Abstract: Fibroblast growth factor 2 (FGF2), a member of the FGF family, positively regulates bone formation and osteoblast differentiation. Bone sialoprotein (BSP) is highly expressed during early bone formation and may play a role in primary mineralization of bone. In the present study, FGF2 (10 ng/mL) was found to increase the levels of Runx2 and BSP mRNA at 3 and 12 h in human osteoblast-like Saos2 cells. Transient transfection assays were performed using chimeric constructs of the human BSP gene promoter ligated with a luciferase reporter gene. FGF2 (10 ng/mL, 12 h) induced the luciferase activities of the -84LUC and -927LUC constructs in Saos2 cells. The results of gel shift assays showed that FGF2 (10 ng/mL) increased the binding of nuclear protein to the FGF2 response element (FRE) and the activator protein 1 (AP1) binding site. Antibodies against Dlx5, Msx2, Runx2 and Smad1 blocked FRE-protein complex formation, and antibodies against CREB1, c-Jun and Fra2 interrupted AP1-protein complex formation. These results indicate that FGF2 increases BSP transcription by targeting the FRE and AP1 elements in the proximal promoter of the human BSP gene. Moreover, the transcription factors Dlx5, Msx2, Runx2, Smad1, CREB1, c-Jun and Fra2 could be key regulators of the effects of FGF2 on human BSP transcription. (J Oral Sci 55, 63-70, 2013)

Keywords: bone sialoprotein; fibroblast growth factor 2; activator protein 1; gene expression.

Introduction

Fibroblast growth factor 2 (FGF2) is expressed by mesenchymal cells and stored in the extracellular matrix (1,2). In cell culture systems, FGF2 has been shown to decrease osteoblast proliferation, and reduce the levels of mRNA for type I collagen, osteocalcin and alkaline phosphatase (3-5). However, intermittent FGF2 treatment increases bone formation in vitro (6). Deletion of the FGF2 gene (Fgf2-/- adult mice) results in a decrease of bone mass and bone formation (7), whereas exogenously applied FGF2 induces recovery of bone nodule formation in Fgf2-/- bone marrow stromal cell cultures in vitro (8). Overexpression of FGF2 (FGF2 transgenic mice) causes a variety of skeletal malformations, including shortening and flattening of long bones and moderate macrocephaly (9). Previously, we have reported that FGF2 activated the tyrosine kinase and mitogen-activated protein kinase (MEK) pathways and induced expression of the bone sialoprotein (BSP) gene in rat osteoblast-like ROS 17/2.8 cells (10). FGF2 increased BSP transcription via FGF2 response element (FRE) in the proximal promoter of the rat BSP gene, which regulates both basal and FGF2-induced BSP transcription (11). We have also reported that the activator protein 1 (AP1) element is another target of FGF2 in the context of BSP transcription (11), and that in addition prostaglandin E₂ induces BSP gene expression through a cAMP response element (CRE) and FRE in the proximal promoter of the rat BSP gene (12).

BSP is a phosphorylated and sulfated non-collagenous glycoprotein found in bone and other mineralizing connective tissues (13-16). Analysis of tissues obtained from 13-, 17- and 21-day fetuses, and from 4-, 14- and
100-day-old animals has indicated that expression of BSP mRNA is restricted to cells actively forming the mineralizing tissues of bone, dentin and cementum (17). BSP gene expression has been observed in breast, lung, thyroid and prostate cancers (18-20). These findings suggest that BSP might be involved in the ectopic calcification of metastatic cancers through its ability to bind to calcium and cells through polyglutamic acid and RGD sequences (15,16,21). Therefore, BSP appears to have an important role in the differentiation of osteoblasts, bone formation and tumor metastasis. Studies focusing on BSP gene transcription have cloned and characterized the promoters of the rat, mouse and human BSP genes (22-26). These promoters include an inverted TATA box (-24 to -19) (27) and an inverted CCAAT box (-50 to -46) (28,29). Furthermore, a CRE (-75 to -68) (12,30), a FRE (-92 to -85) (10,12,31,32), a pituitary specific transcription factor-1 (Pit-1) motif (-111 to -105) (33,34), a homeodomain protein binding site (HOX; -199 to -192) (35,36), a transforming growth factor beta (TGF-β) activation element (-499 to -485) (35,37) and a glucocorticoid response element (GRE; -920 to -906) overlapping with AP1 (-921 to -915) have been characterized (14,38).

