Enhancement of Fibronectin Fibrillogenesis and Bone Formation by Basic Fibroblast Growth Factor via Protein Kinase C-Dependent Pathway in Rat Osteoblasts

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ABSTRACT

Fibronectin (Fn) is involved in early stages of bone formation and basic fibroblast growth factor (bFGF) is an important factor regulating osteogenesis. We found that bFGF enhanced extracellular assembly from either endogenously released or exogenously applied soluble Fn in primary cultured osteoblasts. bFGF increased protein levels of Fn using Western blotting analysis. Protein kinase C (PKC) inhibitors such as H7, 3-[3-[3-(amidinothio)propyl]-1H-indol-3-y]-3-[1-methyl-1H-indol-3-yl]maleimide (Bisindolylmaleimide IX), methanesulfonate (Ro 318220,) or 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo[2,3-a]pyrrolo[3,4-c] carbazole (Gö 6976) antagonized the increase of Fn protein by bFGF. Treatment of osteoblasts with bFGF increased membrane translocation of various isoforms of PKC, including α, β, ε, and δ. However, treatment with antisense of various PKC isoforms demonstrated that α and β isozymes play important roles in the enhancement action of bFGF on Fn assembly. Down-regulation of PKC by prolonged treatment with 1 μM 12-O-tetradecanoylphorbol-13 acetate for 24 h inhibited the potentiating action of bFGF. It has been reported that α5β1 integrin is related to Fn fibrillogenesis, and immunocytochemistry showed that bFGF treatment increased the clustering of α5 integrins. Flow cytometry analysis demonstrated that bFGF increased cell surface expression of α5 and β1 integrins and PKC inhibitors antagonized the increase by bFGF. Local administration of bFGF into the metaphysis of the tibia via the implantation of a needle cannula significantly increased the protein levels of Fn in the area of trabecular spongiosa, which was inhibited by coadministration of PKC inhibitors. Furthermore, local injection of bFGF increased the bone volume of secondary spongiosa in tibia, which was significantly antagonized by PKC inhibitors. These results suggest that bFGF increased bone formation and Fn fibrillogenesis both in vitro and in vivo via PKC-dependent pathway.

The cell functions, including cell adhesion, migration, proliferation, and differentiation, are regulated by the intimate interaction of extracellular matrix (ECM) and cells. Fibronectin (Fn), a unique dimeric glycoprotein, is one of the major ECM components. It is composed of two similar but nonidentical subunits with molecular weights of ~250,000 (Mosher et al., 1992). Fn has been proven to be distributed throughout many tissues as in insoluble form as well as in the serum as in soluble form. The insoluble form of Fn may mediate various kinds of physiological events during embryogenesis, angiogenesis, thrombosis, inflammation, and wound healing (Hay, 1991). It has been demonstrated that Fn is formed in the early phase of osteogenesis (Weiss and Reddi, 1981) and is maintained within mineralized matrix (Grzesik and Robey, 1994). Fn is the earliest bone matrix protein synthesized by osteoblast and precedes collagen synthesis in developing bone (Cowles et al., 1998). With regard to the bone metabolism, Fn is closely related to the mineralization of bone matrix, induction of bone cell differentiation, and the survival of bone cells, although the precise function of Fn in bone is not definitive (Moursi et al., 1997).

The process of assembling extracellular Fn matrix network is closely related to the integrin receptors on the cell surface. Integrins are a family of dimeric transmembrane receptors

ABBREVIATIONS: ECM, extracellular matrix; Fn, fibronectin; H7, 1-(5-isouquinolinesulfonyl)-2-methylpiperazine dihydrochloride; TPA, 12-O-tetradecanoylphorbol-13 acetate; U73122, 1-[6-[17β-methoxyestra-1,3,5(10)-tien-17-y]amino]hexyl]-1H-pyrole-2,5-dione; D609, tricyclodecano-9-y1-xanthogenate; Ro 318220, 3-[3-[3-(amidinothio)propyl]-1H-indol-3-y]-3-[1-methyl-1H-indol-3-yl]maleimide (Bisindolylmaleimide IX), methanesulfonate; Gö 6976, 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo[2,3-a]pyrrolo[3,4-c] carbazole; FGFs, fibroblast growth factors; GF109203X, 3-[1-[3-(dimethylaminopropyl)-1H-indol-3-yl]-4-(1H-indol-3-yl)-1H-pyrole-2,5-dione monohydrochloride; bFGF, basic fibroblast growth factor; PBS, phosphate-buffered saline; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; ELISA, enzyme-linked immunosorbent assay; PVDF, polyvinylidene difluoride; PKC, protein kinase C; BMD, bone mineral density; BMC, bone mineral content; PI-PLC, phosphoinositide-specific phospholipase C; PC-PLC, phosphatidylycholine-specific phospholipase C.
containing α and β chains (Wu et al., 1993; Dzamba et al., 1994). Different combinations of α and β chains form different kinds of cell transmembrane receptors. They will bind various kinds of ECM molecules. Previous studies have shown that human and rat osteoblasts express α2, α5, α3, αv, and β1 chains. The α2β1 receptor binds collagen and laminin, α5β1 receptor binds Fn, and αvβ5 contains receptors for vitronectin (Wayner et al., 1991; Clove et al., 1992). The integrins provide a site for cell attachment and cell interactions that may induce reorganization of cytoskeleton and influence many cellular physiological events, including proliferation, differentiation, survival, and migration (Rosales et al., 1995; Lauffenburger and Horwitz, 1996). Furthermore, integrins are involved in the signal transduction of translating the strain in the organic matrix to the biochemical signals in the bone cells (Juliano and Haskill, 1993). However, the role of cytokines in the cell-matrix interactions in osteoblasts has not been extensively studied.

