

## Interactions between Fibronectin and Osteoblast are Important in Osteoblast Differentiation and Mineralized Nodule Formation

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Fibronectin (FN), one of bone extracellular matrix (ECM), is detected in the periosteum and the synthesis of FN is sharply increased during the early stage of osteoblast differentiation. In addition, factors known to regulate osteoblast differentiation affect both FN expression and osteoblast attachment to FN. These reports suggest that FN may play an important role in osteoblast recruitment, differentiation, and ECM organization. In the present study, we investigated the possible role of FN in osteoblast differentiation and bone formation by observing the effects of disruption of FN-osteoblast interactions. To disrupt FN-osteoblast interactions, we added to culture media 120 kDa FN fragments (120FN) which contain central cell binding domain. In addition, we observed the effects of several inhibitors of possible signaling molecules that are known to be involved in integrin signalings and compared with the effect of FN-cell interaction disruption. 120FN reduced the expression levels of osteoblast differentiation associated genes such as alkaline phosphatase and osteocalcin and significantly suppressed mineralized nodule formation in fetal rat calvarial cell cultures. The majority of inhibitory effects seem to be occurred during early phase of osteoblast differentiation and nodule initiation, though 120 FN also delayed the nodule maturation. Among inhibitors of signaling pathways, cytochalasin B and genistein, inhibitors of actin filament rearrangement and tyrosine kinase, respectively, markedly inhibited mineralized nodule formation. Caffeic acid and aristolochic acid, inhibitors of 5-lipoxygenase and phospholipase A<sub>2</sub>, respectively, exhibit little inhibitory effect, if any. U73122 and NPC-15437, inhibitors of phospholipase C and protein kinase C, respectively, significantly delayed

nodule initiation and maturation, though the decrease in the number of mineralized nodules formed at the end of culture was not that much. These findings suggest that the interactions between osteoblasts and FN may play an important role in the regulation of progressive differentiation and finally mineralized nodule formation of osteoblasts. In addition, these results raise the possibility that cytoskeleton rearrangement together with tyrosine kinase activation may be the important part of signaling from FN-osteoblast interactions.

**Keywords:** Fibronectin-osteoblast interactions, Osteoblast differentiation, Mineralized nodule formation

### Introduction

Osteogenesis involves the recruitment of mesenchymal cells to the osteoblast lineage and their progressive differentiation to produce a mineralized extracellular matrix (ECM). Structural ECM proteins, local growth factors stored in the ECM, and ECM-degrading proteases and their inhibitors are all potential regulators of osteoblast differentiation and function. One ECM protein that may influence osteogenesis is fibronectin (FN). In chicken osteoblast culture, FN was synthesized and accumulated in the ECM during proliferation and early differentiation period and FN synthesis was reduced as cells matured (Winnard *et al.*, 1995). In rat calvarial osteoblast culture, expression of FN increased during the early stages of differentiation (Stein *et al.*, 1996). In addition, the hormones known to regulate osteoblast differentiation have been shown to affect both FN expression and osteoblast attachment to FN, suggesting a possible regulatory role of FN on osteoblast differentiation (Eielson *et al.*, 1994; Franceschi *et al.*, 1987; Shalhoub *et al.*, 1992).

FN is a heterodimeric glycoprotein that has several cell-

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and matrix-binding domains (reviewed in Yamada, 1991). Sequences in at least three different regions of FN bind to the cell surface via two classes of transmembrane receptors: integrins and cell surface proteoglycans. The arginine-glycine-aspartic acid (RGD) sequence, present in FN type III repeat (FNIII) 10 of the central cell binding domain, recognizes several members of the integrin family such as  $\alpha 5\beta 1$  and several  $\alpha v$ -containing integrins. The carboxy terminal heparin-binding region of FN (FNIII 11-14) contains several sequences that bind to transmembrane cell surface proteoglycans and low-affinity binding activity for the  $\alpha 4\beta 1$  integrin. Finally the variably spliced IIIc3 connecting sequence contains high-affinity binding sequence for the  $\alpha 4\beta 1$ .

