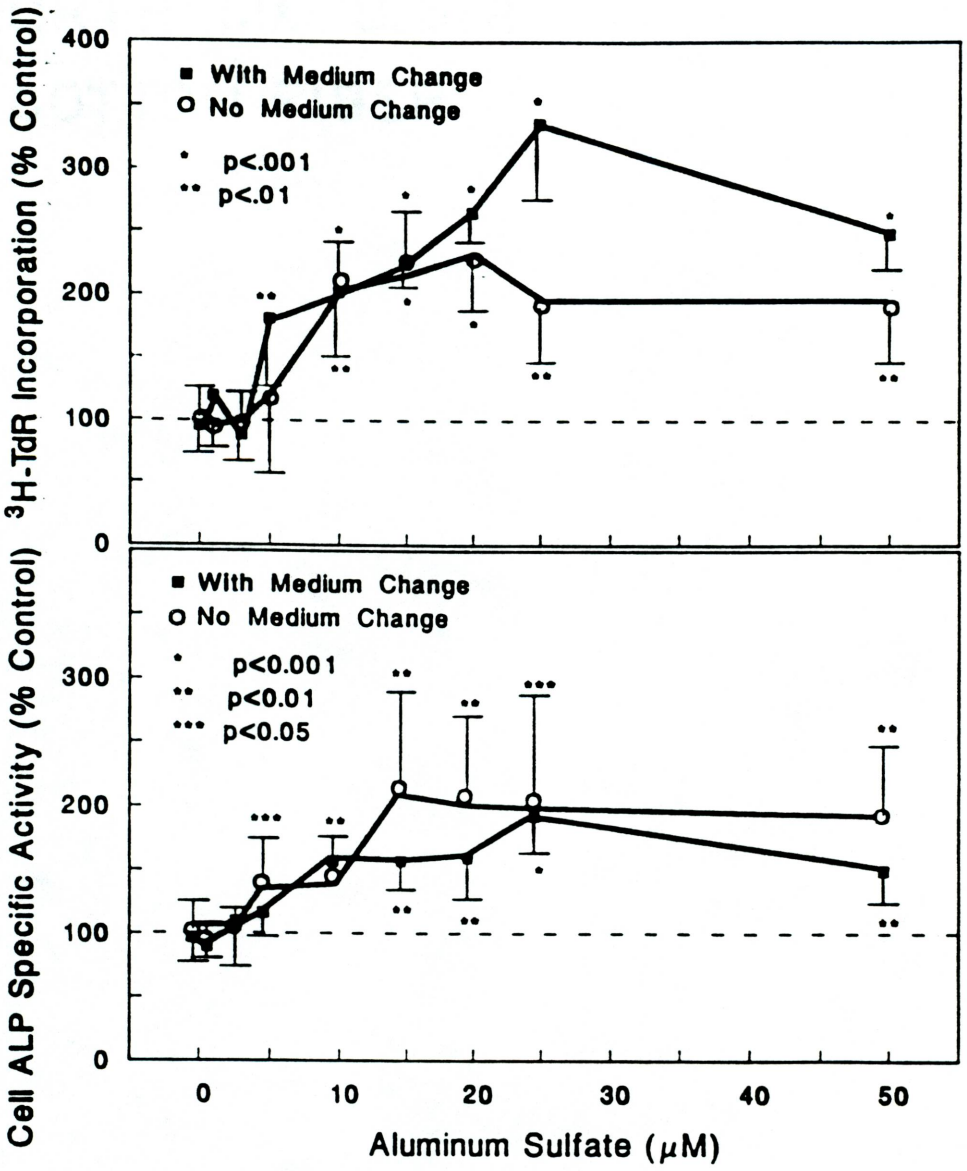

A
B
Osteocalcin Secretion
(ng/well)
Cellular Alkaline Phosphatase Activity
(mU/mg protein)
+ 1,25(OH)2D3
*p ⟨ 0.01
**p ⟨ 0.02
***p ⟨ 0.05
*p ⟨ 0.001
**p ⟨ 0.020
Aluminum Sulfate (μM)
0
2
4
6
8
10
200
400
600
800
1000
25
50
75
100
125
150
175
200

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.

IV. Effect of medium change on the mitogenic activity of aluminum ion.

Because no attempt was made in this study to remove contaminating fluoride from reagents and glassware, one might argue that the observed mitogenic actions of aluminum could be due to low concentrations of fluoride. To assess this possibility, the mitogenic activity of aluminum was compared to that of fluoride. This laboratory has previously shown that the osteogenic actions of fluoride were abolished by medium changes, and that the mitogenic activity of fluoride depended on a coincubation with growth factors (*i.e.*, in the CM (101,108)). My studies showed that removal of endogenous growth factors by medium change immediately before the addition of aluminum 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 stimulation 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 phosphatase activity), suggesting that there were interactions between aluminum ion and

Figure 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 TE85 osteosarcoma 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 TE85 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.