Fibroblast growth factors (FGFs) are a family of polypeptides that are important factors controlling cell proliferation, differentiation, and survival in cells. bFGF is a 16.5-kDa heparin binding growth factor that influences the proliferation and differentiation of various cell types in vitro (Gospodarowicz et al., 1987). The skeleton is an important target tissue for FGFs; these factors are involved in bone development, growth, remodeling, and repair (Rodan et al., 1989). Mutation in the genes for human FGF receptor 1 (FGFR1), FGFR2, and FGFR3 causes a variety of disorders in the development of the skeleton (Ornitz, 2000). The cellular actions of FGFs are known to be mediated by interations with FGFRs, a family of tyrosine kinase receptors. Binding of bFGF to its receptor results in autophosphorylation of the receptor (Ullrich and Schlessinger, 1990), activation, and ultimately the induction of transcription regulatory proteins (Mohammadi et al., 1991; Hall et al., 1991). bFGF is a constituent of the bone matrix, and cultured osteoblasts produce bFGF (Baylink et al., 1993; Hurley et al., 1994). In addition, it is well known that bFGF expression is increased during the repair of bone fracture (Bolander, 1992). PKC isoforms of α, β, ε, and δ have been identified in osteoblasts (Yang et al., 2002). It has recently been reported that PKC pathway plays a central role in the bFGF-stimulated expression and transactivation activity of Runx2, a key transcription factor in osteoblast differentiation (Kim et al., 2000). bFGF may thus play an important role in bone remodeling and fracture healing. Both stimulatory and inhibitory effects of exogenous bFGF on collagen synthesis have been reported in osteoblastic cell cultures (McCarthy et al., 1989). Little is known about the effects of bFGF on Fn assembly. We here investigated the regulatory action of bFGF on Fn fibrillogenesis and bone formation. The results suggest that PKC is involved in the regulatory action of bFGF.

Materials and Methods

Materials. 1-(5-Isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H7), 12-O-tetradecanoylphorbol-13-acetate (TPA), and trichloroacetaldehyde were obtained from Sigma-Aldrich (St. Louis, MO). Genistein, herbinycin A, U73122, D609, Ro 318220, and GF109203X were from Calbiochem (San Diego, CA). bFGF and soluble human Fn were purchased from Invitrogen (Carlsbad, CA).

Primary Osteoblast Cultures. Primary osteoblastic cells were obtained from the calvaria of the 18-day-old fetal rats. In brief, the pregnant rats were anesthetized using intraperitoneal injection of trichloroacetaldehyde (400 mg/kg). The calvaria of fetal rats were then dissected from fetal rats with aseptic technique. The soft tissues were removed under dissecting microscope. The calvaria were divided into small pieces and were treated with 0.1% type I collagenase (Sigma Chemical, St. Louis, MO) solution for 10 min at 37°C. The next two 20-min sequential collagenase digestions were then pooled and filtered through 70 µm nylon filters (Falcon; BD Biosciences, San Jose, CA). The cells were grown on the plastic cell culture dishes in 95% air/5% CO2 with Dulbecco’s modified Ca2+-free Eagle’s medium (Invitrogen) which was supplemented with 20 mM HEPES and 10% heat-inactivated fetal calf serum, 2 mM-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (pH adjusted to 7.6). The cell medium was changed twice a week. The characteristics of osteoblasts were confirmed by morphology and the expression of alkaline phosphatase.

Immunocytochemistry. Osteoblasts were grown on glass coverslips. Cultures were rinsed once with phosphate-buffered saline (PBS), and fixed for 15 min at room temperature in phosphate buffer containing 4% paraformaldehyde. Cells were then rinsed three times with PBS. After blocking with 4% BSA for 15 min, cells were incubated with rabbit anti-rat Fn (1:100; Invitrogen) for 1 h at room temperature. Cells were then washed again and labeled with FITC-conjugated goat anti-rabbit IgG (1:150; Leinco Tec. Inc., St. Louis, MO) for 1 h. Finally, cells were washed, mounted, and examined with the use of a Zeiss confocal microscope (LSM 410) as soon as possible. The mean fluorescence under 10 to 15 cells (approximately three to five fields per culture) was measured with the use of the Zeiss confocal microscope (LSM 410). The focus of the z-axis was on the substratum of the monolayer cells. The value for contrast and offset adjustment of confocal microscope was fixed so that the variation of the relative fluorescence of control experiments is rather small. When the α5 integrin was examined, the cells were fixed with acetone for 30 s. After fixation, cells were washed with PBS and incubated with 4% BSA for 1 h. Cells were then incubated with rabbit anti-rat α5 (1:500; Chemicon, Temecula, CA) for 3 h and FITC-conjugated goat anti-rabbit IgG for 1 h at room temperature.