Previous studies have demonstrated that cell interaction with FN could initiate signals that affected cytoskeletal organization, cell motility, tissue-specific gene expression, and matrix remodeling (Homandberg *et al.*, 1993; Huhtala *et al.*, 1995; Werb *et al.*, 1989). Integrins are likely to transduce those signals generated by FN. Osteoblasts are known to express integrin receptors for FN:  $\alpha 3\beta 1$ ,  $\alpha 4\beta 1$ ,  $\alpha 5\beta 1$  and  $\alpha v\beta 3$  (Clover *et al.*, 1992; Grzesik and Robey, 1994; Brighton and Albelda, 1992). Although the mechanisms by which FN-integrin interactions generate cell regulatory signals are not well understood, integrins have been shown to regulate various functions of cells as follows; i) the levels of the intracellular ions  $H^+$ ,  $Ca^{2+}$ , and  $K^+$ , ii) reorganization of the cytoskeleton, iii) lipid metabolism including lipid hydrolysis via activation of the phospholipase C (PLC) and phospholipase  $A_2$ , iv) lipoxygenase (LOX) and protein kinase C (PKC) activation, v) protein phosphorylation through activation of focal adhesion kinase and mitogen activated protein kinase, and vi) finally gene expression (reviewed in Schwartz *et al.*, 1995).

In the present study, we investigated the possible role of FN in osteoblast differentiation and bone formation by observing the effects of disruption of FN-osteoblast interactions. To disrupt FN-osteoblast interactions, we added to culture media 120 kDa FN fragments (120FN) which contain central cell binding domain. In addition, we observed the effects of several inhibitors of possible signaling molecules which are known to be involved in integrin signalings, such as cytoskeleton organization, tyrosine kinases, LOX,  $PLA_2$ , PLC and PKC and compared with the effect of FN-cell interaction disruption.

## Materials and Methods

### Materials

Media, fetal bovine serum (FBS), collagenase and other cultural reagents were obtained from Gibco laboratories (Grand Island, NY, USA) and plastic culture wares from Nunc (Roskilde, Denmark). [ $\alpha$ - $^{32}P$ ]-dCTP, and megaprime DNA labeling system were purchased from Amersham (Arlington Heights, IL, USA), TRI reagent from Molecular research center (Cincinnati, OH, USA), and ExpressHyb<sup>TM</sup> Hybridization

solution from CLONTECH laboratories (Palo Alto, CA, USA). 120FN was purchased from Gibco laboratories (Grand Island, NY, USA), U73122 and NPC-15437 from RBI (Natick, MA, USA), and aristolochic acid, caffeic acid, cytochalasin B, genistein and all other reagents were, unless otherwise stated, from Sigma (St. Louis, MO, USA).

### Cultures of osteoblastic cells

Osteoblastic cells were isolated from rat calvaria of 19th day of gestation (Sprague-Dawley) by sequential enzyme digestion protocol as previously described (An *et al.*, 1989). Cells were cultured in  $\alpha$ -minimum essential medium ( $\alpha$ -MEM) supplemented with 10% FBS, and incubated at 37°C in 95% humidified air plus 5%  $CO_2$ . Approximately 80% confluent primary cells were subcultured and assays were performed with cells of passage number 2.

### Treatment of inhibitors

To determine whether there is a functional role for FN-osteoblast interactions in the progressive differentiation of osteoblasts, 120FN consisting of approximately FN type III repeats 3-11 (20  $\mu g/ml$ ) was added to cultures. 120FN is a highly purified 120 kDa chymotryptic fragment and is known to have cell attachment activity but inhibit FN binding to cells and FN-induced activities like anti-FN antibody (Ginsberg *et al.*, 1985; Moursi *et al.*, 1996). As inhibitors of signal transduction pathways, aristolochic acid (1  $\mu M$ ), genistein (10  $\mu M$ ), cytochalasin B (0.25~1  $ng/ml$ ), caffeic acid (20~50  $\mu M$ ), NPC-15437 (1  $\mu M$ ) or U73122 (1  $\mu M$ ) was added. These agents were added from the third day of culture with every media change until termination of the experiments.

