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Effects of the Combination of Fibroblast Growth Factor-2 and Bone Morphogenetic Protein-2 on the Proliferation and Differentiation of Osteoprecursor Cells*

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Abstract

Objectives. Fibroblast growth factor (FGF) plays a critical role in bone growth; FGF-2 is known to be an important regulator of osteoblast activity because it stimulates osteoblast replication and decreases differentiation markers. Bone morphogenetic protein-2 (BMP-2) has been shown to be an active inducer of osteoblast differentiation and stimulates expression of mineralization-associated genes.

Material and Methods. The dose-dependent impact of FGF-2 and BMP-2 on the cellular proliferation and differentiation of osteoprecursor cells was evaluated. The alkaline phosphatase activity (ALP) test was performed to assess differentiation, and protein expressions related to bone formation were measured using the Western blot analysis.

Results. Cultures grown in the presence of FGF at 20 ng/mL showed significantly increased value when compared with control group and cultures loaded with FGF-2 at 20 ng/mL, and BMP-2 at 100 ng/mL showed significant decrease in cellular proliferation when compared with cultures loaded with FGF-2 at 20 ng/mL. The ALP activity increased when cells were treated with 10 and 100 ng/mL BMP-2, with relative ALP activity of 213.1% and 312.5%, respectively, when ALP activity of the uncontrolled control was considered 100%. However, when 100 ng/mL BMP-2 was combined with 20 ng/mL FGF-2, the relative increase reached up to 392.2%, but this did not reach a statistically significant increase when compared with 100 ng/mL BMP-2 alone.

Conclusions. Within the limits of this study, BMP-2 significantly enhanced osteoblast differentiation but combined delivery of FGF-2 and BMP-2 did not produce synergistic effects on osteoblast differentiation under the current experimental condition (*Adv Clin Exp Med* 2014, 23, 3, 463–467).

Key words: bone morphogenetic protein 2, differentiation, fibroblast growth factor 2, osteoblast, proliferation.

Fibroblast growth factor (FGF) plays a critical role in bone growth and development, affecting osteogenesis and chondrogenesis [1]. FGF-2 is known to be an important regulator of osteoblast activity because it stimulates osteoblast replication and decreases differentiation markers such as alkaline phosphatase and type 1 collagen [2, 3]. Bone morphogenetic protein-2 (BMP-2) has been shown to be an active inducer of osteoblast differentiation and stimulates expression of mineralization-associated genes [4–6].

A previous study showed that sequential supplementation of FGF-2 followed by BMP-2

showed high alkaline phosphatase activity and abundant bone matrix formation [7]. Controversial opinions exist regarding the combined effects of FGF-2 and BMP-2 in differentiation of the osteoblast. In some reports, FGF-2 augmented recombinant human BMP-2-induced osteoinductive activity [8]. In other reports, combined delivery of FGF-2 and BMP-2 resulted in an inhibitory effect on osteogenesis [9].

This study aimed at examining the dose-dependent impact of FGF-2 and BMP-2 on the differentiation of osteoprecursor cells. In addition, impact of the molecule on cell viability was evaluated.

* This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT & Future Planning (2014003106).

The alkaline phosphatase activity (ALP) test was performed to assess differentiation and protein expressions related to bone formation, including osteopontin, and estrogen receptor- α was measured using the Western blot analysis to evaluate the underlying mechanism.

Material and Methods

Cell Culture and Cellular Proliferation

MC3T3-E1 murine calvarial osteoprecursor cells were plated at a density of 1.0×10^4 cells/mL/well in twelve-well plates. The cultures were maintained in α -minimum essential medium (α MEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen) and antibiotics (100 U/mL of penicillin and streptomycin 100 μ g/mL, Invitrogen). Cells were stimulated with BMP-2 and FGF-2 at final concentrations of 10 ng/mL to 100 ng/mL for BMP-2 and 2 ng/mL to 20 ng/mL for FGF-2, respectively.

The effects of BMP-2 and FGF-2 on the cellular proliferation of the preosteoblasts were assessed after 24 h. The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reagents were then added at a final concentration of 0.5 mg/mL, and the cells were incubated for 1 hour at 37°C [10]. Rinsing with phosphate-buffered saline (PBS, pH 7.4) was followed by the addition of dimethyl sulfoxide (DMSO; Sigma). After complete dissolution with gentle shaking, aliquots were transferred into 96-well plates and absorbance was recorded at 560 nm and 670 nm with the microplate spectrophotometer system (BioTek, Winooski, VT).