To observe Fn assembly apart from Fn synthesis by rat osteoblasts, human soluble Fn (30 µg/ml) was added to the cultures for overnight. Rat osteoblasts also used the exogenous human soluble Fn to form fibrillar Fn underneath the cells. After washout of residual soluble Fn, fixation, and blocking with 4% BSA for 15 min, cells were incubated for 1 h at room temperature with mouse anti-human Fn (1:50; BD Transduction Laboratories, Lexington, KY), which does not recognize endogenously released rat Fn. Cells were then washed again and labeled with FITC-conjugated goat anti-mouse IgG (1:150, Jackson ImmunoResearch, West Grove, PA) for 1 h. Finally, cells were washed, mounted, and examined with a Zeiss fluorescence microscope.

Quantification of Extracellular Immobilized Fn by ELISA. The level of extracellular immobilized Fn was also determined by an enzyme-linked immunosorbent assay (ELISA). After treatment with bFGF at 37°C, the cells were washed twice with PBS and fixed at room temperature with 1% paraformaldehyde for 30 min. After washing with PBS, they were then blocked with 1% BSA in PBS for 15 min before being incubated successively with rabbit anti-rat Fn antibody (1:150) for 1 h and horseradish peroxidase-labeled anti-rabbit antibody (1:1000) for 30 min. After each incubation, the cells were washed two times with PBS. O-Phenylenediamine dihydrochloride substrate [0.4 mg/ml in phosphate-citrate buffer, pH 5.0, 24.3 mM citric acid, 51.4 mM Na2HPO4, 12 H2O, and 12% H2O2 (v/v)] was then applied to the cells for 30 min, and 3 M sulfuric acid added to stop the reaction. The absorbance was measured at 450 nm by an ELISA reader (Bio-Tek, Winooski, VT). Each assay was performed in triplicate. In pretreatment experiments, cells were incubated with various kinds of inhibitors before addition of bFGF.
Oligonucleotide Transfection. Osteoblasts were grown to confluence on 24-well dishes for quantification of extracellular Fn by ELISA. The complete medium was replaced with Opti-MEM (Invitrogen) containing the antisense or phosphorothioate oligonucleotide (5 μg/ml) that had been preincubated with Lipofectin (10 μl/ml; Invitrogen) for 30 min. The cells were washed after 24 h of incubation at 37°C and washed before the addition of medium containing bFGF. All antisense oligonucleotides were synthesized and HPLC-purified by MBio, Inc. (Taipei, Taiwan).

Sequences are as follows: PKC-α, AAAACGTAGCCATG; PKC-β, AAGATGGCTGACCCGGCTCGC; PKC-δ, GTCGCTATGGAGCCTTTT; PKC-ε, TTGAAACTACCATG (Lu et al., 1998; Tinsley et al., 2004).

Flow Cytometric Analysis. Osteoblasts were plated in six-well (35-mm) dishes. The cells were then washed with PBS and detached with trypsin at 37°C. Cells were fixed for 10 min in PBS containing 1% paraformaldehyde. After rinsing in PBS, the cells were incubated with rabbit anti-rat α5 or β1 integrin antibody (1:100; Chemicon, Temecula, CA) for 1 h at 4°C. Cells were then washed and incubated with FITC-conjugated secondary IgG for 45 min and analyzed by flow cytometry using FACScalibur (CellQuest software; BD Biosciences).

Western Blotting Analysis. Osteoblasts were plated on six-well (35-mm) dishes. Cells were incubated with bFGF for different time intervals as indicated under Results and then washed with PBS, lysed for 30 min at 4°C with radioimmunoprecipitation assay buffer (200 μg/ml protein; composition, 150 mM NaCl, 50 mM Tris-HCl, 1 mM EGTA, 1% Nonidet P-40, 0.25% deoxycholate, 1 mM sodium fluoride, 50 mM sodium orthovanadate, 5 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml leupeptin, pH 7.5). After centrifuging for 15 min at 10,000g, the supernatant was subjected to Western blotting. Equal protein (30 and 80 μg for the measurement of Fn and PKC, respectively) was applied in each lane, and electrophoresis was performed under denaturing conditions on a 7.5% polyacrylamide-SDS gel and transferred to an Immobilon polyvinylidene difluoride (PVDF) membranes at 4°C overnight. The blots were blocked with 4% BSA for 1 h at room temperature and then probed with rabbit anti-rat antibodies against Fn (1:1500) for 1 h at room temperature. After three washes, the blots were subsequently incubated with a donkey anti-rabbit peroxidase-conjugated secondary antibody (1:2000; Amersham Biosciences, Piscataway, NJ) for 1 h at room temperature. The blots were visualized by enhanced chemiluminescence. The same blot was also probed with mouse anti-rat α-tubulin antibody (1:1000; Oncogene Science, Cambridge, MA).