### Mineralized nodule formation *in vitro*

Osteoblastic cells were plated at a density of 30,000 cells/ $cm^2$  in 4-well plate and cultured in  $\alpha$ -MEM supplemented with 10% FBS. After confluence (3 days), the medium was further supplemented with freshly prepared ascorbic acid (50  $\mu g/ml$ ) and  $\beta$ -glycerophosphate (10 mM) to trigger differentiation. Media were changed every second or third day and cultures were maintained up to indicated days. At the end of cultures, cell layer was fixed with 10% neutral buffered formalin and stained *in situ* by the von Kossa technique for mineral deposits. Numbers of mineralized nodules were then counted at 100X magnification using a microscope.

### RNA isolation and northern blot analysis

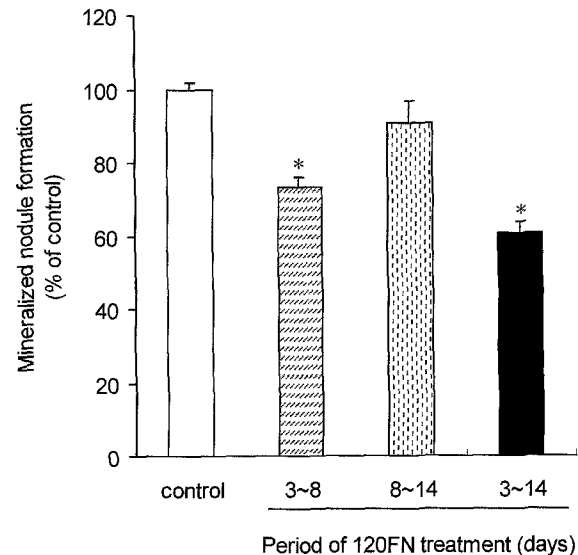
To observe the effect on the osteoblast differentiation, the expression levels of osteoblast differentiation marker genes were observed. Osteoblastic cells were plated at a density of 30,000 cells/ $cm^2$  in 60 mm tissue culture dishes and cultured in  $\alpha$ -MEM supplemented with 10% FBS. After confluence (3 days), the medium was further supplemented with ascorbic acid (50  $\mu g/ml$ ) and  $\beta$ -glycerophosphate (10 mM) to trigger differentiation. Media were changed every second

or third day and cultures were maintained up to indicated days. At the end of culture, total cellular RNAs were isolated using TRI reagent. Thirty microgram of each RNAs was separated on a 1.2% (wt/vol) formaldehyde-denaturing agarose gel and transferred to a nylon membrane. The RNA blots were prehybridized for 30 min at 60°C in ExpressHyb™ hybridization solution, and then hybridized for 1 h at 60°C with a complementary DNA to human pro- $\alpha$ 1(I)-collagen, human fibronectin, rat alkaline phosphatase, rat osteopontin, or rat osteocalcin followed by  $\beta$ -actin probe labeled with  $^{32}$ P-dCTP. The blots were washed twice with wash solution 1 (2X SSC, 0.05% SDS) and wash solution 2 (0.1X SSC, 0.1% SDS) and exposed to film (X-OMAT AR) with enhancer screens. Densitometric analysis of the steady-state expression of mRNA was performed using phosphorimaging analysis system (Fuji BAS 1500, Japan).

## Results and Discussion

When osteoblasts isolated from fetal rat calvaria are plated at near confluent density, they proliferate and differentiate in culture over a 14,16 day period to form multilayered sheets of cells that include prominent mineralized nodules (Bellows *et al.*, 1986; Stein *et al.*, 1996). In this study, osteoblastic cells formed confluent monolayers within 3 days. By 6~8 days, discrete clusters of cells appeared that possessed a markedly rounder shape than surrounding cells (nodule initiation). These clusters of round cells subsequently became increasingly phase-dense over the next week as observed by phase contrast microscope (nodule maturation), and mineral deposition was evident by 14 day, as shown by von Kossa staining.