Alkaline Phosphatase Activity Assays

The ALP assay for osteoblast differentiation was performed after two days. MC3T3-E1 murine calvarial preosteoblasts were grown in an osteogenic differentiation medium (α MEM supplemented with 50 μ g/mL ascorbic acid [Sigma, St. Louis, MO]) and 10 mM β -glycerolphosphate (Sigma) to induce osteogenic differentiation. Cells were lysed with a buffer containing 10 mM Tris-HCl (pH 7.4) and 0.2% Triton X-100 and were then sonicated for 20 s at 4°C. Samples were incubated with 10 mM p-nitrophenylphosphate as a substrate in 100 mM glycine buffer (pH 10.5) containing 1 mM MgCl_2 at 37°C in a water bath. Total protein content was measured in comparison with a series of bovine serum albumin as internal standards. The

absorbance at 405 nm was measured using a microplate reader, and ALP activities were normalized with respect to total protein content [11, 12].

Western Blot Analysis

MC3T3-E1 cells were washed with ice-cold PBS and solubilized with lysis buffer. The lysates were centrifuged at 14,000 rpm for 20 min at 4°C. The supernatants were boiled in a sodium dodecyl sulfate sample buffer containing β -mercaptoethanol. Equal amounts of cell extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred onto polyvinylidene fluoride microporous membrane (Immobilon-P membranes; Millipore Corporation, Billerica, MA). Membranes were then blocked in 0.1% (v/v) phosphate-buffered saline and Tween 20 containing 5% (w/v) powdered milk. The membrane was immunoblotted with the mouse antibodies against osteopontin (Santa Cruz Biotechnology, Santa Cruz, CA) and estrogen receptor- α (Cell Signaling Technology, Inc., Danvers, MA). The membrane was incubated with horseradish peroxidase-conjugated secondary antibody and then the washed blot was developed using enhanced chemiluminescence detection kits [13, 14].

Statistical Analysis

Data were represented as means \pm standard deviation of the experiments. One-way analysis of variance (ANOVA) was performed to determine differences between groups using a commercially available program (SPSS 12 for Windows, SPSS Inc., Chicago, IL). The level of significance value considered was 0.05.

Results

Cellular Proliferation

Cultures grown in the presence of FGF at 20 ng/mL showed significantly increased value when compared with the control group ($P < 0.05$) (Fig. 1). Cultures loaded with FGF-2 at 20 ng/mL and BMP-2 at 100 ng/mL showed a significant decrease in cellular proliferation when compared with cultures loaded with FGF-2 at 20 ng/mL ($P < 0.05$).

Alkaline Phosphatase Activity Assays

The ALP activity increased when cells were treated with BMP-2, with highest value at 100 ng/mL, when compared with the non-loaded