Protein Content of Fn in the Tibia of Young Rats. Male Sprague-Dawley rats weighing 76 to 90 g were used. Implantation of a cannula (22 gauge) was done from the posterolateral side into the proximal tibial metaphysis in both limbs of rats anesthetized with trichloroacetaldehyde. The cannula had its outer end in the subcutaneous tissue. bFGF (300 ng/ml, 10 μl) was percutaneously injected into the proximal tibia through the cannula (once per day) for 5 consecutive days. The same volume of vehicle was injected into the contralateral side for comparison. Rats were then sacrificed, and tibiae were frozen in liquid nitrogen. The regional trabecular spongosia and bone marrow (as shown in Fig. 8A) were removed, homogenized, and sonicated in lysis radioimmunoprecipitation assay buffer. After centrifugation for 15 min at 10,000g, the supernatant was then obtained for Western blotting analysis. For immunohistochemical staining, tibiae were fixed, decalcified, and embedded in paraffin. Serial sections (5 μm) were cut longitudinally, and endogenous peroxidase activity was inactivated by treatment with 3% H2O2 in methanol for 20 min. The sections were then treated with normal goat serum to block nonspecific binding, followed by incubation with rabbit anti-rat Fn antibody (1:300) overnight at 4°C. The sections were then treated with normal goat serum to block nonspecific binding, followed by incubation with rabbit anti-rat Fn antibody (1:300) overnight at 4°C. The sections were then treated with normal goat serum to block nonspecific binding, followed by incubation with rabbit anti-rat Fn antibody (1:300) overnight at 4°C. The sections were then treated with normal goat serum to block nonspecific binding, followed by incubation with rabbit anti-rat Fn antibody (1:300) overnight at 4°C. The sections were then treated with normal goat serum to block nonspecific binding, followed by incubation with rabbit anti-rat Fn antibody (1:300) overnight at 4°C. The sections were then treated with normal goat serum to block nonspecific binding, followed by incubation with rabbit anti-rat Fn antibody (1:300) overnight at 4°C.
proximal tibia were photographed using an IX70 microscope (Olympus, Tokyo, Japan). Measurement of bone volume was performed on the secondary spongiosa, which is located 1.0 to 3.0 mm distal to the epiphyseal growth plate and is characterized by a network of larger trabeculae. Bone volume was calculated using image analysis software (Image Pro Plus 3.0) and expressed as percentage of bone area. All measurements were done in a single-blind fashion. All protocols complied with institutional guidelines and were approved by the Animal Care Committee of Medical College, National Taiwan University.

Statistics. The values given are means ± S.E.M. The significance of difference between the experimental group and control was assessed by Student’s t test. The difference is significant if the p value is less than 0.05.

Results

bFGF Enhanced Fn Fibrillogenesis in Cultured Osteoblasts. The fibrillogenesis from the endogenously released Fn by the primary cultured rat osteoblasts was studied using immunocytochemistry. Osteoblasts from days 3–5 were changed to serum-free medium and incubated with bFGF (30 ng/ml) for 24 h. Immunostaining of Fn was examined in 4% formaldehyde-fixed and nonpermeabilized cells. The mean immunofluorescence intensity underneath a cell group of 10 to 15 cells was measured using confocal microscope. As shown in Fig. 1A, osteoblasts are able to form Fn network underneath the cell using endogenously released Fn. Extracellular assembly of Fn fibril increased in response to 24-h treatment of bFGF (Fig. 1B). The mean fluorescence intensity underneath 10 to 15 cells was 42.5 ± 3.3 and 84.1 ± 4.5 (n = 26–33; n represents the field number) for control and

![Figure 1A](image1a.png)

![Figure 1B](image1b.png)

Fig. 2. Up-regulation of protein levels of Fn by bFGF through PKC-dependent pathway. A, osteoblast cultures were treated for 24 h with different concentrations of bFGF. The cultures were washed with ice-cold PBS, and protein samples for Western blotting analysis were collected by the direct addition of lysis buffer to cultures without trypsin digestion. Compared with control, bFGF dose-dependently increased the protein levels of Fn. B, PKC inhibitors Go 6976 (0.1 μM), Ro 318220 (1 μM), and H7 (10 μM) antagonized the increase of Fn by bFGF when examined at a concentration of 30 ng/ml. The quantitative data are shown in C and D. *, p < 0.05 compared with control; #, p < 0.05 compared with bFGF-treated group.