To determine whether there is a functional role for FN-osteoblast interactions in osteoblast differentiation and mineralized nodule formation, we added 120FN to confluent osteoblast culture. In cultures treated with 120FN, mineralized nodule formation was significantly inhibited (Fig. 1). Although those inhibitory effects were most significant when 120FN was added from day 3 to the end of the culture, the majority of inhibitory effects seems to be occurred during early phase of osteoblast differentiation and nodule initiation period



**Fig. 1.** Effects of 120FN on the mineralized nodule formation in rat calvarial osteoblastic cell cultures. Osteoblastic cells were plated at a density of 30,000 cells/cm<sup>2</sup> in 4-well plates and cultured in  $\alpha$ -MEM supplemented with 10% FBS. After confluence (3 days), the medium was further supplemented with ascorbic acid and  $\beta$ -glycerophosphate to trigger differentiation. 120FN, 120 kDa fragment of FN containing central cell binding regions, was added to cultures for indicated period. At day 14, cells were stained by the von Kossa technique and the numbers of mineralized nodules were counted at 100X magnification. Data represent mean  $\pm$  S.E. (N = 4~8) and were expressed as a percentage of control. \* $p < 0.01$ , compared to control.

(3~8 days). Similar results were exhibited in table 1 that when nodule counting was performed at day 10, inhibitory effect of 120FN was more conspicuous, suggesting that cell-FN interaction might be important in nodule initiation. In addition, 120 FN also significantly decreased the ratio of mineralized to total nodules (unmineralized cellular nodule + mineralized nodules) such that only 13.8% was mineralized in FN120 treated cultures while 90% was mineralized in control cultures at day 10 (Table 1). This data indicate that nodule maturation was also delayed in addition to suppression of nodule initiation by interruption of FN-osteoblast interaction. Our results were similar to the reports that polyclonal anti-

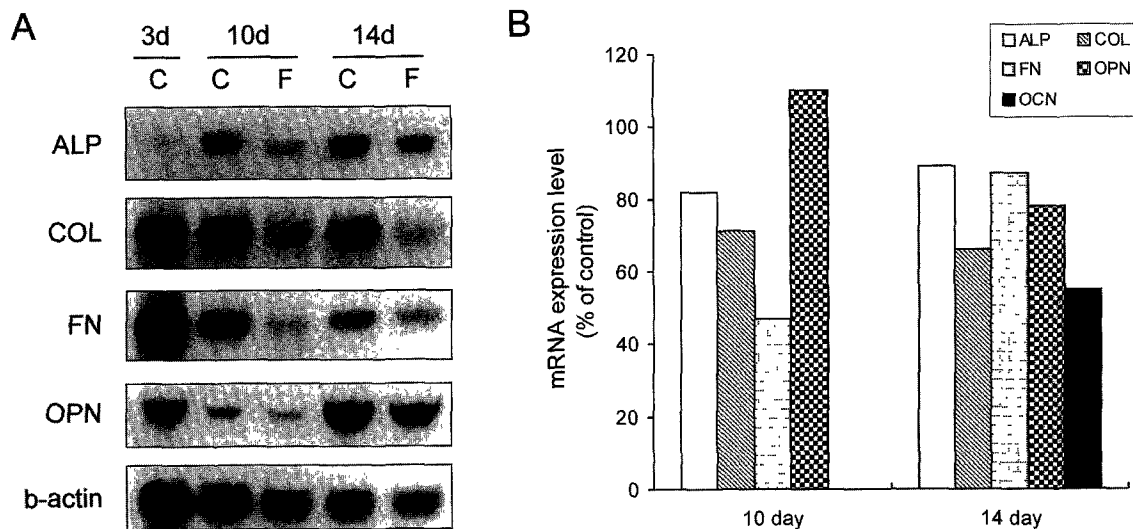
**Table 1.** Effects of 120FN on the nodule initiation and maturation in rat calvarial osteoblastic cell cultures

	Number of total nodules (% of control)		Number of mineralized nodules (mineralized/total $\times$ 100%)	
	Control	120FN	Control	120FN
10 day	123.6 $\pm$ 5.5 (100.0 $\pm$ 4.4)	43.5 $\pm$ 11.9* (35.2 $\pm$ 9.6)	111.3 $\pm$ 4.7 (90.0 $\pm$ 3.8)	6.0 $\pm$ 5.5* (13.8 $\pm$ 12.6)
14 day	191.3 $\pm$ 11.3 (100.0 $\pm$ 5.9)	138.5 $\pm$ 6.6* (72.4 $\pm$ 3.4)	191.3 $\pm$ 11.3 (100.0)	138.5 $\pm$ 6.6 (100.0)

