Abstract. Fibroblast growth factor (FGF) and type β transforming growth factor (TGFβ) are potent modulators of proliferation and differentiation in many types of cells. TGFβ acts in an autocrine manner, and the regulation of TGFβ gene expression is one of the crucial events in the control of cellular functions. This study examines FGF regulation of TGFβ1 gene expression in osteoblast-like cells. Bovine basic FGF (bFGF) increased the steady-state level of 2.5-kb TGFβ1 mRNA two- to threefold in rat osteosarcoma (ROS17/2.8) cells in a dose-dependent manner, starting at 0.1 ng/ml. The increase of the message was detectable within 3 h after the addition of bFGF, peaked at 6 h, and lasted at least up to 48 h. This effect was blocked by a protein kinase inhibitor, K252a, indicating the involvement of phosphorylation. bFGF increased the rate of TGFβ1 gene transcription estimated by nuclear run-on assay, while the stability of TGFβ1 mRNA was not altered. bFGF increased the TGFβ activity in the conditioned media, estimated by DNA synthesis inhibition assay using mink lung epithelial (CCL-64) cells. Parathyroid hormone reduced the abundance of TGFβ1 mRNA in ROS17/2.8 cells and opposed the bFGF effect on TGFβ1 mRNA. bFGF also increased the steady-state level of TGFβ1 mRNA in mouse calvaria-derived MC3T3E1 and human osteosarcoma SaOS-2 cells. These findings indicate that FGF enhances the expression of TGFβ1 gene in osteoblast-like cells and point to the tight relationship of the two growth factors involved in the control of cellular functions.

Fibroblast growth factor (FGF) and type β transforming growth factor (TGFβ) are potent modulators of proliferation and differentiation in a wide variety of cells (Gospodarowicz et al., 1987a, Thomas and Gimenez-Gallego, 1986; Thomas, 1987, 1988; Massague, 1985, 1987; Sporn et al., 1986, 1987; Sporn et al., 1986, 1987). FGF and TGFβ coordinate regulation of the extracellular matrix accumulation and are implicated in tissue repair, angiogenesis, and early embryogenesis (Slack et al., 1987; Gospodarowicz et al., 1987a,b; Kimelman and Kirschner, 1987; Weeks and Melton, 1987; Schweigerer, 1988). On the other hand, the two growth factors interact each other in certain cases, such as the regulation of plasminogen activator activity (Saksela et al., 1987; Gospodarowicz et al., 1987a,b). The diverse directions of their interaction appear to be due to the bifunctional nature of TGFβ (Roberts et al., 1985; Sporn et al., 1986, 1987; Centrella et al., 1987a). The diverse directions of their interaction appear to be due to the bifunctional nature of TGFβ (Roberts et al., 1985; Sporn et al., 1986, 1987; Centrella et al., 1987a). These features of the two growth factors reveal their close relationship in modulation of cell functions, especially proliferation and extracellular matrix accumulation.

TGFβ and FGF are relatively abundant in bone (Hauschka et al., 1986; Seyedin et al., 1985, 1986) where they are produced by osteoblasts (Globus et al., 1989; Robey et al., 1987). In osteoblast-rich cultures or osteoblast-like cells, FGF stimulates proliferation and expression of osteocalcin (in bovine cells) and osteopontin, while it inhibits the expression of alkaline phosphatase, collagen, osteocalcin (in rat cells), and parathyroid hormone (PTH)-responsive adenylyl cyclase activity (Canalis and Raisz, 1980; Canalis, 1980, 1985; Guenther et al., 1986; Globus et al., 1988; Rodan et al., 1987, 1989; Centrella et al., 1987b). TGFβ, on the other hand, either stimulates or inhibits proliferation and expression of alkaline phosphatase; enhances expression of collagen, osteonectin, and osteopontin genes; and inhibits osteocalcin gene expression in osteoblast-like cells (Centrella et al., 1986, 1987a,b; Noda and Rodan, 1986, 1987; Pfeilshifter et al., 1987; Globus et al., 1988; Noda et al., 1988a; Noda, 1989). In addition, high doses of TGFβ1 and TGFβ2 stimulate periosteal bone formation in vivo (Noda and Camilliere, 1989). Thus, both FGF and TGFβ potently modulate the functions of osteoblasts in an autocrine manner.