![Figure 2A](image2a.png)

![Figure 2B](image2b.png)

Fig. 3. bFGF increased extracellular Fn assembly from exogenously-applied soluble Fn. Culture medium of osteoblasts was replaced by serum-free medium containing soluble human Fn (30 μg/ml) in the presence or absence of bFGF (30 ng/ml). Immunocytochemistry was performed 24 h later using mouse anti-human Fn antibody, which does not recognize endogenously-released Fn from rat osteoblasts (A). Compared with control (B), treatment with bFGF markedly enhanced extracellular assembly of Fn (C). Bar, 10 μm.
bFGF-treated cells, respectively. These results suggest that bFGF increased Fn fibrillogenesis in cultured rat osteoblasts. Western blotting was used to examine the effect of bFGF on the protein levels of Fn. Osteoblasts from days 3–5 were changed to serum-free culture medium and treated with bFGF for 24 h. The cultures were then washed with ice-cold PBS, and protein samples were collected by the addition of lysis buffer without trypsin digestion. The result from Western blotting may contain both soluble cytosolic Fn and extracellular immobilized Fn. As shown in Fig. 2A, bFGF concentration-dependently increased protein levels of Fn 24 h after incubation with cells. H7 (10 μM), a nonspecific kinase inhibitor that also targets PKC, as well as PKC inhibitor Ro 318220 (1 μM), antagonized the increase of Fn protein level by bFGF. In addition, Gö 6976 (0.1 μM), which is an inhibitor of classic PKC isoforms (Gschwendt et al., 1996), also antagonized the increase of Fn protein level by bFGF when examined at a concentration of 30 ng/ml (Fig. 2B). These results indicate that bFGF may increase Fn fibrillogenesis via PKC-dependent pathway.

Regulation of Fn Assembly from Exogenous Origin by bFGF. Increase of Fn fibril formation by bFGF may result from the effect on Fn synthesis and/or extracellular Fn assembly. To simply look at the effect of bFGF on Fn assembly apart from Fn synthesis, soluble human Fn (30 μg/ml) was applied into cultures for 24 h in the presence or absence of bFGF (30 ng/ml). The cells are still able to use exogenous soluble Fn to form Fn fibril even if the cells are attached to the dishes. The cultures were then washed with plain culture medium to remove the remaining soluble form of human Fn, and immunocytochemistry of immobilized Fn was performed using mouse anti-human Fn monoclonal antibody, which does not recognize endogenously released rat Fn. The cultured osteoblasts showed no significant immunoreactive staining without addition of exogenous soluble human Fn (Fig. 3A), even in the presence of bFGF (30 ng/ml) (data not shown). Taking advantage of this antibody specificity, we are able to observe the assembly of Fn matrix by the application of exogenous soluble human Fn. Compared with control, bFGF (30 ng/ml) markedly enhanced the formation of Fn fibrils in a PKC-dependent manner.

Fig. 4. Involvement of various PKC isoforms in the potentiation of Fn expression by bFGF. Treatment of osteoblasts with bFGF (30 ng/ml) for 30 min decreased cytosolic and increased membrane translocation of PKC isoforms, including α, β, ε, and δ (A). Expression of extracellular Fn was measured by ELISA in this experiment. Pretreatment with the tyrosine kinase inhibitors genistein (30 μM) and herbimycin A (3 μM), the PI-PLC inhibitor U73122 (3 μM), and the PKC inhibitors Ro 318220 (1 μM), H7 (10 μM), GF109203X (1 μM), and Gö 6976 (0.1 μM), but not PC-PLC inhibitor D609 (30 μM), antagonized the potentiation of Fn expression by bFGF (B). Treatment of osteoblast with antisense (AS) directed against different isoforms of PKC for 24 h, specifically reduced cell content of respective PKC isoforms (C). The antisense of PKC α and β but not of ε and δ inhibited the enhancement of bFGF on Fn expression (D). Results are expressed as the mean ± S.E.M. of three independent experiments performed in triplicate. * p < 0.05 compared with control; # p < 0.05 compared with bFGF-treated group.
fibril from exogenously-applied soluble Fn (Fig. 3, B and C).
The mean fluorescence intensity underneath 10,000 cells was
23.5 ± 2.8 and 48.6 ± 5.6 (n = 16–21) for the control and
bFGF-treated group, respectively.