120FN was added to osteoblastic cell cultures from day 3 at a concentration of 20  $\mu$ g/ml and replenished with every medium change. At day 10 and 14, cells were stained by the von Kossa technique and the number of unmineralized and mineralized nodules was counted. Number of total nodule = number of unmineralized nodules + number of mineralized nodules.

Data represent mean  $\pm$  S.E. (N=4~8).

\* $p < 0.01$ , compared to control.



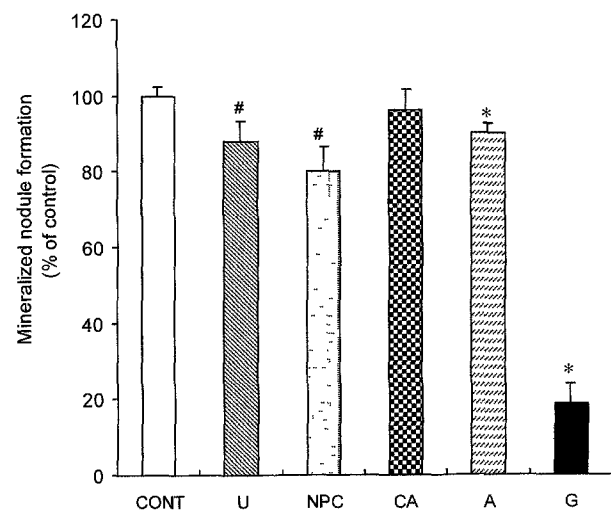
**Fig. 2.** Effect of 120FN on the expression levels of osteoblast differentiation associated genes. Total RNAs were prepared and analyzed by northern blotting as described in Materials and Methods. (A) Northern blot analysis demonstrating the mRNA expression of alkaline phosphatase (ALP), type I collagen (COL), fibronectin (FN), and osteopontin (OPN). (B) Densitometric analysis of northern blot. Results were presented as a percentage of the control values obtained from cultures at the time points indicated after normalization to  $\beta$ -actin mRNA levels. OCN; osteocalcin.

FN antibody and FN fragments that contain central cell binding region reversibly inhibited mineralized nodule formation (Moursi *et al.*, 1996) and anti- $\alpha 5$  integrin subunit antibody reduced nodule formation (Moursi *et al.*, 1997).

To determine whether interfering with FN-osteoblast interactions also alters the gene expression pattern of osteoblasts, we observed the steady state expression of mRNA for genes characteristic of early (alkaline phosphatase, FN, type I collagen) or late (osteopontin, osteocalcin) stages of differentiation. As shown in Fig. 2, 120FN more or less decreased mRNA expression. FN expression was also reduced to 50% of control at day 10. These results were partly similar to the report that inhibition of FN-osteoblast interactions selectively suppressed alkaline phosphatase and osteocalcin expression but did not influence osteopontin mRNA level (Moursi *et al.*, 1996).

These results suggest that the interaction between osteoblasts and endogenously released FN may be important in the regulation of progressive differentiation and finally mineralized nodule formation of osteoblasts. Previously, it has been demonstrated that FN-cell interactions participate in FN fibrillogenesis which is important in formation of the ECM (McDonald *et al.*, 1987; Akiyama *et al.*, 1989). Taken together, it is suggested that direct interactions between FN and cells be requisite to properly perform the role in matrix organization in bone matrix as well as in regulation of osteoblast differentiation and bone formation.

Although the mechanisms by which FN-integrin interactions generate cell regulatory signals is not well understood, integrins have been shown to induce reorganization of cytoskeleton and changes in the levels and activities of various intracellular signaling molecules (reviewed in Schwartz *et al.*, 1995).