Cell ALP Specific Activity (% Control)
^{3}H-TdR Incorporation (% Control)
400
300
200
100
0
■ With Medium Change
O No Medium Change
* p<.001
** p<.01
■ With Medium Change
O No Medium Change
* p<0.001
** p<0.01
*** p<0.05
0
10
20
30
40
50
Aluminum Sulfate (μM)

endogenous growth factors in the stimulations shown in these two parameters, even though the effects of aluminum ion did not require the presence of growth factors.

V. Interaction of fluoride and aluminum on bone cell proliferation.

As a further test of the hypothesis that aluminum might be acting in concert with fluoride, we reasoned that 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 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, the effects of various doses of fluoride on the stimulation of TE85 cell proliferation by aluminum sulfate were tested (Fig. 7). There were two noteworthy observations: (a) fluoride did not significantly shift or alter the dose-dependent curves of aluminum ion; and (b) fluoride potentiated the stimulation of [^{3}H]thymidine incorporation by aluminum ion, *i.e.*, the maximal stimulation was higher when both effectors were present than each alone. To further analyze this apparent interaction between fluoride and aluminum ion, the data of Figure 7 were 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 sulfate 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 ion, is consistent with the interpretation that the rate-limiting steps for the mitogenic actions of fluoride and aluminum are different.

Figure 7. Interaction of the mitogenic activity of aluminum and fluoride on human TE85 osteosarcoma 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.*, neither aluminum nor fluoride). The 100% of the control value (indicated by the dashed line) was 4891 cpm incorporated per well.

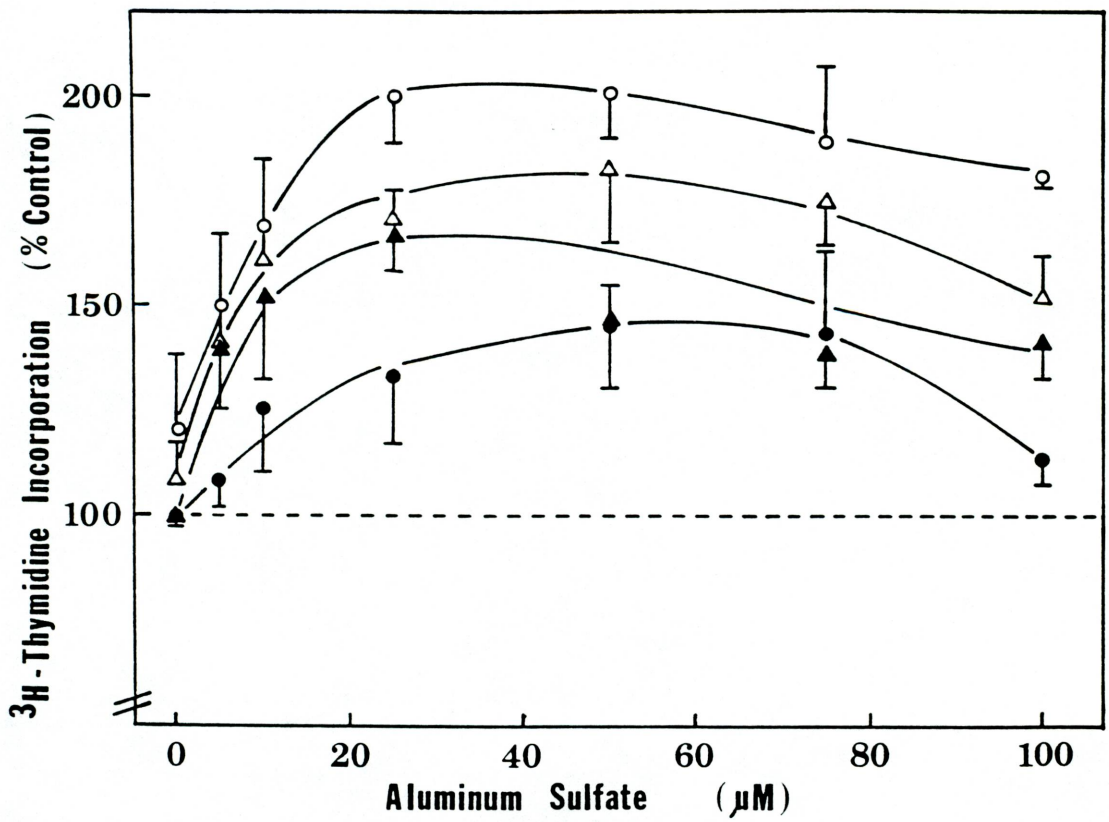
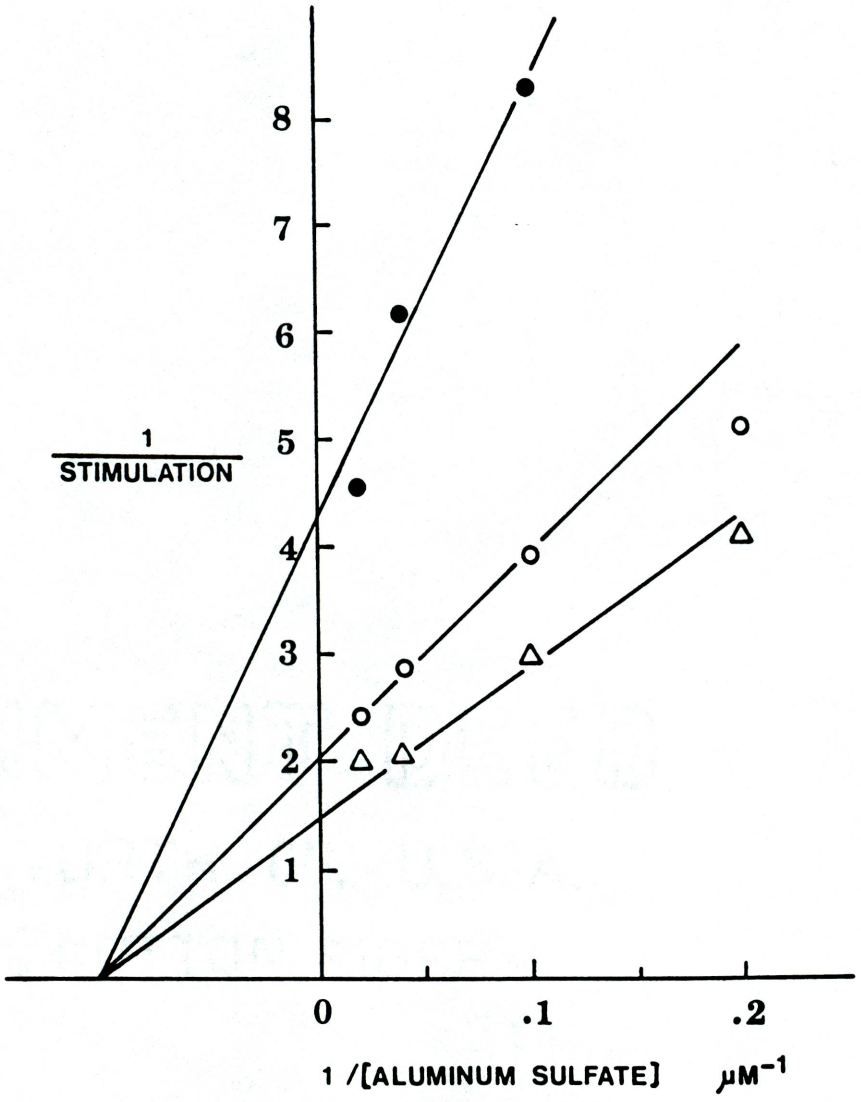