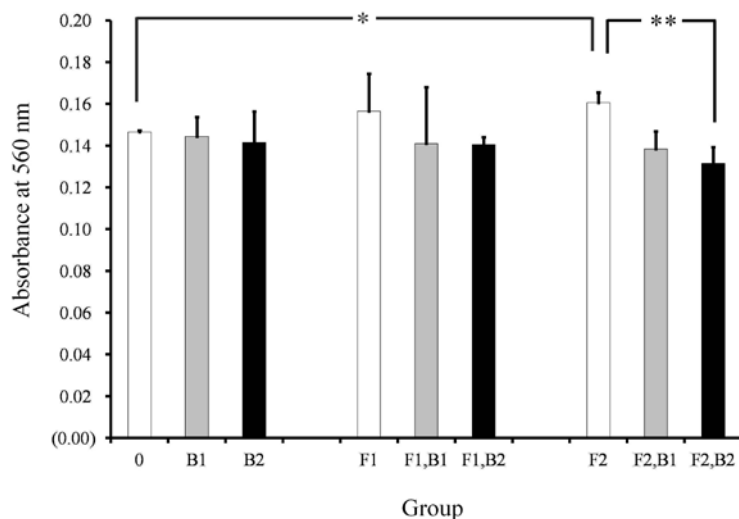


Fig. 1. Determination of cellular proliferation using the MTT assay

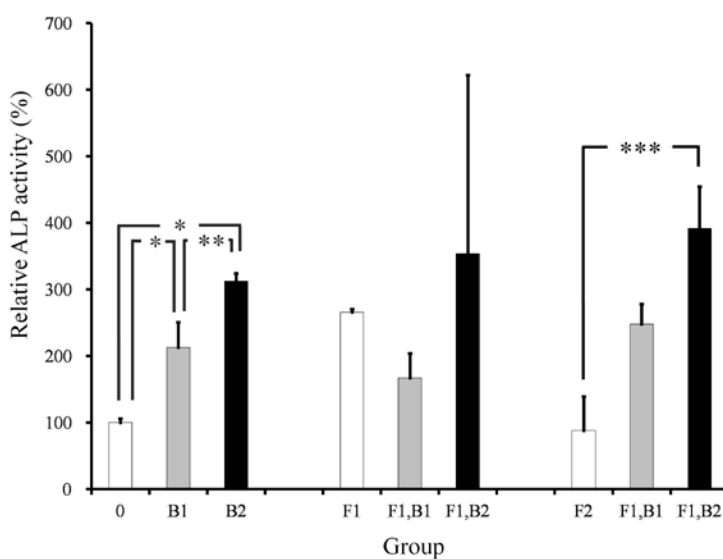


Fig. 2. Relative value of ALP activity.
 *A statistically significant increase was observed when compared with the control (non-loaded group) ($P < 0.05$). **A significant difference was seen when compared with the 10 ng/mL BMP-2 group ($P < 0.05$). ***A significant difference was seen when compared with the 2 ng/FGF-2 group ($p < 0.05$)

control ($P < 0.05$) (Fig. 2). The ALP activity increased accordingly when 10 ng/mL and 100 ng/mL BMP-2 was added to the FGF-2 loaded groups at 20 ng/mL concentration, with a significant difference at 100 ng/mL BMP-2 ($P < 0.05$).

Western Blot Analysis

Western blot analysis was performed to detect protein expression following treatment with FGF-2 and BMP-2 (Figs 3, 4). The results seemed to show that the addition of BMP-2 decreased the expression of osteopontin, with significant difference at 100 ng/mL (Fig. 3). Osteopontin expression decreased accordingly when 10 ng/mL and 100 ng/mL BMP-2 was added to the 2 ng/mL FGF-2 loaded groups, with a significant difference at 100 ng/mL BMP-2 ($P < 0.05$).

The results showed that the addition of BMP-2 decreased the expression of estrogen receptor- α (Fig. 4). Estrogen receptor- α expression decreased when 10 ng/mL and 100 ng/mL BMP-2 was added to the 2 ng/mL FGF-2 loaded groups.

Discussion

This report examines the combined effects of FGF-2 and BMP-2 on cellular proliferation, differentiation, and protein expression of osteoblast progenitor cells under predetermined concentrations (2 and 20 ng/mL FGF-2; 10 and 100 ng/mL BMP-2). In addition, evaluations were conducted to identify whether combinations of FGF-2 and BMP-2 produce effects additively, synergistically, or competitively.

The MTT assay was used in this study to evaluate osteoblast proliferation because it is considered a more sensitive assay than the trypan blue assay [15]. The trypan blue assay is based on the principle that live cells possess intact cell membranes that exclude penetration of the dye, but the MTT assay assesses cellular proliferation through the determination of mitochondrial dehydrogenase activity [10]. Significant increase of cellular proliferation was only achieved with the 20 ng/mL FGF. The inhibitory effect of BMP-2 on cellular proliferation was seen when 20 ng/mL FGF-2 was

