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Effects of Fluoride on the Osteoblastic Activities and Osteoclast Generation *In Vitro*

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In an effort to assess the effects of F on overall bone metabolism, cultures of osteoblastic cells isolated from fetal rat calvaria for bone formation *in vitro*, and of mouse bone marrow cells to generate osteoclast-like cells were conducted. Considerable number of mineralized nodules was developed in osteoblastic cell cultures maintained in the presence of ascorbic acid and β-glycerophosphate up to 21 days. The number of mineralized nodules was not increased significantly by continuous treatment of cultures with 10 μM NaF, a clinically effective osteogenic concentration. When cultures were treated with pulses of 48 hr-duration before apparent mineralization was occurring, 1.8-fold increase in their number was detected. Addition of 10 μM NaF with onset of mineralization exhibited an inhibitory tendency on the formation of mineralized nodules. In the subsequent study, NaF stimulated [^{3}H]thymidine incorporation in osteoblastic cells both under serum-supplemented and serum-free conditions. Alkaline phosphatase activity of osteoblastic cells was not changed by NaF over the concentration ranges examined. Rather a dose-dependent inhibition appeared with extension of treatment duration. Osteoclast-like cell generation in cultures of mouse bone marrow cells induced by PGE_2 and PGE_2 plus IL-6 was significantly inhibited by NaF at the relatively higher concentrations, but not at 10 μM. And IL-6 production in osteoblastic cells was also decreased by NaF only at higher concentrations. These results taken together suggest that F enhances bone formation and the stimulatory effect of F on the proliferation of osteoblastic cells is probably most relevant to its mechanism underlying augmented bone formation.

Key words : fluoride, bone formation, osteoblast, osteoclast generation

Introduction

Fluoride (F) has been used to treat the patients with metabolic bone disease for more than 30 years, since Rich and Ensinck (1961) first introduced the use of sodium fluoride in the treatment of postmenopausal women suffering from primary osteoporosis with vertebral crush fractures. Although many therapeutic agents have been investigated thereafter, clinical studies demonstrated that F is the single most effective agent currently available for increasing bone volume and decreasing fracture frequency in osteoporotic skeleton (Briancon and Meunier, 1981; Riggs *et al.*, 1994). F was initially used to treat osteoporosis because it might decrease bone resorption by reducing the solubility of apatite crystal (Grynpas, 1990). However, subsequent studies have shown that augmentation of bone volume induced by F administration was attributed to an increased bone formation rather than a suppression of resorption. Its anabolic effect on bone has been documented in several clinical studies by demonstrating an increase in spinal bone mineral density (Riggs *et al.*, 1994) and by histomorphometric evidence of increased bone volume in bone biopsies (Bovin *et al.*, 1990). Animal studies have also shown that F increased the matrix apposition, osteoid surface and thickness, and the number of active osteoblasts (Turner *et al.*, 1989; Chavassieux, 1990). These *in vivo* findings suggest that F enhances osteoblastic activity at tissue level, though Harrison *et al.* (1990) described that F delayed mineralization in ectopic bone formation induced by implantation of demineralized cortical bone in rats. In contrast, the effect of F on the parameters associated with bone formation *in vitro* has been divergent. Farley *et al.* (1983, 1988, 1990) found that F at concentration similar to those active *in vivo*, increased [^{3}H] thymidine incorporation, cell number and alkaline phosphatase activity in isolated embryonic chick

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calvarial cells and organ cultures maintained in serum-free medium. Such stimulatory effects of F have been further demonstrated in cultures of several osteoblastic cells including human bone cells derived from trabecular explants (Khokher and Dandona, 1990), human bone marrow stromal cells (Kassem *et al.*, 1993) and osteosarcoma cells (Reed *et al.*, 1993). But conflicting findings have also been described. Chavassieux *et al.* (1993), and Kopp and Robey (1990a, b) observed no effect of F on the proliferation or total protein synthesis in cultured human bone cells prepared from either adult or fetal tissues. The reasons for the discrepancy concerning the effect of F on bone cell proliferation remain unclear.