200
150
100
^{3}H-Thymidine Incorporation (% Control)
0
20
40
60
80
100
Aluminum Sulfate (μM)

Figure 8. Analysis of interaction between fluoride and aluminum with a plot analogous to the Lineweaver-Burk kinetic plot. The results of Figure 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.


8
7
6
1
STIMULATION
5
4
3
2
1
0
.1
.2
1 /[ALUMINUM SULFATE] μM⁻¹

VI. Effects of aluminum on cAMP production.

Since fluoroaluminate is a potent stimulator of adenylate cyclase through its action on the G-proteins (85,86), we also measured the effects of aluminum ion on cAMP production by TE-85 cells. Table 2 shows that mitogenic concentrations of aluminum did not significantly increase the cellular cAMP production in these cells. PTH and 10 mM sodium fluoride both significantly stimulated the cellular cAMP production in these cells, indicating that the adenylate cyclase system in these cells was responsive to stimulation.

VII. Effects of aluminum ion on IGF's release from TE85 cells.

To test the hypothesis that the mitogenic action of aluminum ion is mediated by the local bone production of IGFs, the concentrations of IGFs in the CM of TE85 cells treated with or without mitogenic doses of aluminum sulfate for 48-hours were measured (Fig. 9). The basal concentration of IGF-I produced by TE85 cells was very low (*i.e.*, at the low detection limit of our RIA), and treatment with aluminum ion appeared to enhance the release of IGF-I into CM by these cells. However, the effective dose range was very narrow. In this experiment, 25 μM of aluminum ion was the only effective concentration. In a repeat experiment, the optimal dose of aluminum ion was between 25 and 50 μM (data not shown). On the other hand, the IGF-II concentration in the CM of the unstimulated TE85 cells was at least an order of magnitude higher than that of IGF-I. Treatment with the same doses of aluminum ion significantly stimulated the release of IGF-II by these cells in a biphasic dose-dependent manner. The optimal dose of aluminum ion in each of three experiments ranged from 50 to 75 μM. Equivalent concentrations of sodium sulfate (up to 200 μM) were also tested, but sodium sulfate had no effects on either [^{3}H]thymidine incorporation, or cell alkaline phosphatase specific activity (data not shown). Thus, the observed effects were due to aluminum ion and not to the counter anion, sulfate.