To determine the molecular mechanism of FGF2 regulation of the human BSP gene, we used Saos2 human osteosarcoma-like cells to analyze the effects of FGF2 on BSP transcription. We found that FGF2 induces human BSP gene transcription via the FRE and AP1 elements in the human BSP gene promoter.

Materials and Methods

Materials
Alpha minimum essential medium (α-MEM), fetal calf serum (FCS), lipofectamine, penicillin, streptomycin and TrypLE Express were obtained from Invitrogen (Carlsbad, CA, USA). PGL3-basic vector and pSV-β-galactosidase (β-Gal) control vector were purchased from Promega Co. (Madison, WI, USA). An EXScript RT reagent kit and SYBR Premix Ex Taq were purchased from Takara (Tokyo, Japan). All chemicals used were of analytical grade.

Cell culture
The human osteosarcoma cell line, Saos2 (generously provided by Dr. Jaro Sodek, University of Toronto, Canada), was cultured in 10% FCS containing α-MEM. The cells were grown to confluence in 60-mm culture dishes and then cultured in α-MEM without serum and incubated with or without FGF2 (10 ng/mL) for 3, 6, 12 and 24 h. Nuclear proteins were extracted by addition of extra proteinase inhibitors (the extraction buffer contained 0.42 M NaCl, 0.2 mM EDTA, 1.5 mM MgCl₂, 1 mM dithiothreitol, 25% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 2 μg/mL leupeptin, 2 μg/mL pepstatin, 1 μg/mL aprotinin, pH 7.9).

Real-time PCR
One microgram of total RNA was used as a template for synthesis of cDNA using an EXScript RT Reagent Kit. Quantitative real-time PCR was performed using the following primer sets (purchased from Takara, Tokyo, Japan): BSP forward, 5'-CTG-GCACAGGGTATACAGGGTTAG-3'; BSP reverse, 5'-ACTGGTGCCGTATGCTTG-3'; Runx2 forward, 5'-ATGTTGTTTGTTCAGCAGCA-3'; GAPDH forward, 5'-GCACGGTCAAGGCTGAGAAC-3'; GAPDH reverse, 5'-ATGGTGTTGGAAGCGCAGC-3'. The amplification reactions were performed in a final volume of 25 μL containing 2×SYBR Premix Ex Taq (12.5 μL), 0.2 μM forward and reverse primers (0.5 μL) and 50 ng cDNA (5 μL). To reduce variability between replicates, PCR premixes that contained all reagents except for cDNA were prepared and aliquoted into 0.2-mL tubes. The thermal cycling conditions were 1 cycle at 95°C for 10 s, and 40 cycles at 95°C for 5 s and at 60°C for 30 s. Post-PCR melting curves confirmed the specificity of single-target amplification; fold expression of human BSP relative to GAPDH was determined in quadruplicate, and that of Runx2 relative to GAPDH was determined in triplicate.

Transient transfection assays
Exponentially growing Saos2 cells were used for transient transfection assays. Twenty-four hours after plating, cells at 40-60% confluence were transfected using Lipo-fectamine reagent. The transfection mixture included 1 μg of a luciferase (LUC) construct (10) and 2 μg β-gal plasmid as an internal control. Two days after transfection, Saos2 cells were transferred to medium lacking serum for 12 h, FGF2 was then added, and the cells were cultured for a further 12 h prior to harvesting. The luciferase assays were performed in accordance with the supplier’s protocol (picaGene, Toyo Inki, Tokyo, Japan) using a BLR201 luminescence reader (Hitachi Aloka Medical, Ltd, Mitaka, Japan) to measure the luciferase activities.