Most of the previous works have focused on the possibility that F directly modulates the activity of bone cells, in particular that of osteoblast. From these studies, it is generally considered that augmentation of bone volume after treatment with F is attributed to an unbalanced coupling between resorption and formation in favor of formation. However, it has not yet been determined at which stage in osteoblast development F is acting, or whether its principal mode of action is upon stimulation of proliferation or differentiation of osteoblast. On the other hand, its effect on the osteoclastic bone resorption cannot be excluded since bone volume *in vivo* is maintained by remodeling process in which initial resorptive phase is closely linked to ensuing formative phase. While limited information is available regarding this issue, Okuda *et al.*, (1990) reported inhibitory effect of F on the number and activity of isolated osteoclasts. These findings suggested that observed net increase of bone mass *in vivo* may involve the effects of F on the generation and activity of osteoclasts as well as of osteoblasts.

Recently, model systems for *in vitro* bone formation in cultures of osteoblastic cells, and for the generation of osteoclasts in bone marrow cell cultures have been established. In one of these systems, enzymatically isolated fetal rat osteoblastic cells maintained up to 21 days in the presence of ascorbic acid and β-glycerophosphate, formed discrete three-dimensional mineralized nodules (Bellow *et al.*, 1986; An *et al.*, 1989; Kim *et al.*, 1990). Stein and co-workers (Dworrezky *et al.*, 1990; Lian *et al.*, 1992; Lian and Stein, 1993) have characterized this model system further with respect to the developmental sequence of osteoblasts. They have proposed that fetal rat calvarial osteoblasts *in vitro* undergo developmental sequence of growth and differentiation that can be divided into three distinct stages-proliferation, matrix maturation and mineralization-according to the temporal expression of genes encoding osteoblast phenotype makers. And osteoclast-like (OC-like) cells retaining the characteristics of osteoclast such as multinuclearity and positive staining for tartrate-resistant acid phosphatase (TRAP) could be generated in cultures of mouse bone marrow cells when treated with prostaglandin E_2 (PGE_2) or with 1,25-dihydroxycholecalciferol (Ko *et al.*, 1990; Lee *et al.*, 1992). The present study was therefore undertaken to investigate the effects of F on the bone formation and osteoclast generation *in vitro* employing these systems. Effects of F on the proliferation and phosphatase activities in fetal rat osteoblastic cells were also assessed in an effort to determine the mechanism whereby F stimulates bone formation.

Materials and Methods

Materials

Media, fetal bovine serum (FBS) and other cultural reagents were obtained from Gibco laboratories (Grand Island, NY, USA) and plastic culture wares from Nunc (RosKlide, Denmark). [^{3}H] thymidine was purchased from Amersham (Arlington Heights, IL, USA). Recombinant human interleukin-1β (IL-1β), recombinant human tumor necrosis factor-α (TNF-α), and enzyme-linked immunosorbent assay (ELISA) kit for human interleukin-6 (IL-6) were obtained from Genzyme (Cambridge, MA, USA). NaF, PGE_2 and all other reagents were, unless otherwise stated, purchased from Sigma (St. Louis, MO, USA).

Isolation and cultures of osteoblastic cells

Calvaria from fetal rat (Sprague-Dawley) at 19 days of gestation were dissected and subjected to sequential enzyme digestion protocol as previously described (An *et al.*, 1989; Kim *et al.*, 1990). Briefly, fetal frontal and parietal bone were digested consecutively five times with enzyme mixture containing 0.1% collagenase, 0.05% trypsin and 0.5 mM EDTA for predetermined periods. Immediately after digestion, isolated cells were washed with Hank's balanced salt solution two times and viable cells were counted with

trypan blue exclusion method. Calvarial cells, released in later stages, which exhibit osteoblastic characteristics, were pooled, plated in α-minimum essential medium (α-MEM) supplemented with 10% FBS, and incubated at 37°C in 95% humidified air plus 5% CO_2.