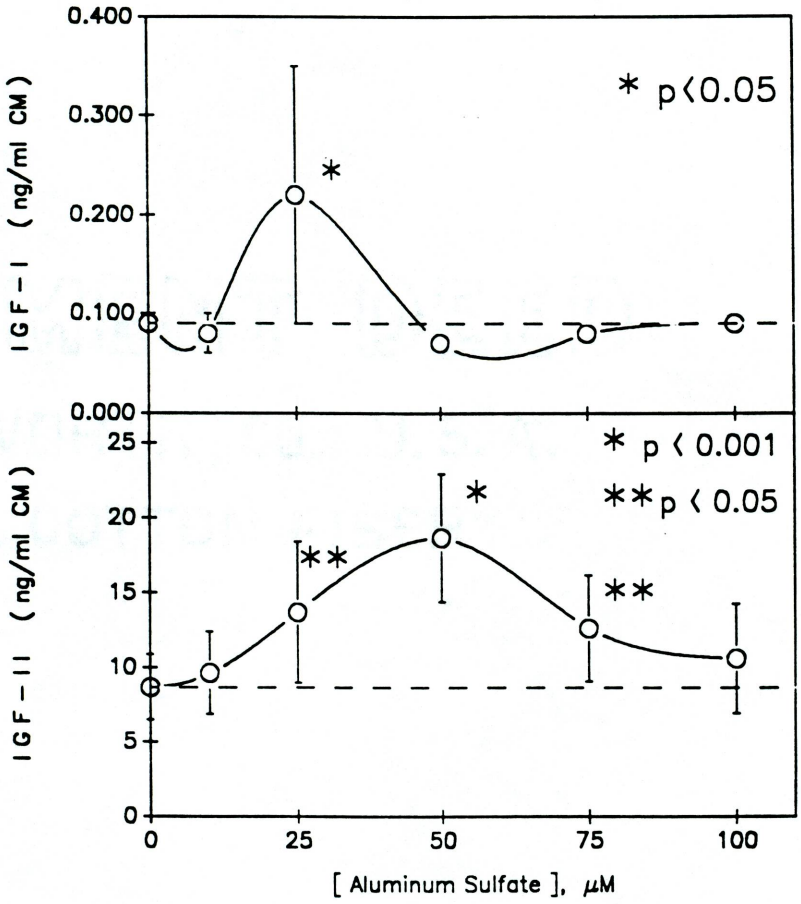
Table 2. Effect of the mitogenic concentrations of aluminum on cAMP production in TE85 cells. Confluent TE85 cell cultures were treated with effectors in fresh serum-free DMEM containing 1 mM isobutylmethylxanthine at room temperature for 15 minutes. cAMP content in the cell extract after extraction with ether to remove trichloroacetate 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 $Al_2(SO_4)_3$	86.30 ± 43.27	n.s.
100 μM $Al_2(SO_4)_3$	58.80 ± 17.68	n.s.
150 μM $Al_2(SO_4)_3$	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 Anaylsis of Variance 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.

Figure 9. Effects of aluminum ion on the secretion from IGFs of human TE85 osteosarcoma cells. TE85 cells were plated at a density of 5,000 cells per cm^2 in 3 ml of DMEM containing 10% bovine calf serum in 6-well plates. When the cells reached approximately 40% confluence, the cultures were changed to serum-free DMEM for 24 hours, after which the medium was changed to fresh serum-free DMEM, and various concentrations of aluminum were added half an hour later. The cells were allowed to incubate for 48 hours. The CM from each well was then collected in plastic test tubes precoated with 1% bovine serum albumin in DMEM. One ml of each CM was then dried by vacuum centrifugation, and resuspended in 50 μl of assay mixture. IGF-I and -II were assayed as described in Methods. The top panel shows the IGF-I concentrations in CM; and the bottom panel indicates the IGF-II concentrations in CM. The dotted lines represent the IGF concentrations in CM of the untreated control cells.


0.400
0.300
0.200
0.100
0.000
IGF-I (ng/ml CM)
* p<0.05
*
25
20
15
10
5
0
IGF-II (ng/ml CM)
* p < 0.001
**p < 0.05
*
**
**
0
25
50
75
100
[Aluminum Sulfate], μM

VIII. Effects of an inhibitory IGF binding protein (*i.e.*, IGFBP-4) on the aluminum ion-dependent stimulation of cell proliferation of TE85 cells.

As a further test of the hypothesis that the aluminum-dependent stimulation of human bone cell proliferation was mediated by an increase in IGF secretion, we measured the effects of adding an inhibitory IGF binding protein, IGFBP-4 (131), on aluminum-induced human bone cell proliferation. [Mohan and coworkers have previously demonstrated that IGFBP-4 was a specific and potent inhibitor of IGF-I and -II stimulated in human bone cell proliferation (131)]. Figure 10 shows that IGFBP-4 at 300 ng/ml significantly inhibited the basal proliferation of the TE85 cells by $17.8 \pm 11.0\%$, and that aluminum sulfate at 100 μM stimulated the [^{3}H]thymidine incorporation into TE85 cells by $47 \pm 21.9\%$ ($p < 0.001$). Addition of 300 ng/ml of IGFBP-4 significantly blocked the aluminum-induced stimulation of [^{3}H]thymidine incorporation by $27.7 \pm 8.6\%$ ($147.0 \pm 21.9\%$ of controls vs. $106.3 \pm 11.9\%$ of controls, $p < 0.001$), which is larger than the inhibitory effect of IGFBP-4 on the basal cell proliferation rate.