Regulation of the expression of TGFβ gene is one of the crucial events in the control of cellular function by this autocrine factor. TGFβ1 expression is stimulated by TGFβ1 itself, EGF, PDGF (Schilling et al., 1988), and tumor promoter 12-tetradecanoyl-phorbol-13 acetate (Akhurst et al., 1988).
The expression of TGFβ1 as well as FGF is also regulated developmentally (Gospodorovicz et al., 1987a,b; Sporn et al., 1986, 1987). We examined the FGF regulation of TGFβ1 gene expression and found that FGF enhances the expression of TGFβ1 gene in rat, mouse, and human osteoblast-like cells.

**Materials and Methods**

Bovine brain-derived basic FGF (bFGF) and acidic FGF, porcine platelet-derived TGFβ1, and neutralizing antibody against TGFβ1 were purchased from R & D Systems, Inc. (Minneapolis, MN). Recombinant bFGF and synthetic human PTH (1-34) were obtained from Bachem Inc. (Torrance, CA). [α-32P]dCTP (3,000 Ci/mmol) and [α-32P]UTP (800 Ci/mmol) were purchased from Amersham Corp. (Arlington Heights, IL). A simian TGFβ1 cDNA (Sharple et al., 1987) was kindly provided by Dr. Paricio (Oncogene Science, Inc., Seattle, WA).

**Cell Culture**

ROS17/2.8 cells were grown in 9.5- or 150-cm² Costar (Cambridge, MA) or Nunc (Roskilde, Denmark) tissue culture dishes in modified Ham’s F12 medium supplemented with 5 % FBS and 100 μg/ml kanamycin as described previously (Noda and Rodan, 1987). MC3T3E1 cells were kindly provided by Dr. Kodama (Ohs University, Fukushima, Japan) and were maintained as described previously in a α-minimum essential medium supplemented with 60 μg/ml kanamycin and 10 % FBS (Sudo et al., 1983). SaOs2 cells were obtained from American Type Culture Collection (Rockville, MD) and were cultured in RPMI-1640 media supplemented with 1 % glutamine, 100 μg/ml kanamycin, and 10 % FBS.

**RNA Isolation**

ROS17/2.8, MC3T3E1, or SaOs2 cells were grown to confluence in 150-cm² dishes and were treated for an indicated period of time with bFGF or other agents. Cytoplasmic RNA was extracted as described previously (Greenberg and Ziff, 1984; Noda and Rodan, 1987). Cells (1-3 x 10⁷) were rinsed with cold PBS, scraped in PBS, and centrifuged at 500 g at 4°C. The cell pellets were resuspended in 1 ml of NP-40 lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl2, 0.5 % [vol/vol] NP-40), incubated on ice for 5 min, and then spun at 500 g at 4°C for 2 min. The supernatants were mixed with vinylid ribonucleoside complex (final concentration 10 mM), vortexed, and spun at 12,000 g for 4°C for 10 min. The supernatants were mixed with equal volume of digestion buffer (0.2 M Tris-HCl, pH 7.4, 0.44 M NaCl, 2 % SDS, 25 mM EDTA) containing proteinase K (final concentration 200 μg/ml), incubated at 37°C for 60-120 min, phenol/chloroform (1:1) extracted, and ethanol precipitated. RNA was quantified by spectrometry at the wavelength of 260 nm.