Bone formation in vitro

Fetal osteoblastic cells were plated at 5×10^4 cells/35 mm dish in α-MEM supplemented with 15% FBS, 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate. Media were changed every second or third day and cultures were maintained up to 21 days in the presence of 10 μM NaF. In some experiments, cells were pulsed with 10 μM NaF only for 48 hrs on the days indicated instead of its continuous presence during the culture periods. To initiate mineralization at desired time, cells were maintained in medium without β-glycerophosphate and supplemented it alone or in combination with F thereafter. At the end of cultures, cell layer was fixed with neutral buffered formalin and stained *in situ* by the von Kossa technique for mineral deposits. Numbers of mineralized nodules were then counted at 40X magnification using a dissecting microscope.

Cell proliferation

As an index of cell proliferation, DNA synthesis rate was evaluated by measuring the incorporation of [^{3}H]thymidine into trichloroacetic acid (TCA)-insoluble fraction. Osteoblastic cells, maintained at density of 1×10^4 cells/well in 24-well plates were treated with NaF for 24 hrs under serum-free or serum-supplemented conditions. During the final 4 hrs, cells were pulsed with 5 μCi of [^{3}H] thymidine and incorporation was terminated by aspiration of medium. Cells were then fixed for 10 min at 4°C and washed 4 times with ice-cold 5% TCA. Precipitated radioactivity was solubilized in 0.5 M NaOH and measured with liquid scintillation counter.

Alkaline and acid phosphatase activity

Phosphatase activity was measured by spectrophotometry using *p*-nitrophenyl phosphate (15 mM) as a substrate. NaF-treated cells were collected in 0.5 ml distilled water and sonicated. Aliquot of the cell homogenate was incubated at 37°C for 30 min in 0.1 M glycine-NaOH buffer (pH 10.3) for alkaline phosphatase or in 0.1 M sodium citrate buffer (pH 4.8) for acid phosphatase, respectively. The optical density of *p*-nitrophenol, a reaction product, was read at 410 nm. Total protein content in cell homogenate was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

Osteoblastic IL-6 production

Osteoblastic MG-63 cells (purchased from American Type Culture Collection), human osteosarcoma-derived cell line, were subcultured into 35 mm tissue culture dishes at 2.6×10^5 cells/dish in MEM supplemented with 10% FBS. The cells were allowed to attach for 24 hrs and then washed with Hank's balanced salt solution and placed in fresh serum-free medium. After 24 hrs, the cells were treated with 1.0 ng/ml of IL-1β, 20 ng/ml of TNF-α or 10^{-6} M PGE_2 alone and in combination of F for 48 hrs. Each treatment was quadruplicated. The conditioned media (1.5 ml/dish) were collected, and used for IL-6 assay. Cell layers were washed with PBS, lysed in 0.5 ml of 0.1 N NaOH and used to determine the protein concentration using BCA (bicinchoninic acid) protein assay reagents. The assay for IL-6 was performed using ELISA. Recombinant human IL-6 was used as a standard and all assays were performed in duplicate according to protocol provided with the kit. Briefly described, 96-well microtiter plate was coated with 6 μg/ml of capture antibody overnight, washed, and blocked with 4% bovine serum albumin in PBS for 2 hrs. Then, the plate was incubated with diluted IL-6 standard and samples for 1 hr, washed, incubated with 0.6 μg/ml of biotinylated second antibody for 1 hr, washed, and incubated with horseradish peroxidase conjugated streptavidin for 15 min. Finally it was incubated in tetramethylbenzidine substrate solution for 10 min and the reaction was stopped by adding 1 M H_2SO_4. ELISA plate was read by a Titertek Multiskan Plus (Flow Laboratories, VA, USA). The data were corrected for mg protein of cells and expressed as the amount of IL-6 (ng) per mg protein.