IX. Effects of aluminum ion on secretion of IGFBPs by human bone cells.

The effects of a mitogenic dose of aluminum sulfate on the secretion of IGFBPs by TE85 cells were also examined. IGFBPs were measured by Western ligand blot analysis. Figure 11 shows that human TE85 cells secreted mostly the 38.5 and 41.5 kD IGFBP-3 and the 25 kD IGFBP-4. TE85 cells also secreted an unidentified IGFBP with an apparent molecular size of approximately 30 kD. Treatment with 50 μM aluminum sulfate appeared to greatly inhibit the secretion of IGFBP-3 and -4 from TE85 cells. Laser densitometric analyses of the autoradiographs of the Western ligand blots revealed that this dose of aluminum ion reduced the IGFBF-3 level in the CM by approximately 35% at each time point and the IGFBP-4 level in the CM by approximately 30% at each time point. The inhibitory effects of aluminum ion could be

Figure 10. Effect of IGFBP-4 on the mitogenic activity of aluminum ion on TE85 cells. The mitogenic activity was assessed by stimulation of [^{3}H]thymidine incorporation. The cells were incubated with 300 ng/ml IGFBP-4 alone, 100 μM aluminum sulfate alone, or both effectors for 20 hours. The dotted line indicates the 100% value of [^{3}H]thymidine incorporation in the vehicle-treated control cells.

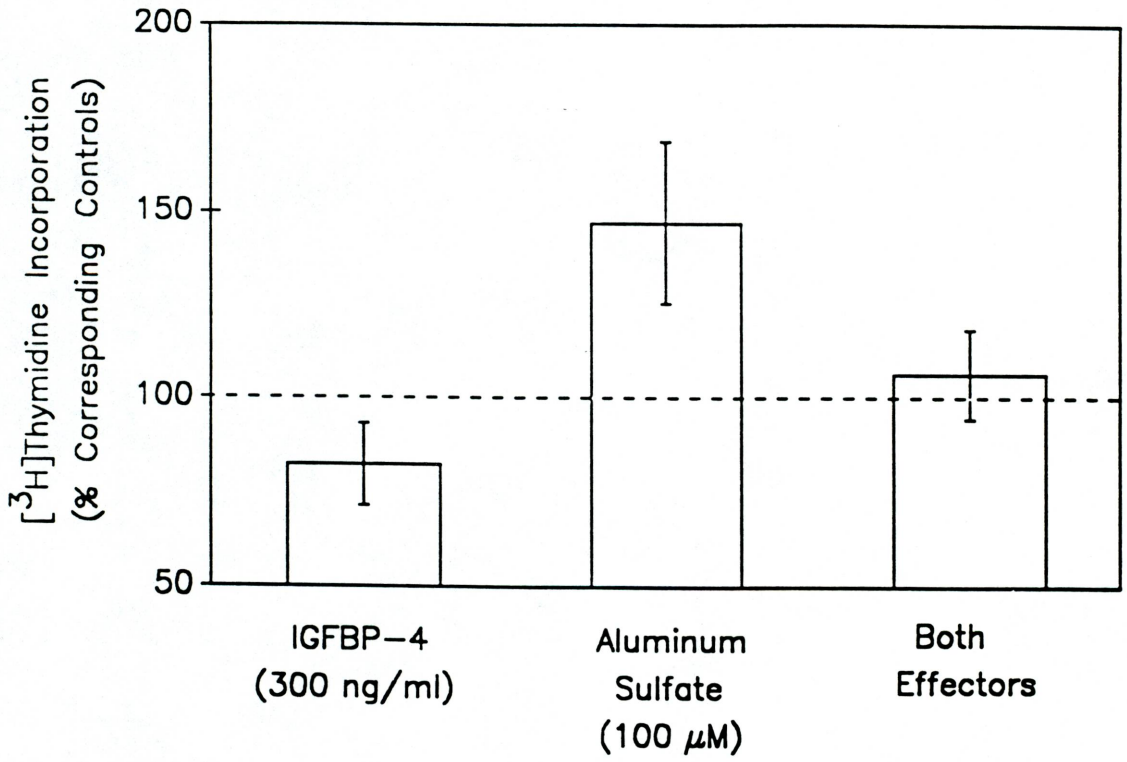
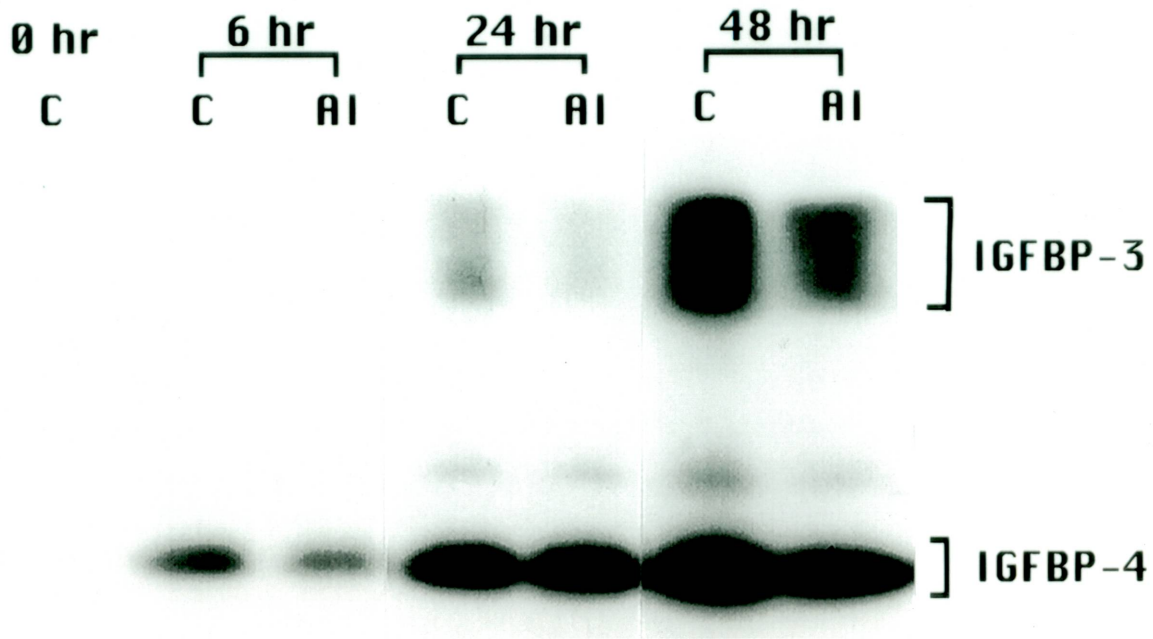