bFGF Increased Fn Fibrillogenesis in a PKC-Dependent Pathway. We then investigated which isoform of PKC is involved in the stimulatory effect of bFGF. PKC isoforms, including α, β, ε, and δ, have been identified in osteoblasts (Yang et al., 2002). Incubation of osteoblasts with bFGF (30 ng/ml) for 30 min increased membrane translocation of PKC isoforms, including α, β, ε, and δ (Fig. 4). To examine the intracellular signaling pathway of bFGF, ELISA was used to detect extracellular immobilized Fn. Pretreatment of osteoblasts with the tyrosine kinase inhibitors genistein (30 μM) and herbimycin A (3 μM), the PI-PLC inhibitor U73122 (3 μM), but not the PC-PLC inhibitor D609 (30 μM), antagonized the potentiating effect of bFGF (Fig. 4B). In addition, the PKC inhibitors such as Ro 318220, H7, GF109203X, and Gö 6976 antagonized the increase of Fn fibrillogenesis by bFGF (Fig. 4B). To examine which PKC isoforms are involved in the potentiation of Fn fibrillogenesis by bFGF, isoform-specific antisense oligonucleotides were used. As shown in Fig. 4C, treatment of antisense against PKC α, β, ε, and δ for 24 h selectively decreased the specific isoform and left the other isoforms unaffected in cell lysate. It was demonstrated that antisense of PKC isoforms α and β isoforms but not ε and δ antagonized the potentiating action of bFGF in Fn fibrillogenesis. The PKC-dependent effects of bFGF on the assembly of Fn in osteoblasts were further examined by the down-

Fig. 5. Down-regulation of PKC antagonizes the enhancement of Fn assembly by bFGF. Osteoblasts were incubated with 1 μM TPA for 24 h to down-regulate PKC. Culture medium of osteoblasts was then replaced with serum-free medium containing soluble human Fn (30 μg/ml) in the presence or absence of bFGF (30 ng/ml). Immunocytochemistry was performed 24 h later using mouse anti-human Fn antibody, which does not recognize endogenously released Fn from rat osteoblasts. Compared with control (A), prolonged treatment with TPA markedly inhibited extracellular assembly of Fn (B). Down-regulation of PKC by long-term treatment of 1 μM TPA markedly inhibited the potentiating action of bFGF on Fn assembly (C). The time-dependent biphasic action of TPA on Fn assembly and the inhibition of bFGF action by prolonged treatment with 1 μM TPA were shown in (D). Data are presented as mean ± S.E.M. (n). *, p < 0.05 compared with control. Bar, 10 μm. n = 17 to 25.

Fig. 6. Effects of bFGF on the clustering of α5 integrins. Osteoblast cultures were treated with bFGF (30 ng/ml) for 24 h. Immunocytochemistry was performed and fluorescent images were obtained from confocal microscope. Compared with control (A), treatment with bFGF markedly enhanced the clustering of α5 integrins (B). Bar, 10 μm.
regulation of PKC. We have shown previously that treatment with high concentration of TPA (1 μM) for 24 h is able to down-regulate these isoforms of PKC (Yang et al., 2002). Exogenous soluble human Fn (30 μg/ml) was then applied to the cultures for the assembly of Fn network by osteoblasts in the absence or presence of bFGF (30 ng/ml). As shown in Fig. 5, prolonged treatment with high concentration of TPA to down-regulate PKC exerted an inhibitory effect on the assembly of exogenous Fn underneath the cells (Fig. 5B). The potentiation of Fn fibrillogenesis by bFGF was also antagonized by PKC down-regulation (Fig. 5, C and D), suggesting that PKC is involved in the potentiating action of bFGF. Because PKC activation by TPA enhanced Fn fibrillogenesis (Yang et al., 2002), the time-dependent effect of high concentration (1 μM) of TPA was shown in Fig. 5D. Incubation with 1 μM TPA within 2 h increased extracellular Fn assembly, whereas the incubation time longer than 8 h decreased the assembly.

**Effect of bFGF on the Distribution of Integrin.** The assembly of extracellular Fn matrix underneath the cells may be related to integrins (Wu et al., 1993). Integrins are a family of heterodimeric transmembrane receptors that contain α and β subunits. The different combination of α and β chains forms different receptors for the various kinds of ECM molecules. α5β1 integrin is a specific receptor for Fn. We have previously found that application of GRGDS (50 μg/ml) or triflavin (2.8 μM), an Arg-Gly-Asp (RGD)-dependent disintegrin (Huang et al., 1991), inhibited Fn assembly and the enhancing effect of BMP-4, indicating that RGD motif is involved in Fn fibrillogenesis (Tang et al., 2003). We further used immunocytochemistry to visualize the localization of integrins. The α5 staining for the control shows a punctate pattern (Fig. 6A). However, treatment with bFGF for 24 h greatly enhanced the clustering of α5 integrin, and the cells often show a staining of fibrillar pattern (Fig. 6B). We then used flow cytometry to investigate the effect of bFGF on the cell surface expression of integrins. As shown in Fig. 7, incubation with bFGF for 24 h significantly enhanced the fluorescence intensity of α5 and β1 integrins (Fig. 7, A and B). The increase of cell surface expression of integrins by bFGF was antagonized by PKC inhibitors Gö 6976, Ro 318220, and H7 (Fig. 7, C and D). However, PKC inhibitor alone slightly reduced the expression of integrin, suggesting that a basal activity of PKC is involved in the regulation of integrin activity in cultured osteoblasts (Fig. 7, C and D).