**Northern Blot Analysis**

Total cytoplasmic RNA (10 or 20 μg) was subjected to electrophoresis through 1 % agarose-formaldehyde (0.44 M) gel and electrophoretically transferred to nylon filters (Hybond N; Amersham Corp.) (Thomas, 1980). Complementary DNA probes for simian TGFβ1 (Sharple et al., 1987) and rat β-actin (LK 280) were digested to liberate inserts. The inserts were purified and labeled with [α-32P]dCTP by using random oligonucleotide primers (Amersham Corp.) and Klenow fragment as described by Feinberg and Vogelstein (1984) to a specific activity >10⁶ cpm/μg. Membranes with bound RNA were irradiated for 2 min by ultraviolet light to cross-link the RNA to the filters. Then the filters were prehybridized overnight at 42°C in 50 % formamide, 5 × SSC (5 × SSC = 0.15 M NaCl, 15 mM sodium citrate), 5 × Denhardt’s solution, 0.1 % SDS, and 200 μg/ml sonicated herring sperm DNA. Hybridization was carried out for 16-24 h in fresh prehybridization buffer to which each 32P-labeled probe was added at 10⁶ cpm/ml. Filters were washed three times in 2 × SSC, 0.1 % SDS at room temperature for 5 min each, washed once in 0.1 % SDS, 0.1 × SSC at 65°C for 30 min, and exposed to x-ray films (Eastman Kodak Co., Rochester, NY) with intensifying screens.

**In Vitro Nuclear Transcription (Nuclear Run-on) Assay**

Isolation of nuclei, in vitro transcription, and hybridization were carried out essentially as described previously (McKnight and Palmiter, 1979; Grouding et al., 1981; Harrison et al., 1989) with minor modifications (Noda et al., 1988a; Noda, 1989). Nuclei (2-3 x 10⁷) were isolated by gentle homogenization of cells on ice in a buffer containing 0.44 M KCl, 10 mM Tris-HCl, pH 7.4, 1.5 mM MgCl₂, 0.25 % (vol/vol) NP-40. The isolated nuclei were incubated at 25°C for 20 min in a buffer containing 50 mM Tris-HCl, pH 7.4; 100 mM ammonium sulfate; 1.8 mM DTT; 1.8 mM MnCl₂; 80 U RNasin; 0.3 mM each ATP, GTP, and CTP; and 100 μCi of [α-32P]UTP (800 Ci/mmol; Amersham Corp.) followed by sequential digestions with DNase I and proteinase K. RNA was extracted by phenol/chloroform and was precipitated in ethanol. After centrifugation at 12,000 g for 10 min, the pellets were dissolved in 6 M guanidium hydrochloride followed by addition of 0.5 vol ethanol and were reprecipitated at −20°C overnight. After centrifugation at 12,000 g for 10 min at 4°C, the pellets were rinsed with cold 80 % ethanol, dried, and resuspended in TE buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA). Isolated 32P-labeled transcripts (final concentration 2-3 x 10⁶ cpm/ml) were hybridized to plasmids (2 μg each) bound to nitrocellulose filter. Prehybridization (12-16 h) and hybridization (72 h) were carried out in 5× SSC, 50 % formamide, 5 × Denhardt’s solution, 10 mM EDTA, 100 mM Tris-HCl, pH 7.4, 20 μg/ml tRNA, 10 μg/ml polyadenic acid, 10 μg/ml sonicated herring sperm DNA, and 0.1 % SDS at 42°C. Filters were washed three times in 2 × SSC, 0.1 % SDS at room temperature for 5 min each and once in 0.2 × SSC, 0.1 % SDS at 60°C for 15 min. The filters were then autoradiographed at −70°C using intensifying screens. Quantitation of the signals was carried out by densitometric scanning.

**Mink Lung Epithelial (CCL-64) Cell Assay**

Mink lung epithelial (CCL-64) cells were kindly provided by Dr. Weatherbee (R & D Systems) and were maintained in DME supplemented with 10 % FBS. DNA synthesis inhibition assay was carried out as described by Danielpour et al. (1989) with minor modification. Briefly, CCL-64 cells in logarithmic growth phase were trypsinized, resuspended in 10 % FBS, washed once with assay medium (DME supplemented with 0.2 % FBS), resuspended again in assay medium, plated at 8 x 10⁴ cells/100 μl in 0.2-cm² wells (96-well plate; Costar), and cultured for 2 h. After aliquots (5 μl) of conditioned media corresponding to the same number of the ROS cells (prepared as described below) were added, CCL-64 cells were cultured overnight (16-20 h) followed by pulse-labeling with [3H]thymidine (6.7 Ci/mmol; New England Nuclear, Boston, MA) at final concentration of 1 μCi/ml for 2 h. The cells were fixed with 100 μl of methanol/acetic acid (3:1 [vol/vol]), washed three times with 80 % methanol, and were harvested and centrifuged at 12,000 g for 5 min at 4°C to remove cell debris. Supernatant was transferred to a new tube and was heated at 95°C for 5 min before the addition to CCL-64 cell culture.