Gel mobility shift assays
Confluent Saos2 cells in T-75 flasks were transferred to
α-MEM lacking serum for 12 h and then incubated for 3, 6 and 12 h with FGF2 (10 ng/mL) to prepare the nuclear extracts. Double-stranded oligonucleotides encompassing the inverted CCAAT (-64 to -41, 5'-CGTGACAGTGATTTGGCTTTGGAAA-3'), FRE (-102 to -83, 5'-TTTTCTGGTGAGAATCCACG-3') and AP1 (1) (-158 to -129, 5'-CGTTTCTTGTATTCAAATGGCCTGTGT-3') in the human BSP promoter were prepared. For gel shift assays, the double-stranded oligonucleotides were end-labeled with [γ-32P]ATP using T4 polynucleotide kinase. Nuclear proteins (3 µg) were incubated for 20 min at room temperature with 0.1 µM radiolabeled double-stranded oligonucleotide. After incubation, the protein-DNA complexes were resolved by electrophoresis in 5% non-denaturing acrylamide gels (38/2 acrylamide/bis acrylamide) at 200 V. After electrophoresis, the gels were dried and placed in contact with an imaging plate for autoradiography and analyzed using an image analyzer. Supershift gel shift analyses were performed using antibodies against c-Jun (sc-44), c-Fos (sc-253), JunD (sc-74), Fra-2 (sc-604), Mx2 (sc-15396), Smad1 (sc-7965) (Santa Cruz Biotechnology, Inc.), CRE-binding protein 1 (CREB1; P43, Rockland), Dlx5 (AB5728, Chemicon), and Runx2 (AML3, Calbiochem). Each antibody was added to the reaction mixture and incubated for 3 h at 4°C before electrophoresis under the same conditions as those described above.

Statistical analysis

Quadruplicate or triplicate samples were analyzed for each experiment, and experiments were replicated to ensure consistency of the responses to the drugs. The significance of differences between controls and treatments was determined using one-way analysis of variance (ANOVA).

Results

Effects of FGF2 on expression of BSP and Runx2 mRNAs in Saos2 cells

Previously we have reported that FGF2 increased the level of BSP mRNA in rat osteoblast-like ROS 17/2.8 cells (10). To study the regulation of human BSP transcription by FGF2, we performed real-time PCR using total RNA extracted from human osteoblast-like Saos2 cells. FGF2 (10 ng/mL) was used to determine the time courses of Runx2 and BSP mRNA expression. Treatment of Saos2 cells with 10 ng/mL FGF2 significantly increased the expression of Runx2 mRNA at 3 h, and the level of BSP mRNA became maximal at 12 h (Fig. 1).

Transient transfection analysis of Rat BSP promoter constructs

To further characterize the effects of FGF2 on activation of the human BSP gene, various sized stretches of the
human BSP gene promoter ligated to a luciferase reporter gene (-43LUC to -927LUC) were transiently transfected into Saos2 cells, and the subsequent transcriptional activities were determined in the presence or absence of FGF2. The results of luciferase analyses indicated that FGF2 at 10 ng/mL increased the transcription of human BSP at 12 h after transfection for constructs between -84LUC and -927LUC (Fig. 2). Included within the DNA sequence unique to the human BSP promoter region is an inverted CCAAT box (ATTGG; nts -54 to -50), together with cAMP response element 1 (CRE1; nts -79 to -72), FRE (nts -96 to -89), AP1 (nts -148 to -142), HOX (nts -200 to -191) and CRE2 (nts -674 to -667) (TGACCATCA).