Mouse bone marrow cell culture

Four to six-week-old mice (ICR) were sacrificed by cervical dislocation, and tibiae and femurs were aseptically removed and dissected free from adhering tissues. The bone ends were cut and marrow was flushed with 1 ml of α-MEM using 25-

gauge needle. The collected marrow cells were washed twice and cultured in 0.5 ml of α-MEM supplemented with 10% FBS at 1.5×10^6 cells/well in 24-well plates. Medium was replaced every third day, and cultures were maintained for 8 days.

Osteoclast generation in vitro

Mouse marrow cells were cultured in the presence of 10^{-6} M PGE_2 or 100 ng/ml IL-6 to generate OC-like cells. Varying concentration of NaF was further added to medium and maintained throughout the culture period to assess its effect on osteoclast generation. After cultures, cells adherent to the well surface were rinsed with phosphate-buffered saline (pH 7.4), fixed with ethanol-acetone (50:50, v/v) for 1 min, dried at room temperature for 10 min, and stained for TRAP, a marker enzyme of osteoclast. Staining for TRAP was performed as described (Ko *et al.*, 1990; Lee *et al.*, 1992). In brief, fixed cells were incubated for 20 min at room temperature in an acetic buffer (0.1 M sodium acetate, pH 5.0) containing naphthol AS-MX phosphate as a substrate, and fast red violet LB salt as a stain for the reaction product in the presence of 20 mM sodium tartrate. TRAP-positive cells appeared as dark red cells. Number of TRAP-positive, multinucleated OC-like cells which contain 3 or more nuclei were scored under light microscope.

Results

Effect of NaF on Mineralized Nodule Formation

At the beginning of the culture, fetal rat calvarial cells exhibited spindle-shaped appearance but became polygonal, and multilayered, three dimensional nodular structure was observed around day 7. When calvarial cells were cultured with ascorbic acid and β-glycerophosphate, considerable number of mineralized nodules was developed after 21 days. The number of mineralized nodules was not changed significantly by the continuous presence of 10 μM NaF during the whole culture period. In contrast to this, pulsed NaF treatment enhanced the formation of mineralized nodules (Table 1). As shown in Fig. 1, their number was increased to the extent of 1.8-fold greater than that of control group by pulse of 10 μM NaF for 48 hrs at day 0, 7 and 14. Cultures maintained in the presence of ascorbic acid for 15 days but without β-glycerophosphate failed to develop mineralized nodules.

Table 1. Effect of NaF on the formation of mineralized nodules in cultures of fetal rat osteoblastic cells[a]

NaF	Treatment	Number of Mineralized Nodules
None	–	145±49
10 μM	Continuous (day 0-21)	168±21
10 μM	48hr-pulse at day 0	268±18*
10 μM	48hr-pulse at day 7	264±27*
10 μM	48hr-pulse at day 14	263±21*

[a] Fetal rat calvarial cells were cultured in the presence of 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate for 21 days.

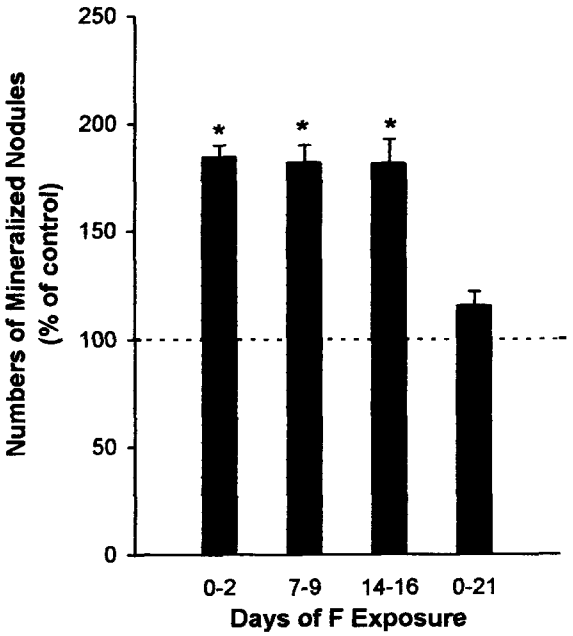

Fig. 1. Effect of continuous or pulsed exposure of fetal rat osteoblastic cells to 10 μM NaF on mineralized nodule formation. Data represent mean±S.E. (N=5) and expressed as the ratio to the corresponding mean of control. *Significanty different from control, $p<0.05$

When β-glycerophosphate was added to these cultures, mineralization of nodules was restored. Simultaneous addition of 10 μM NaF at that time and maintaining its concentration by the end of cultures, reduced the number of mineralized nodules although such an inhibition was not significant statistically (Table 2).