**Preparation of Conditioned Medium**

Confluent ROS17/2.8 cells in 9.5-cm² dishes were rinsed three times with PBS, cultured in 1 ml serum-free F12 medium overnight in the absence or the presence of 1 ng/ml bFGF, rinsed three times with PBS, and cultured in 1 ml assay medium for 20 h with or without 1 ng/ml bFGF. A part of media prepared with or without 1 ng/ml bFGF was kept at 4°C and was used as unconditioned media. The cells were trypsinized and counted by a counter (Coulter Electronics Inc., Hialeah, FL), and the conditioned media were harvested and centrifuged at 12,000 g for 5 min at 4°C to remove cell debris. Supernatant was transferred to a new tube and was heated at 95°C for 5 min before the addition to CCL-64 cell culture.

**Results**

Bovine bFGF (10 ng/ml) increased the steady-state level of TGFβ1 mRNA two- to threefold after 48 h in rat osteoblastic osteosarcoma (ROS17/2.8) cells (Fig. 1 A). This effect was observed within 3 h after the addition of bFGF (Fig. 1 B) and lasted at least up to 48 h (Fig. 1 C). β-Actin mRNA levels on the same Northern filters served as control (Fig. 1, B and C). The similar levels of β-actin mRNA in control and FGF-treated cultures indicated that the observed increase of TGFβ1 mRNA was not due to the changes in poly-A' or ribosomal RNA fractions. The abundance of TGFβ1 mRNA relative to actin mRNA was calculated as Ac (or Af) = TGFβ1/actin, where Ac and Af represent control and FGF-
Figure 1. Time course of bFGF effect on TGFβ1 mRNA. Confluent ROS17/2.8 cells were cultured for 48 h (A) or for the indicated periods of time in hours in the absence (lanes C) or the presence (lanes F) of 10 ng/ml bFGF (B and C). Cytoplasmic RNA was isolated as described in Materials and Methods, and 10 μg was loaded on each lane for Northern blot hybridization to 32P-labeled TGFβ1 probe. The same filters were rehybridized with 32P-labeled β-actin probe later. The positions of TGFβ1, β-actin (AC), and 18S ribosomal RNA are indicated. (B) Early time course of bFGF effect on TGFβ1 mRNA level. (C) Effect of prolonged treatment with bFGF on TGFβ1 mRNA level. The figures represent one of two similar experiments.

Figure 2. Dose response of the bFGF effect on the steady-state level of TGFβ1 mRNA. Confluent ROS17/2.8 cells were treated with indicated doses of bFGF for 36 h. Cytoplasmic RNA was isolated as described in Materials and Methods and was used for Northern blot hybridization to 32P-labeled probe. The position of TGFβ1 and 18S ribosomal RNA are indicated. (A) Dose dependence of bFGF effects on TGFβ1 mRNA level. (B) Quantitation of TGFβ1 mRNA bands in A. The relationship between the relative magnitude of the response (Y) and dose (X) fits the equation, Y = 1.46 x log₁₀X + 6.76, in which the regression coefficient was statistically significant (p < 0.05) as judged by F test. The figures represent one of three similar experiments.

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Figure 3. Effect of K252a on bFGF-induced accumulation of TGFβ1 mRNA levels. Confluent ROS17/2.8 cells were treated with vehicle (lane C), 10 ng/ml bFGF (lane F), 1 μM K252a (lane K), or bFGF and K252a (lane KF). After 10 h, cytoplasmic RNA was extracted and subjected to Northern blot analysis (10 μg/lane) as described in Materials and Methods. The positions of TGFβ1, β-actin (AC), and 18S ribosomal RNA are indicated. The figure represents one of two similar experiments.