**bFGF Enhanced Fn Formation and Bone Volume of Tibia in Young Rat.** Trabecular bone is composed of a lattice or network of branching bone spicules. The spaces between the bone spicules contain bone marrow. bFGF (300 ng/ml, 10 μl, once per day) was locally administered into tibia for 5 consecutive days via an implantation of a needle cannula (22 gauge) in young rats weighing 76 to 90 g (Fig. 8A).

![Fig. 7.](image-url)

*Fig. 7.* Increase of the cell surface expression of α5 and β1 integrins by bFGF using flow cytometric analysis. Compared with control, treatment with bFGF (30 ng/ml) for 24 h significantly enhanced the fluorescence intensity of α5 and β1 integrins (A and B). PKC inhibitors Gö 6976 (0.1 μM), Ro 318220 (1 μM), and H7 (10 μM) antagonized the increase of cell surface expression of α5 (C) and β1 (D) integrins by bFGF. Data are presented as mean ± S.E.M. (n = 4). *, p < 0.05 compared with control; #, p < 0.05 compared with bFGF-treated group.
The protein from trabecular bone and bone marrow surrounding the injection site (as shown in Fig. 8A, left) was then isolated on day 6 for Western blotting analysis of Fn. Local injection of bFGF significantly increased the protein level of Fn. Coadministration of PKC inhibitors such as Go6976 (10 pmol), Ro 318220 (100 pmol), or H7 (1 nmol) antagonized the increase of Fn by bFGF (Fig. 8, B and C). We further examined the long-term effect of bFGF on the bone formation by local injection of bFGF into tibia for 7 consecutive days, and the rats were sacrificed later on day 14. The vehicle was injected into contralateral side for comparison. Compared with the vehicle-injected side (Fig. 9A; arrow shows the hole of the injection site), bFGF significantly increased bone volume of the secondary spongiosa (Fig. 9B). Trabecular bone in the secondary spongiosa increased by 72.5% after local administration of bFGF. The immunohistochemistry also showed that Fn predominantly localized around trabecular bone, and bFGF increased the staining of Fn (Fig. 9, C and D). In addition, BMD and BMC increased after long-term application of bFGF (Table 1). Coadministration of PKC inhibitors H7, Ro 318220, or GF109203X inhibited the potentiating action of bFGF (Table 1). Furthermore, H7, Ro 318220, or GF109203X alone slightly decreased the bone volume, suggesting that basal PKC activity is involved in the regulation of bone formation (Table 1).

**Discussion**

bFGF has been demonstrated to be able to increase the amount of bone formation (Gonzales et al., 1990), to enhance the bone formation during fracture healing (Kawaguchi et al., 1994), and to stimulate proliferation of osteoblastic cells (Rodan et al., 1987). In the present study, immunocytochemistry was used to investigate the Fn fibrillogenesis from either endogenously released or exogenously applied Fn. We previously demonstrated that rat osteoblasts can synthesize and secrete Fn to form fibril matrix network underneath the cells (Yang et al., 2002). The Fn network is an important factor for the differentiation, expression of physiological function, and survival of osteoblasts (Globus et al., 1998). In this study, we further identify Fn as a target protein for bFGF signaling pathway in cultured osteoblasts. We also show that
potentiation of Fn fibrillogenesis by bFGF requires an activation of PKC signaling pathway.

bFGF stimulated Fn fibrillogenesis using endogenous or exogenous origin of Fn in a concentration-dependent manner. Furthermore, bFGF increased the protein levels of Fn as demonstrated by Western blotting analysis. Several isoforms of PKC exist in primary cultured osteoblasts, including α, β, ε, and δ (Yang et al., 2002). All of these PKC isoforms possess a phorbol ester-binding site and are capable of being activated by TPA. These PKC isoforms are down-regulated in osteoblasts in response to prolonged treatment of high concentration of TPA (Yang et al., 2002). PKC inhibitors such as H7, Ro 318220, GF109203X, and Gö 6976 antagonized the potentiating actions of bFGF. The membrane translocation of PKC isoforms, including α, β, ε, and δ, was increased by bFGF in a manner similar to that of TPA. However, prolonged treatment with a high concentration of TPA caused a down-regulation of PKC and inhibited Fn fibrillogenesis (Yang et al., 2002). The enhancement of Fn fibrillogenesis by bFGF was inhibited by PKC down-regulation, suggesting that bFGF acts in a PKC-dependent pathway. It has been reported that bFGF stimulates PLC and results in the activation of PKC and the formation of IP3 in MC3T3-E1 cells (Kim et al., 2000, Suxuki et al., 2000). The PI-PLC inhibitor U73122, but not the PC-PLC inhibitor D609, inhibited the increase of Fn expression by bFGF, suggesting that the PI-PLC pathway is involved in PKC activation by bFGF. In addition, genistein and herbimycin A also inhibit the potentiating action of bFGF. These results indicated that both PKC and tyrosine kinase activity are required for the effect of bFGF. It has also been found that bFGF increases N-cadherin expression in human calvaria osteoblasts via activation of PKC pathways (Debiais et al., 2001). We here show that bFGF is able to increase membrane translocation of various PKC isoforms, including α, β, ε, and δ in primary osteoblastic cultures. We have previously demonstrated that PKC increases and PKA inhibits the Fn fibrillogenesis (Yang et al., 2002). Here, we further define a ligand that can increase Fn formation via PKC activation. Treatment with antisense oligonucleotides directed against PKC isoforms α and β but not PKC ε and δ antagonized the potentiating action of bFGF in the Fn expression, indicating that α and β isoforms are much more important to mediate the action of bFGF in osteoblasts. bFGF has been reported to enhance TGFβ gene expression in osteoblastic cells (Noda and Vogel, 1989). However, we previously found that TGFβ inhibited Fn assembly (Tang et al., 2003), suggesting that enhancement of Fn fibrillogenesis by bFGF does not result from the release of TGFβ.