200
150
100
50
[3H]Thymidine Incorporation
(% Corresponding Controls)
IGFBP–4
(300 ng/ml)
Aluminum
Sulfate
(100 μM)
Both
Effectors

Figure 11. Effect of aluminum ion on the secretion of IGFBPs into CM by TE85 cells. The procedure for the IGFBP assay is described in the Methods. This figure shows the autoradiograph of the Western ligand blot. Laser densitometric analyses indicate that the reduction of IGFBP-3 level induced by 50 μM aluminum at 6-, 24-, and 48-hour incubation was 24%, 36%, and 34%, respectively. The reduction of IGFBP-4 level induced by aluminum at 6-, 24-, and 48-hour incubation was 21%, 25%, and 25%, respectively. This experiment has been repeated twice with similar results. These cells also secreted an identified IGFBP with an apparent molecular size slightly larger than that of IGFBP-4.

Effects of Aluminum ion on IGFBP production by Human Bone Cells



observed within the first 6-hours of exposure and remained evident even after a 48-hour treatment. Similar results were obtained in two repetitive experiments.

CHAPTER 4: DISCUSSION

The present study demonstrates that low concentrations, in the micromolar range, of aluminum stimulated the proliferation, measured by the incorporation of [^{3}H]thymidine into cellular DNA and differentiation, determined by increased cellular alkaline phosphatase specific activity, a recognized marker of osteoblastic differentiation (149), in osteoblast-like cells derived from chicken or human bone. The stimulations were highly reproducible (*i.e.*, observed in every experiment), but the extent of stimulation and the optimal dose of aluminum 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 only 150% of control in others. The optimal doses of aluminum in various experiments also differed from 10 to 75 μM. The cause for this variation is unknown. However, 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. These experiments did not take into account the possibility of contaminating aluminum as a secondary determinant of the actual concentration of aluminum in our experiments.

My findings were consistent with previous studies (48,49) which showed that the mitogenic action of aluminum, unlike that of fluoride (45,95,100), was not specific for bone cells. Since the mitogenic concentrations of aluminum also influenced the 1,25 dihydroxyvitamin D_3-dependent stimulation of osteocalcin secretion, an index of osteoblastic function (150), by human osteoblasts and these concentrations of aluminum stimulated collagen synthesis by human osteoblast-line cells, the results of my studies provide strong evidence that aluminum can directly affect the activity of human osteoblast-line cells.