Figure 4. The apparent half-life of TGFβ1 mRNA, estimated by analyzing the decay of the message level after the treatment with DRB, was ~6 h and was not altered by bFGF (Fig. 4B). bFGF increased two- to threefold the rate of the transcription of TGFβ1 gene, estimated by nuclear run-on assay (Fig. 5). The rate of the β-actin gene transcription served as control and was not altered by the treatment with bFGF.
to that of purified TGFβ1 at 0.1 ng/ml added to CCL-64 cells in a 50-μl aliquot (~30% inhibition) (Fig. 6 C). bFGF itself in unconditioned media did not inhibit DNA synthesis in CCL-64 cells (Fig. 6 A). Similar results were also obtained by colony formation assay in soft agarose using NRK-49F cells (data not shown).

PTH acts as a calcitropic agent in bone metabolism and opposes TGFβ action on the expression of several phenotypic genes in osteoblast-like cells (Noda et al., 1988b; Noda and Rodan, 1989). 48 h of treatment with PTH reduced the TGFβ1 mRNA level (Fig. 7 A) and opposed the bFGF effect when added together (Fig. 7 B).

Treatment with bovine bFGF for 48 h also increased the steady-state level of TGFβ1 mRNA in normal mouse calvaria-derived MC3T3E1 cells and human osteoblastic osteosarcoma-derived SaOS2 cells (Fig. 8), suggesting the generality of the bFGF effects on TGFβ1 gene expression in osteoblastic cells.

**Discussion**

The results from this study show that FGF enhances the expression of the TGFβ1 gene. Both FGF and TGFβ1 are found in a wide variety of tissues and act coordinately or counteract each other's effects in modulation of the function of many cells. FGF stimulation of the TGFβ1 gene expression may confer either a positive or negative feedback mechanism in regulation of cellular functions. In bone, both TGFβ1 and FGF are secreted by osteoblasts (Robey et al., 1987; Globus et al., 1988), deposited in bone matrix (Hauschka et al., 1986; Globus et al., 1989), and potently regulate bone cell function. The accumulated FGF in bone matrix appears to
Figure 7. PTH inhibition of bFGF-induced accumulation of TGFβ1 mRNA. (A) Confluent ROS17/2.8 cells were treated with vehicle (lane C) or $10^{-7}$ M human PTH (1-34) (lane P) for 48 h. (B) Confluent ROS17/2.8 cells were treated with vehicle (lane C), 10 ng/ml bFGF (lane F), $10^{-7}$ M human PTH (1-34) (lane P), or bFGF and PTH (lane PF) for 48 h. Cytoplasmic RNA was extracted and was used for Northern blot analysis as described in Materials and Methods. The positions of TGFβ1, β-actin (AC), and 18S ribosomal RNA are indicated. The figures represent one of two similar experiments.

be stored in an active form (Globus et al., 1989) while most of the secreted TGFβ1 is accumulated in a latent form (Pircher et al., 1986; Nakamura et al., 1986; Wakefield et al., 1988; Miyazono et al., 1988). It is still to be determined how the latent TGFβ1 is activated after FGF enhancement of TGFβ1 expression and accumulation in extracellular matrix.