Gel mobility shift assays

To identify transcription factors that bind to the inverted CCAAT, FRE and AP1 and mediate the effects of FGF2 on gene expression, double-stranded oligonucleotides were end-labeled and incubated with nuclear proteins (3 μg) from confluent Saos2 cells that had been cultured with FGF2 (10 ng/mL). With nuclear proteins from control cultures of Saos2 cells, shifts of FRE and AP1 DNA-protein complexes were evident (Fig. 4, lanes 5 and 9). After stimulation with FGF2 (10 ng/mL) for 3, 6 and 12 h, FRE and AP1-protein complexes were increased at 3 h and maintained almost the same levels of intensity at 6 and 12 h (Fig. 4, lanes 6-8, lanes 10-12).

When the inverted CCAAT was used as a probe, a strong band for NF-Y binding, identified from previous reports (28,29), was observed. However, the intensities of these bands did not change after exposure to FGF2 (Fig. 4, lanes 1-4). Competition experiments in which a 40-fold molar excess of FRE and AP1 double-stranded oligonucleotides reduced the amount of complex formation (Fig. 5, lanes 3 and 9) demonstrated that these DNA-protein complexes represented specific interactions. In contrast, mutated FRE (mFRE), HOX and mutated HOX (mHOX) did not compete with FRE DNA-protein complex formation (Fig. 5, lanes 4-6), and mutated AP1 (mAP1), CRE1 and CRE2 did not reduce DNA binding to the AP1 (Fig. 5, lanes 10-12). To further analyze the proteins in the complexes formed with FRE and AP1, we performed supershift gel shift assays using antibodies against several transcription factors. Addition of antibodies against Dlx5, Msx2, Runx2 and Smad1 disrupted FRE-protein complex formation (Fig. 6, lanes 3-6), and antibodies against CREB1, c-Jun and Fra2 partially disrupted AP1-protein complex formation (Fig. 6, lanes 9, 10, and 13).
Our studies have shown that FGF2 increased the expression of the BSP gene in human osteoblast-like Saos2 cells by targeting FRE and AP1 elements in the gene promoter. Runx2 is a crucial transcription factor for osteoblast differentiation. Runx2-deficient mice die just after birth without breathing, and display an absence of bone due to arrested osteoblast differentiation (39). In the present study, FGF2 induced the expression of Runx2 and BSP mRNA at 3 h, and the level of BSP mRNA became maximal at 12 h in Saos2 cells (Fig. 1). These results suggest that Runx2 might regulate BSP gene expression.

From luciferase assays, we initially located the FGF2 response region between -184 and -60 in the human BSP gene promoter (Fig. 2), a stretch that encompasses the CRE1, FRE and AP1(1) motifs (Fig. 3). The specific response elements in -184LUC, -116LUC and -84LUC are AP1(1), FRE and CRE1. Involvement of the FRE and AP1(1) elements was confirmed by gel shift assays in which nuclear proteins forming complexes with FRE and AP1(1) were increased by FGF2 (10 ng/mL) in Saos2 cells (Fig. 4). The results of supershift gel assays using antibodies (Fig. 6) indicated that FGF2 induced expression of the human BSP gene through targeting of FRE by Dlx5, Msx2, Runx2 and Smad1, and through targeting of AP1(1) by CREB1, c-Jun and Fra2 in the human BSP gene promoter. There was no additional increase in the activity of -184LUC, which contains an AP1(1) element, in comparison with that of -116LUC (Fig. 2), suggesting that FGF2-induced transcription of BSP through AP1(1) might be weaker than that through FRE. A 40-fold molar excesses of double-stranded CRE1 and CRE2 oligo-nucleotides was unable to compete for AP1(1)-protein complex formation in the human BSP gene promoter. There was no additional increase in the activity of -184LUC, which contains an AP1(1) element, in comparison with that of -116LUC (Fig. 2), suggesting that FGF2-induced transcription of BSP through AP1(1) might be weaker than that through FRE. A 40-fold molar excesses of double-stranded CRE1 and CRE2 oligo-nucleotides was unable to compete for AP1(1)-protein complex formation (Fig. 5, lanes 11 and 12), suggesting that the components of AP1(1), and CRE1 and CRE2 binding proteins are different.