Effect of NaF on osteoblastic cell proliferation

NaF stimulated proliferation of fetal rat calvarial cells as demonstrated by [^{3}H]thymidine incorporation assay. At the concentration of 10 μM under serum-free condition, NaF produced a modest stimulatory effect which was not statistically significant. Higher concentration ranging from 100 to 500 μM increased [^{3}H]thymidine incorporation

Table 2. Effect of simultaneous addition of NaF and β-glycerophosphate at day 15 on the mineralized nodule formation in fetal rat osteoblastic cell cultures[a]

NaF	β-glycerophosphate	Number of Mineralized Nodules
None	10 mM	100.0±6.1
10 μM	10 mM	85.2±3.0

[a] Cultures were maintained in the presence of 50 μg/ml ascorbic acid for 15 days but without β-glycerophosphate, and then β-glycerophosphate was added to initiate mineralization of nodules thereafter. 10 M NaF was added simultaneously and maintained during the remaining culture period (21 days). Data represent mean±S.E. (N=5).

with approximately 20-30% magnitude when compared with control.

In contrast, maximal stimulatory effect was noted at 10 μM NaF over the range of concentration examined when calvarial cells were maintained in serum-supplemented medium (Fig. 2).

Effect of NaF on phosphatase activity in osteoblastic cells

No significant changes in both of alkaline and acid phosphatase activity were observed when fetal calvarial cells were treated with various concentrations of NaF for 48 hrs under serum-free condition. In the case of alkaline phosphatase, similar negative responses were noted under different experimental conditions; treatment of cultures for 24 hrs or at higher cell density, 5×10^5 cells/35 mm dish.

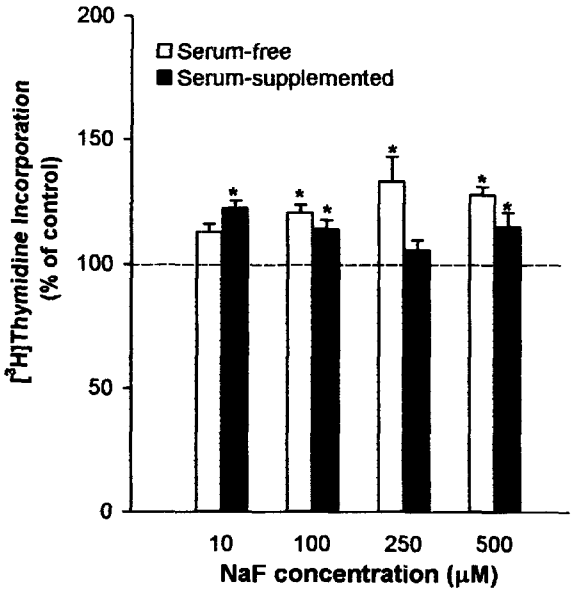

Fig. 2. Effect of NaF on [^{3}H]thymidine incorporation in cultures of fetal rat osteoblastic cells. Data represent mean±S.E. (N=5) and expressed as the ratio to the corresponding mean of control. *Significantly different from control, p<0.05.