In vitro nuclear run-on assays indicated that FGF stimu-

Figure 6. Increase of the TGFβ activity in conditioned medium of ROS17/2.8 cells by treatment with bFGF. Confluent ROS17/2.8 cells were cultured in the presence or the absence of 1 ng/ml bFGF. Preparation of the conditioned media and mink lung epithelial (CCL-64) cell assay were carried out as described in Materials and Methods. The numbers of ROS 17/2.8 cells after culture in the absence or the presence of bFGF were 927,000 ± 39,000 (mean ± standard error) and 936,000 ± 43,000 cells per well, respectively. (A) Unconditioned media or conditioned media were heated at 95°C for 5 min and cooled before the addition to CCL-64 cell cultures in an aliquot of 50 μl, which corresponds to 46,000 ROS cells. (B) Conditioned media were incubated with 200 μg/ml neutralizing antibody against TGFβ for 1 h before the addition to CCL-64 cells (thick columns). The neutralizing antibody at the same concentration completely blocked the inhibitory activity of purified TGFβ1 at 1 ng/ml. The antibody by itself did not affect the DNA synthesis in CCL-64 cells (thin columns). (C) TGFβ1 at the indicated concentrations in a 50-μl aliquot of assay media was added to CCL-64 cell cultures (thin columns). Thick columns indicate the effects of conditioned media. Triplicate wells were used for each condition. The data are expressed as mean ± standard error and represent the results from one of three similar experiments. The significance of the difference was evaluated by t test. (*) p < 0.05 against control. NS, no significant difference.
lates TGFβ1 gene expression at least in part through transcriptional control. The promoter region of human TGFβ1 gene is rich in G and C, contains 11 CCGCC repeats, 7 Spl binding sites, and has sequences similar to fat-specific element 2, nuclear factor 1, and 12-tetradecanoyl-phorbol-13 acetate responsive element, respectively, but contains neither TATA nor CAAT boxes (Kim et al., 1989a). TGFβ1 has been shown to stimulate collagen gene expression by acting through the nuclear factor 1 site (Rossi et al., 1988); however, TGFβ1 enhancement of the expression of itself is not mediated through nuclear factor 1 site (Kim et al., 1989a, b), suggesting the presence of diverse pathways. FGF induces diacylglycerol formation, protein kinase C activation, and ionized calcium mobilization within minutes in Swiss 3T3 cells (Tsuda et al., 1985; Kaibuchi et al., 1986). FGF was shown to enter the nucleolus and stimulate nucleolin production before the enhancement of ribosomal gene transcription in bovine aortic endothelial cells (Bouche et al., 1987). The inhibition of the FGF effect by K252a suggests the involvement of protein kinase activation in the mediation of the FGF effects on TGFβ1 gene expression. Tumor promoter (12-tetradecanoyl-phorbol-13 acetate) appears to activate the collagenase gene through phosphorylation of existing API without de novo synthesis of the protein (Angel et al., 1987). Presence of the sequence similar to 12-tetradecanoyl-phorbol-13 acetate–responsive element in the promoter region of the TGFβ1 gene (Kim et al., 1989a) suggests a similar mechanism in activation of TGFβ1 gene expression by FGF. Additional studies are needed to identify the level(s) where protein kinase(s) might be involved in mediation of FGF effects.

FGF inhibits the expression of collagen and alkaline phosphatase genes in rat osteoblasts or osteoblast-like cells (Canalis and Raiz, 1980; Canalis et al., 1987, 1988; Rodan et al., 1987, 1989) while it enhances the expression of osteocalcin in bone cell lines (Globus et al., 1988) and osteopontin/2ar in mouse calvaria–derived MC3T3E1 (Noda, M., unpublished data), rat osteosarcoma ROST/2.8 cells (Rodan et al., 1989), and fibroblasts (Nomura et al., 1988). FGF also elevates prostaglandin E2 production in cultured fetal calvaria (Canalis et al., 1987). These observations indicate that FGF enhances at least some of the phenotypic expression of osteoblasts in certain conditions. TGFβ enhances the accumulation of extracellular matrix in many cells and tissues (Sporn et al., 1987) and appears to possess features as an anabolic agent in bone metabolism both in vitro and in vivo (Canalis et al., 1988b; Centrella et al., 1987a, b; Noda and Rodan, 1987; Noda et al., 1988a; Noda and Camilliere, 1989). The results from this study suggest that FGF may act indirectly as an anabolic agent in bone via stimulation of the expression of TGFβ1 gene. Alternatively, FGF enhancement of TGFβ1 expression may serve as a negative feedback mechanism against the inhibitory effects of FGF on osteoblastic phenotypes, such as alkaline phosphatase and collagen gene expression.

Both TGFβ and FGF are produced in and affect the functions of a wide variety of cells. Whether the FGF effect on TGFβ expression might be found in cells other than osteoblast-like cells and, if so, how general this FGF effect is still remain to be answered by extensive screening of various types of cells.

FGF- and TGFβ-like factors are involved in mesodermal induction from animal hemisphere in early *Xenopus* embryogenesis (Kimelman and Kirschner, 1987; Weeks and Melton, 1987; Rosa et al., 1988), indicating the coordinate relationship of FGF and TGFβ in vivo. The physiological significance of FGF regulation of TGFβ gene expression in modulation of cellular function in local milieu in vivo remains to be determined.

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References


Globus, R. K., J. Plevot, and D. Gospodarowicz. 1989. Cultured bovine bone cells synthesize basic fibroblast growth factor and store it in their extracellular...