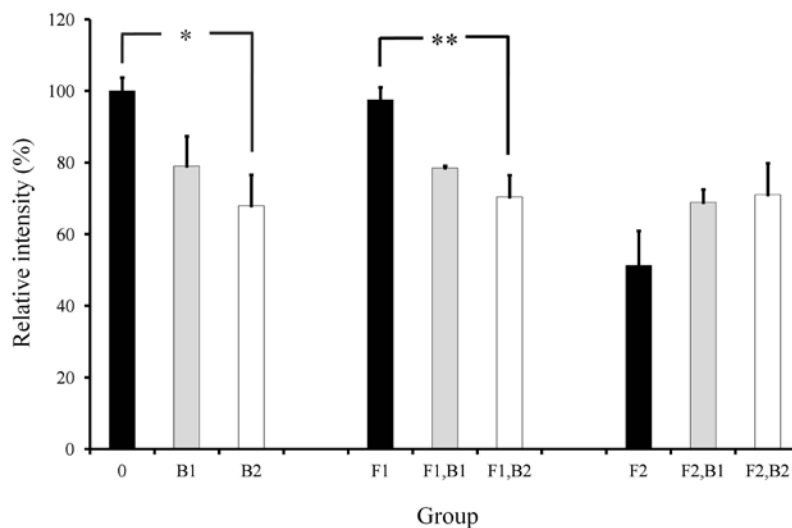


Fig. 3. Western blot analysis to detect the protein expressions of osteopontin. *A significant difference was seen when compared to the control (non-loaded group) ($P < 0.05$). **A significant difference was seen when compared with the 2 ng/FGF-2 group ($p < 0.05$)

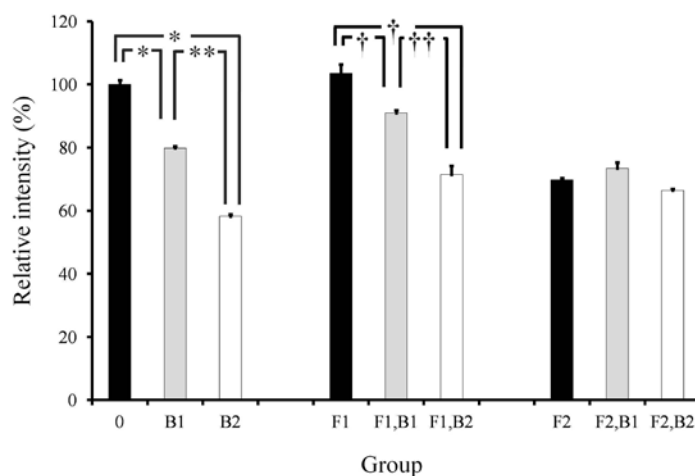


Fig. 4. Quantitative analysis of the protein expressions of estrogen receptor- α by densitometry

administered simultaneously. There could be variations in doses related to the stimulatory or inhibitory effects on cellular proliferation, depending on the culturing period and stage of cells [4, 8, 6, 17].

The ALP activity may be considered an early marker of osteoblastic cell differentiation and this was used to evaluate the osteoblast differentiation [13, 18]. When 10 and 100 ng/mL BMP-2 were loaded alone, the relative increase of the ALP activity was 213.1% and 312.5%, respectively. However, when 100 ng/mL BMP-2 was combined with 20 ng/mL FGF-2, the relative increase reached up to 392.2%, but this did not reach a statistically significant increase when compared with 100 ng/mL BMP-2 alone. This suggests that combined delivery of FGF-2 and BMP-2 did not show synergistic effects on osteoblast differentiation under the current experimental condition.

The Western blot analysis was performed to detect protein expression of osteopontin and estrogen receptor- α to provide information on the possible mechanism. Osteopontin is reported to be an important regulator of bone remodeling, and it may act as a negative regulator in osteogenic

differentiation [13, 19]. This study showed that BMP-2 seemed to produce a tendency toward a dose-dependent increase in OPN expression. It was also seen that addition of BMP-2 affected the expression of estrogen receptor- α .

The combined effects of FGF-2 and BMP-2 on osteoblast differentiation still appear to be controversial [8, 9]. Recent reports showed that different effects were noted from old and young cell cultures [4]. Coadministration of FGF-2 and BMP-2 increased mineralization in cell cultures from elderly mouse and human bones but not in young mouse calvarial cultures. The different responses to combined delivery of FGF-2 and BMP-2 may in part be attributed to the type of cells, the stage of differentiation of the cells, the culturing condition, or the culturing period [20]. Further elucidation of the mechanisms by which FGF-2 and BMP-2 affect osteoblast differentiation may be warranted to improve application of FGF-2 and BMP-2.

Within the limits of this study, BMP-2 significantly enhanced osteoblast differentiation but combined delivery of FGF-2 and BMP-2 did not produce synergistic effects on osteoblast differentiation under the current experimental condition.

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Received: 3.06.2013

Revised: 31.01.2014

Accepted: 9.06.2014