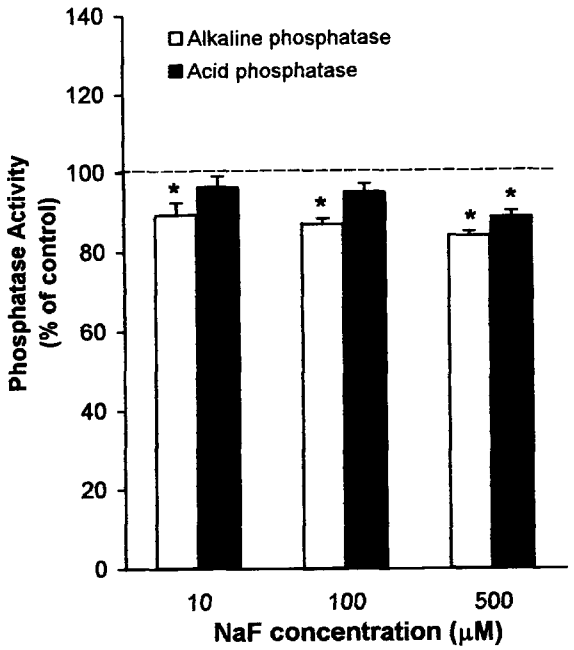

Fig. 3. Changes in phosphatase activity in fetal rat osteoblastic cells induced by NaF treatment for 6 days in the presence of serum. Data represent mean±S.E. (N= 3-4) and expressed as the ratio to the corresponding mean of control. *Significantly different from control, p<0.05.

Alkaline phosphatase activity was however reduced in dose-dependent manner by NaF treatment for 6 days in the presence of serum. Under these conditions, acid phosphatase activity was also inhibited parallel to the NaF concentration and significant reduction was observed at 500 μM (Fig. 3).

Effect of NaF on osteoblastic IL-6 production

Basal level of IL-6 production in MG-63 cells was undetectable, but its production was induced by TNF-α and IL-1β. TNF-α-induced IL-6 production was significantly inhibited by 100 μM NaF while lower concentration had no effects. However IL-1β-induced production was not inhibited even at 100 μM concentration (Fig. 4). PGE_2 or PGE_2 plus NaF did not affect IL-6 production of MG-63 cells at all.

Effect of NaF on osteoclast-like cell generation

Approximately 60 TRAP-positive multinuclear cells (MNC) were generated by the addition of 10^{-6} M PGE_2 in mouse bone marrow cell cultures. IL-6 did not induce the TRAP-positive MNC generation

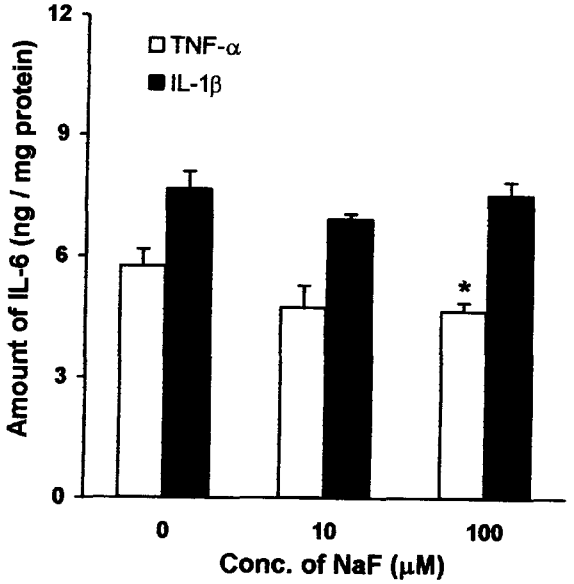

Fig. 4. Effect of NaF on IL-6 production in osteoblastic MG-63 cells. MG-63 cells were treated with TNF-α (20 ng/ml) or IL-1β (1 ng/ml) for 48 hrs. Basal level of IL-6 production was undetectable. Data represent mean±S.E. (N=4). *Significantly different from corresponding control, $p<0.05$.

in itself, but augmented the inductive effect of PGE_2. NaF, at the concentration of 10 μM, did not affect the generation of TRAP-positive MNC. However higher concentration of NaF, 100 and 500 μM, significantly suppressed the OC-like cell generation induced by PGE_2 as well as by PGE_2 plus IL-6 (Fig. 5).