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 (85,86). G proteins have been implicated to be key regulators of cellular signal transduction mechanism (151), an important initial step in the cell proliferation and differentiation processes. However, my results indicated that mitogenic concentrations of aluminum did not stimulate cAMP production in TE-85 cells. 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 (152). In contrast, this study showed that aluminum, not only did not inhibit, but significantly enhanced the 1,25 dihydroxyvitamin D_3-dependent stimulation of osteocalcin production by normal human osteoblasts. Hence, these findings argue against the involvement of G proteins in the osteogenic action of aluminum. It should, however, be emphasized that G proteins can also have other functions, *e.g.*, activation of phospholipase C, protein kinase C, and the phosphoinositide pathway (68), and that aluminum has been shown to stimulate phosphoinositide breakdown in fibroblasts (87). In addition, a recent preliminary report using mouse MC3T3-E1 cells suggested that the mitogenic action of aluminum might involve protein kinase C (50). Thus, we cannot dismiss the possibility that the osteogenic action of aluminum may involve G proteins that do not effect cAMP production.

One mechanistic possibility was also examined that aluminum and fluoride could act through the same mechanism to stimulate the proliferation and differentiation of osteoblasts. In this regard, Lau and Farley have previously demonstrated that the mitogenic actions of fluoride and another transition state analog of phosphate, vanadate, require the presence of a growth factor whose receptor is a tyrosyl protein kinase (100,101,108). Two observations indicated that the mitogenic action of fluoride also required that a growth factor be present: (a) the removal of endogenous growth factors by replacing the conditioned medium with fresh unconditioned medium completely abolished the mitogenic effects of fluoride (101,108); 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 (101,108). My findings that the osteogenic effects of aluminum ion could not be abolished by medium change, indicated that the actions of aluminum, unlike that of fluoride, were independent of bone cell growth factors. The 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 fluoride did not appreciably alter the dose dependent curve of aluminum stimulated 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 steps. Based on these findings, I tentatively conclude that aluminum does not exert its osteogenic actions by inhibiting osteoblastic phosphotyrosyl protein phosphatase activity.

My data clearly showed that aluminum ion also had direct effects on human TE85 osteosarcoma cells, at concentrations that stimulated human bone cell proliferation to: (a) increase the secretion of the IGF-I and -II, in a dose-dependent, biphasic manner, and (b) inhibit the secretion of IGFBPs, especially the inhibitory binding protein IGFBP-4. These effects were reproducible, as similar results were attained in repetitive experiments. The conclusion that mitogenic concentrations of aluminum increased IGF production was further supported by the findings that aluminum increased the steady state level of IGF-I mRNA in TE85 cells during a 24-hour incubation (153). Together, these findings are consistent with the interpretation that mitogenic doses of aluminum ion have significant effects on at least two components of the IGF regulatory system (*i.e.*, the IGFs and the IGFBPs) in human TE85 osteosarcoma cells.

Recent findings from several laboratories, including our own, indicated that bone-derived growth factors, especially IGF-II, play an important regulatory role in human bone cell proliferation and/or differentiation (120). My findings that mitogenic concentrations of aluminum ion increased the synthesis and secretion of IGF-II, therefore, suggest an interesting possibility that the mitogenic activity of aluminum ion on human bone cells could be mediated through the stimulation of the local synthesis and release of IGF-II by bone cells to serve as autocrine/paracrine agents. If this hypothesis is correct, it follows that an inhibition of the action of IGF-II should abolish the aluminum ion-dependent stimulation in cell proliferation of the human bone cells. Ideally, one would like to test whether addition of a specific inhibitory antibody for IGF-II would effectively block the aluminum ion-dependent stimulations of bone cell proliferation. Unfortunately, specific inhibitory antibodies for IGF-II are currently unavailable. However, our laboratory has recently purified an inhibitory IGF binding protein (*i.e.*, IGFBP-4), which specifically inhibits the mitogenic activity of both IGF-I and -II (131). Accordingly, if our hypothesis that the mitogenic action of aluminum ion is mediated via local production of IGFs is correct, addition of an inhibitory dose of IGFBP-4 in the medium should also inhibit the mitogenic activity of aluminum ion on bone cells; and, consistent with this expectation, my data clearly showed that the stimulation of [^{3}H]thymidine incorporation induced by a mitogenic concentration of aluminum was blocked by an inhibitory dose of IGFBP-4. In addition, a previous study indicated that the effect of aluminum ion on the proliferation of mouse MC3T3-E1 cells was to induce cells to enter the S phase from the G_0 phase (50). Since it has been known that the action of growth factors to stimulate cell proliferation is also mediated by an induction of the G_0 to S transition, these observations are also consistent with our hypothesis that the mitogenic action of aluminum might be mediated through the local production of growth factors, *e.g.*, IGFs.