**Fig. 3.** Effects of U73122, NPC-15437, caffeic acid, aristolochic acid, and genistein on the mineralized nodule formation in rat calvarial osteoblastic cells. U73122, NPC-15437, caffeic acid, aristolochic acid, or genistein was added to cultures from day 3 and replenished with every medium change. At day 14, cells were stained by the von Kossa technique and numbers of mineralized nodules were counted. Data represent mean  $\pm$  S.E. of five independent experiments and were expressed as a percentage to mean of control. <sup>\*</sup> $p < 0.01$ , compared to control. <sup>#</sup> $p < 0.05$ , compared to control. U, U73122 (1  $\mu$ M); NPC, NPC-15437 (1  $\mu$ M); CA, Caffeic acid (20  $\mu$ M); A, aristolochic acid (1  $\mu$ M); G, genistein (10  $\mu$ M).

Therefore, we observed the effects of several signaling molecules on mineralized nodule formation. Aristolochic acid, an inhibitor of PLA<sub>2</sub>, slightly decreased mineralized nodule formation (Fig. 3) but did not significantly delayed nodule initiation and maturation (data not shown), implying

**Table 2.** Effects of U73122, NPC-15437, and caffeic acid on the nodule initiation and maturation in rat calvarial osteoblastic cell cultures

	Number of total nodules (% of control)				Number of mineralized nodules (mineralized/total × 100%)			
	Control	U73122	NPC-15437	Caffeic acid	Control	U73122	NPC-15437	Caffeic acid
10 day	123.6 ± 5.5 (100.0 ± 4.4)	39.8 ± 9.1* (32.2 ± 7.4)	32.2 ± 5.2* (26.1 ± 4.2)	91.5 ± 9.0* (74.0 ± 7.2)	111.3 ± 4.7 (90.0 ± 3.8)	0*	13.0 ± 4.0* (40.2 ± 12.3)	39.3 ± 9.8* (42.9 ± 10.8)
14 day	191.3 ± 11.3 (100.0 ± 5.9)	174.7 ± 2.0 (91.3 ± 1.1)	118.3 ± 7.2* (61.8 ± 3.7)	165.3 ± 7.6‡ (86.4 ± 4.0)	191.3 ± 11.3 (100.0)	174.7 ± 2.0 (100.0)	118.3 ± 7.2 (100.0)	165.3 ± 7.6 (100.0)

U73122, NPC-15437, and caffeic acid was added to cultures from day 3 at a concentration of 1  $\mu$ M, 1  $\mu$ M and 20  $\mu$ M, respectively and replenished with every medium change. At day 10 and 14, cells were stained by the von Kossa technique and the number of unmineralized and mineralized nodules was counted.

Number of total nodule = number of unmineralized nodules + number of mineralized nodules.

Data represent mean ± S.E. (N=4~8).

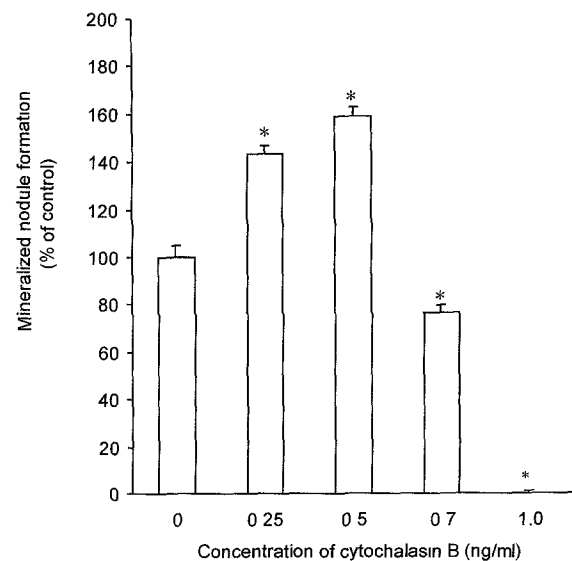
\*p<0.01, compared to control.