FGF2 is a powerful regulator of cartilage and bone growth and osteoblast differentiation. The levels of FGF2 protein and mRNA are increased by parathyroid hormone (40), prostaglandins (41) and TGF-β (42) in osteoblast-like cells. Moreover, FGF receptors (FGFRs) play an important role in a wide variety of biological activities that require bone growth and differentiation. FGF2 and forskolin (FSK) synergistically increase osteo-

**Discussion**

Our studies have shown that FGF2 increased the expression of the BSP gene in human osteoblast-like Saos2 cells by targeting FRE and AP1 elements in the gene promoter. Runx2 is a crucial transcription factor for osteoblast differentiation. Runx2-deficient mice die just after birth without breathing, and display an absence of bone due to arrested osteoblast differentiation (39). In the present study, FGF2 induced the expression of Runx2 and BSP mRNA at 3 h, and the level of BSP mRNA became maximal at 12 h in Saos2 cells (Fig. 1). These results suggest that Runx2 might regulate BSP gene expression.

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calcitonin transcription via an osteocalcin FRE (OCFRE: GCAGTCA), which is juxtaposed to Runx2 (Osf2, Cbfa1) (43,44). In a previous study, we identified a FRE (-92 to -85, GGTGAGAAA) in the rat BSP gene promoter that is juxtaposed with a putative Runx2 site (10). This BSP FRE is a rare element, because FGF2 alone was able to increase the expression of the BSP gene. We have previously reported that the API1 element overlapping with GRE in the rat BSP gene promoter is another target of FGF2 (11,14). API1 mediates signaling of the FGF, but not receptor, during mesoderm induction, and API1/Jun is a key signaling molecule in the development of posterior structure (45). FGF2 increases the transcription of matrix metalloproteinase 1 in NIH3T3 fibroblasts via API1 (46), and induces binding of nuclear proteins from osteoblast-like ROS17/2.8 cells to consensus API1 (10). These findings indicate that API1 may be a crucial response element for FGF2-induced transcription.

BMP2 has been reported to induce Dlx2 and Dlx5 gene expression within 30 min in mouse osteoblast-like cells (47). BMP2-induced expression of Runx2 is mediated by Dlx5, and TGF-β opposes the BMP2-induced differentiation of osteoblasts induced by suppression of Dlx5 (48). Dlx5 regulates the transcription of osteocalcin by antagonizing the repression of Msx2 (49). TGF-β signaling from the cell membrane to the nucleus is mediated via Smad transcription factors (50). Smads interact with the MMP1 API1 site and mediate the repression of MMP-1 gene expression induced by TGF-β1 (51). FRE (GGTGAGAAA) and API1 (TTATTC) in the human BSP gene promoter (Fig. 3) are very similar to the consensus API1 (TGAGTCA) and consensus CRE (TGACGTCA) sequences, suggesting that the proteins binding to FRE are Dlx5, Msx2, Runx2 and Smad1, and that those binding to API1 are CREB1, c-Jun and Fra2. CRE-binding proteins have a basic region adjacent to a leucine zipper motif (52) that is responsible for their dimerization and DNA binding (53). CREB, CRE modulator (CREM) and ATFs can bind to CRE as homodimers or heterodimers (54), and in the present study we found that CREB and API1 family transcription factors (c-Jun and Fra2) interacted with API1(1) in the promoter of the human BSP gene (Fig. 6).

In conclusion, we have characterized the human BSP gene promoter that is required for FGF2-regulated transcription. This promoter region contains FRE and API1(1), which are required for the FGF2 response. Furthermore, the transcription factors Dlx5, Msx2, Runx2, Smad1, CREB1, c-Jun and Fra2 appear to be key regulators of the effects of FGF2 on BSP gene expression.

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