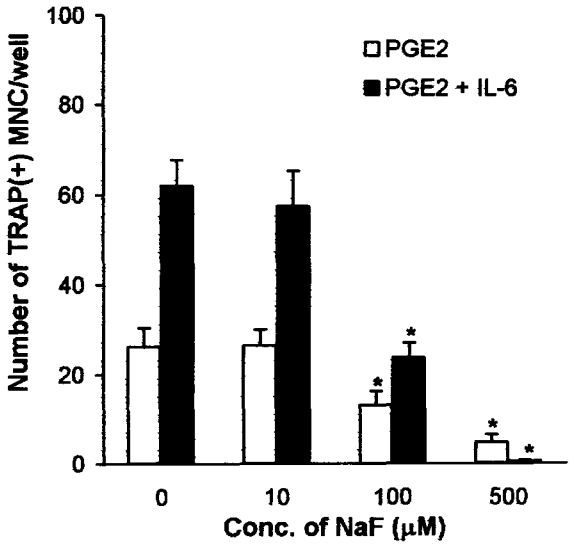

Fig. 5. Effect of NaF on PGE_2- and PGE_2 plus IL-6-induced osteoclast-like cell generation in mouse bone marrow cultures. Mouse bone marrow cells were cultured in the presence of PGE_2 (10^{-6} M) alone or in combination of PGE_2 and IL-6 (100 ng/ml). IL-6 alone had no inducing effect on osteoclast-like cell formation. Data represent mean±S.E. (N=4). *Significantly different from corresponding control, $p<0.05$

Discussion

In the present study, fetal rat osteoblastic cells maintained in the presence of ascorbic acid and β-glycerophosphate for 21 days developed von Kossa-positive mineralized nodules. But their numbers were not changed significantly by the continuous exposure of cultures to 10 μM NaF throughout the culture period. This finding was consistent with the observations of Bellow *et al.* (1990) describing the stimulatory effect of F on the mineralized nodule formation at 500 μM, but no significant effect at clinically effective concentration (5-30 μM). Thus possible enhancing effect of NaF on the bone formation *in vitro* was assessed with different strategy and to define the mechanism whereby F stimulates bone formation, if any. For this Osteoblastic cells were pulsed for 48 hrs with 10 μM NaF at particular time point that is roughly corresponding to the distinct stage in their developmental sequence of growth and differentiation as defined previously (Dworresky *et al.*, 1990; Lian *et al.*, 1992; Lian and Stein, 1993). When fetal rat osteoblastic cells were exposed to pulses of 48 hr-duration with 10 μM NaF on the day 0, 7 or 14 in the present study, approximately 1.8 fold increase in their number of mineralized nodules was detected in all situations contrasting the insignificant effect produced by continuous treatment. These findings support an interpretation that F-induced increase in bone mass seen *in vivo* may be a result of stimulated bone formation. The current data also reflect that stimulatory effect of F is its specific action depending on the differentiated state of target cells rather than a generalized one over the several stages in developmental sequence of osteoblast lineage. And the significant difference appeared between pulse on day 14 (prior to typical mineralization period) and continuous treatment (including mineralization period) with NaF led to speculate that effect on mineralization process may not be involved in its stimulatory action. To provide the evidence for this assumption, β-glycerophosphate was added on day 15 to the cultures maintained in the presence of ascorbic acid but without it during the preceding culture

period to initiate mineralization thereafter. Simultaneous addition of 10 μM NaF along with β-glycerophosphate on day 15 and maintaining its concentration throughout the remaining culture period revealed an inhibitory tendency on the formation of mineralized nodules. This result indicates that effect of F on the mineralization itself is not critical in F-stimulated bone formation *in vitro*. And such a result was further confirmed in a subsequent study-^{45}Ca uptake by mineralizing nodules was significantly inhibited by simultaneous addition of 10, 100 and 500 μM NaF to the extent of 79, 74, and 74% when compared to control. It was shown that initiation phase of mineralization *in vitro* was inhibited over a concentration range of 25-500 μM NaF while unaffected if F was removed before initiation (Bellow *et al.*, 1993). Findings demonstrated in this study fully agree with these observations and suggest that F stimulates bone formation by preferentially affecting the earlier stages in developmental sequence of osteoblasts.