‡p<0.05, compared to control.

that PLA<sub>2</sub> activation through integrin signaling might play a minor role in regulation of osteoblast differentiation and mineralized nodule formation. Caffeic acid (20  $\mu$ M), an inhibitor of LOX, did not significantly decrease the number of mineralized nodules formed at day 14 (Fig. 3), though nodule initiation and nodule maturation seemed to be delayed compared to control (Table 2). When caffeic acid was added at higher concentration (50  $\mu$ M), the number of mineralized nodules was significantly increased to 130% of control (data not shown). U73122 and NPC-15437, inhibitors of PLC and PKC, respectively, decreased mineralized nodule formation at 1  $\mu$ M (Fig. 3). They significantly delayed nodule initiation and nodule maturation when observed at day 10 (Table 2). LOX, PLC and PKC are involved in complex signaling pathways induced by various extracellular regulatory molecules such as hormones and local factors as well as ECM. Therefore, we could not say that the effects of caffeic acid, U73122 and NPC-15437 on mineralized nodule formation and osteoblast differentiation are only due to blocking signaling pathway from FN-osteoblast interaction. Nevertheless, the inability of caffeic acid to suppress mineralized nodule formation and expression of osteoblast differentiation marker genes (data not shown), seems to indicate that LOX activation induced by cell attachment to FN might not be major part of FN signaling in regulation of osteoblast differentiation.

The binding of integrins to the ECM initiates both the localization of cytoskeletal proteins into structures known as focal adhesions and the assembly of actin microfilaments (Burrige *et al.*, 1988). Activation of tyrosine kinases during integrin-mediated cell-matrix adhesion is involved both in the regulation of focal contact assembly and in the initiation of signaling processes at the cell-matrix adhesion interface. Among the most prominent of these tyrosine kinases are focal adhesion kinase and members of Src family. Focal adhesion kinase-associated signaling has been associated with the activation of signaling cascades that include the ERK and Jun kinase pathways, pp130<sup>cas</sup>, and phosphoinositol-3-kinase (Giancotti and Ruoslahti, 1999). In this study, genistein, an inhibitor of protein tyrosine kinases, greatly inhibited the

mineralized nodule formation (Fig. 3). On the other hand, cytochalasin B, an inhibitor of actin filament rearrangement, showed biphasic effects on mineralized nodule formation (Fig. 4). At lower concentration (0.25~0.5 ng/ml), cytochalasin B significantly enhanced nodule formation, while at higher concentrations (0.7~1.0 ng/ml), significantly inhibited nodule formation. Although 1 ng/ml cytochalasin B significantly reduced expression levels of all the differentiation marker genes observed, those effects were more conspicuous on osteopontin and osteocalcin, later differentiation marker genes (data not shown). Considering previous demonstration that cytochalasin B-induced disruption of cytoplasmic F-actin microfilaments associated with focal adhesion resulted in the loss of interaction between fibroblasts and FN mesh



**Fig. 4.** Effects of cytochalasin B on the mineralized nodule formation in rat calvarial osteoblastic cells. Cytochalasin B was added to cultures from day 3 and replenished with every medium change. At day 14, cells were stained by the von Kossa technique and numbers of mineralized nodules were counted. Data represent mean ± S.E. (N=4~8) and were expressed as a percentage to mean of control.

\*p<0.01, compared to control

(Berman, 1994), the reason for the enhancing effect of low concentration cytochalasin B is not clear. In addition, genistein did not exhibit the stimulatory effect on nodule formation even at lower concentrations (data not shown). However, because tyrosine kinases directly participate in a variety of signaling events triggered by growth factors as well as integrin-mediated signaling (Plopper *et al.*, 1995), the effects of cytochalasin B and genistein should not be same. Similarly, there was a report that type I collagen-induced upregulation of alkaline phosphatase and osteopontin gene expression in UMR 106-06 osteoblastic cells was inhibited by genistein but not by cytochalasin (Celic *et al.*, 1998). Anyhow, the inhibitory effect of genistein and cytochalasin B (at higher concentration) suggest that appropriate activity of tyrosine kinases and proper arrangement of actin filament is indispensable to mineralization nodule formation by osteoblasts. In addition, those results raise the possibility that cytoskeleton rearrangement together with tyrosine kinase activation may also be the important part of signalings from FN-osteoblast interactions.

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