Direct osteoblast interactions with the extracellular matrix are mediated by a selective group of integrin receptors, including α5β1, α3β1, αVβ3, and α4β1 (Clover et al., 1992; Grzesik and Robey, 1994). α5β1 integrin, a specific Fn receptor, mediates critical interactions between osteoblasts and Fn required for both bone morphogenesis and osteoblast differentiation (Moursi et al., 1997). Interfering with interactions between Fn and integrin Fn receptors in immature fetal

### TABLE 1

<table>
<thead>
<tr>
<th>BMD</th>
<th>BMC</th>
<th>BVTV</th>
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<tbody>
<tr>
<td>Control</td>
<td>0.090 ± 0.002</td>
<td>0.081 ± 0.002</td>
</tr>
<tr>
<td>bFGF</td>
<td>0.102 ± 0.004</td>
<td>0.111 ± 0.01</td>
</tr>
<tr>
<td>H7</td>
<td>0.087 ± 0.004</td>
<td>0.073 ± 0.005</td>
</tr>
<tr>
<td>Ro 318220</td>
<td>0.087 ± 0.005</td>
<td>0.073 ± 0.006</td>
</tr>
<tr>
<td>GF109203X</td>
<td>0.086 ± 0.004</td>
<td>0.072 ± 0.004</td>
</tr>
<tr>
<td>bFGF + H7</td>
<td>0.093 ± 0.004</td>
<td>0.085 ± 0.007</td>
</tr>
<tr>
<td>bFGF + Ro318220</td>
<td>0.093 ± 0.006</td>
<td>0.091 ± 0.006</td>
</tr>
<tr>
<td>bFGF + GF109203X</td>
<td>0.092 ± 0.005</td>
<td>0.087 ± 0.006</td>
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<tr>
<th>BMTV</th>
<th>bone volume/tissue volume.</th>
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</thead>
<tbody>
<tr>
<td>α</td>
<td>P &lt; 0.05: compared with control groups.</td>
</tr>
<tr>
<td>b</td>
<td>P &lt; 0.05: compared with bFGF-treated groups.</td>
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rat calvarial osteoblasts suppressed formation of mineralized nodules in vitro and delayed expression of tissue-specific genes, including osteocalcin (Moursi et al., 1997). Enhancement of surface expression of α5 and β1 integrins by bFGF is correlated to the increase of Fn assembly by bFGF. Increased surface expression of α5 and β1 integrin by bFGF was antagonized by PKC inhibitors H7, Ro 318220, and Go 6976. On the other hand, prolonged treatment with PKC inhibitor increased Fn fibrillogenesis and the cell surface expression of Fn, suggesting that a basal PKC activity is involved in regulating integrin function in cultured osteoblasts.

Several studies have shown that gene for bFGF is expressed in early embryonic development and have suggested that the growth factor may play an important role in tissue growth and differentiation (Kimelman et al., 1988; Gonzales et al., 1990). Using local injection of bFGF into tibia for 5 consecutive days, we have demonstrated that local administration of bFGF increased the protein level of Fn and bone volume in young rats. The present results suggest that bFGF plays an important role in the developing bone as well. Previous studies have shown that bFGF is a more potent mitogen for fibroblast and preosteoblasts than Fn (McCartney, 1989). The increase of bone formation may be also partially mediated by the increase of proliferation and survival of osteoblasts (Shimoaka et al., 2002); bFGF also increased differentiation marker of osteocalcin (Xiao et al., 2002). Local injection of H7, Ro 318220, or GF109203X alone slightly reduced the bone volume and markedly inhibited the potentiating action of bFGF, indicating that PKC activity plays an important role in the regulation of bone formation.

In conclusion, the present study demonstrated that bFGF increased Fn fibrillogenesis and the cell surface expression of α5 and β1 integrins via a PKC-dependent pathway. Long-term administration of bFGF into tibia of young rats also increased the protein level of Fn and bone volume of secondary spongosia. bFGF may thus play an important role in bone formation via the regulation of Fn matrix network in developing bones.

References


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