Effects of F on the proliferation and alkaline phosphatase activity of osteoblastic cells were therefore examined. NaF increased [^{3}H]thymidine incorporation in calvarial cell cultures under serum-free and serum-supplemented condition. And at clinically effective concentration (10 μM), more significant stimulation was further noted when serum was supplemented. Contrary to the stimulatory effect seen on the proliferation, NaF treatment for 48 hrs under serum-free condition did not induce any significant changes on alkaline phosphatase activity. With extended 6 day-treatment in the presence of serum, a rather inhibitory effect in dose-dependent manner was observed in present study. Therefore, it seems more likely that stimulated proliferation of osteoblastic cells attributed to the F-induced increase in the formation of mineralized nodules by 48 hr-pulse on day 7 and even on day 14. This interpretation can be reconciled with previous findings in several ways; i) fetal rat calvarial cells in cultures still actively proliferate on day 12 (Lian *et al.*, 1992), ii) serum is required for F-induced osteogenesis (Hall, 1987), iii) F-stimulated [^{3}H] thymidine incorporation is dependent on the presence of bone cell mitogen and is inversely correlated with alkaline phosphatase activity, and the *in vitro* mitogenic action of F is greater in less-differentiated osteoprogenitor cell cultures than in alkaline phosphatase-rich, diffentiated osteoblast cell cultures (Farley *et al.*, 1988, 1990; Kassem *et al.*, 1993), iv) fetal rat calvarial cell population used in this study comprise a heterogeneous mixture of cells at various stage of osteoblast differentiation including osteoprogenitors, although they express osteoblastic properties (Woo *et al.*, 1987; Bellow and Aubin, 1990).

The molecular mechanism underlying the mitogenic action of F on osteoblastic cells is unknown. Regarding this, Lau *et al.* (1989) postulated that inhibition of osteoblastic acid phosphatase/phosphotyrosyl protein phosphatase activity can be a biochemical mechanism for mitogenic action of F. Effect of NaF on the acid phosphatase activity was examined in this context, but positive finding was not observed. Apparent inhibition was detectable only by treating calvarial cells with 500 μM NaF for 6 days. Recently, Caverzasio *et al.* (1997) suggested that mitogenic effect of F may be mediated by tyrosine kinase activation, not by inhibition of tyrosine phosphatase. They found that F enhances tyrosyl phosphorylation process in osteoblastic cells without any detectable inhibition of tyrosine phosphatase activity. Further studies would be required to resolve this issue.

Although attention has been directed to the modulating effects of F on the ostoblastic activity, some investigators have suggested that anabolic effect of F on bone metabolism may be partially related to the inhibition of osteoclastic bone resorption (Snow and Anderson, 1986; Marie and Hott, 1986; Okuda *et al.*, 1990). To address this question, effect of NaF on the PGE_2-induced OC-like cell generation in mouse bone marrow cell cultures was assessed. No significant effects on the generation of OC-like cells were found at 10 μM NaF. While their numbers were decreased significantly with simultaneous addition of 100 and 500 μM NaF. To pursue the other possible contribution of F on osteoclast generation, its effects on OC-like cell generation induced by PGE_2 plus IL-6 as well as IL-6 production in osteoblastic MG-63 cells were then determined, since IL-6 is a well-known stimulator of osteoclast formation and involved in osteoporosis and chronic periodontal disease (Lowik *et al.*, 1989; Shin *et al.*, 1996). But similar insignificant results were observed again, suggesting that modulation of osteoclast formation is not so closely associated to its anabolic action.

In summary, the present study demonstrated that F enhances bone nodule formation in osteoblastic cultures. And the current results taken together suggest that stimulatory effect of F on the proliferation of osteoblast lineage cells is probably most relevant to its mechanism underlying the increased bone formation *in vitro* seen at the clinically effective concentration.

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