Recent evidence indicates that IGFBPs may play an important regulatory role in modulating the local activity of IGFs (120). Of particular interest to my studies of human bone cell proliferation is the finding that the level of IGFBP-4 in the CM of human bone cells correlated negatively with bone cell proliferation rate (154). The results of my studies showed that mitogenic doses of aluminum ion, in addition to increasing the production and secretion of IGFs, also reduced the secretion of IGFBP-4 into CM by human osteoblasts. This inhibitory effect of aluminum ion on IGFBP-4 synthesis/secretion can be seen within the first 6 hours of treatment with aluminum ion, suggesting that this effect is probably a direct action of aluminum. Accordingly, we now hypothesize that treatments of human osteoblastic cells with aluminum ion will, not only increase the levels of the IGFs, but will also reduce the concentration of inhibitory IGFBP-4 in the CM. Together, these two actions of aluminum ion could provide a mechanism to increase the bioavailability of IGFs and thus allow for the indirect induction of a mitogenic signal by aluminum ion. Although my studies indicated that human TE85 osteosarcoma cells also produce and secrete IGFBP-3, and that treatment of TE85 cells with aluminum reduced the concentration of IGFBP-3 in the CM, we have not included that effect in our working hypothesis for the mitogenic responce of TE85 cell to aluminum, because the *in vitro* effect of IGFBP-3 on IGF activity has been shown to vary with changes in culture conditions (120). Hence, the significance of the reduction of IGFBP-3 secretion by aluminum ion is not clear at this time, because the physiological function of IGFBP-3 in human bone cells is yet to be determined.

To further assess our hypothesis that the action of aluminum to increase human osteoblastic-line cell proliferation was mediated by an increase in the effective activity of locally synthesized IGFs, the dose-dependencies for the mitogenic activity of aluminum and the aluminum-dependent stimulations of IGF secretion were compared. The result of my studies showed that the dose-responce curve for aluminum-stimulated mitogenic activity correlated more closely with the stimulation of IGF-II secretion than

with the stimulation of IGF-I (Figs. 4 and 9). In addition, TE85 cells synthesize mostly IGF-II and very little IGF-I. Thus, my data lead me to hypothesize that IGF-II, most likely mediates the mitogenic activity of aluminum in human TE85 osteosarcoma cells.

Regardless of whether IGF-I or IGF-II is the agent that mediates the effect, the most significant aspect of my studies was finding that the mitogenic action of aluminum ion is, at least in part, mediated by local bone cell production of IGFs. If my hypothesis that the mitogenic action of aluminum is mediated through IGF-II is correct, it would be more accurate to speak of aluminum ion as an effector of bone cell proliferation, but not a bone cell mitogen *per se*. The action of aluminum is not to directly stimulate mitosis, but rather to stimulate the production of IGF-II, and further enhance the cellular activity of IGF-II reducing the level of IGFBP-4. At the present time, the mechanism by which aluminum stimulates the production and secretion of IGFs and inhibits the secretion of IGFBP-4 is unknown. However, it should be noted that I did not evaluate whether aluminum ion also increases the synthesis and production of other bone-derived growth factors (*e.g.*, TGFß), so that I do not know if other growth factors might also be involved. Nevertheless, my findings are consistent with the premise that the mitogenic action of aluminum ion on human TE85 cells is, at least in part, mediated through effects on the IGF system.

While the findings described in this thesis suggest that the mitogenic activity of aluminum could not be attributed to its counter ions, we can not dismiss the possibility that other group IIIA metals, similar to aluminum, may be able to stimulate bone cell proliferation. Indeed, preliminary studies by Lau indicated that boron, gallium, and indium, like aluminum, all significantly stimulated [^{3}H]thymidine incorporation and increased alkaline phosphatase specific activity in human TE85 osteosarcoma cells; whereas type IIIB metals, (*e.g.*, lanthanum), did not have the same effect (Lau, unpublished observation). However, it is not clear as to whether the mitogenic action of other type IIIA metal is also mediated through a stimulation of IGF production by

bone cells.

The physiologic significance of my findings that micromolar aluminum ion can be mitogenic for bone cells is unclear at the present time. In this respect, the serum concentration of aluminum is normally very low. Even in the case of renal failure patients treated with aluminum, the serum aluminum concentrations are usually between 5-30 μM (1,4-6,18), which are lower than the *in vitro* effective doses. On the other hand, because of the high affinity of aluminum ion for bone mineral, the local concentrations of aluminum in bone could be somewhat higher. For example, the bone aluminum content in normal individuals was determined to be 2.4 ± 1.2 mg/kg dry bone (26). Assuming that the extracellular fluid in bone constitutes 50% of the bone weight, this amount of aluminum ion would be equal to approximately 100 ± 50 μM, which would be well within the *in vitro* osteogenic doses of aluminum ion. This speculation is consistent with the finding that administration of aluminum ion to normal Beagle dogs induced *de novo* bone formation *in vivo* (42-44). However, in the chronic renal failure patients, the bone aluminum content is very high [*i.e.*, it could be as high as 175 mg/kg dry bone weight (26)]. This amount of bone aluminum is roughly equivalent to 10 mM, which is approximately 100-fold higher than the *in vitro* osteogenic dose of aluminum, and was inhibitory in my studies. Accordingly, it can be postulated that while low doses of aluminum could stimulate bone formation, the accumulation of high bone aluminum concentrations could be toxic and may lead to the development of osteomalacia (17,18,23-27).

CHAPTER 5: LITERATURE CITED

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