Bone Morphogenetic Protein-2 and -4 (BMP-2 and -4) Mediates Fraxetin-Induced Maturation and Differentiation in Human Osteoblast-Like Cell Lines

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Fraxetin (7,8-dihydroxy-6-methoxy coumarin), a coumarin derivative, was investigated for its effects on differentiation of osteoblasts. By means of alkaline phosphatase (ALP) activity and osteocalcin ELISA assay, we have shown that fraxetin exhibits a significant induction of differentiation in two human osteoblast-like cell lines, MG-63 and hFOB. Alkaline phosphatase and osteocalcin are phenotypic markers for early-stage differentiated osteoblasts and terminally differentiated osteoblasts, respectively. Our results indicated that fraxetin stimulated osteoblast differentiation at various stages (from osteoprogenitors to terminally differentiated osteoblasts). Induction of differentiation by fraxetin was associated with increased bone morphogenetic protein-2 (BMP-2) and BMP-4 productions. Addition of purified BMP-2 and BMP-4 proteins did not increase the upregulation of ALP activity and osteocalcin secretion by fraxetin, whereas the BMPs antagonist noggin blocked both fraxetin and BMP-2 and BMP-4 mediated ALP activity and osteocalcin secretion enhancement, indicating that BMP-2 and BMP-4 productions are required in fraxetin-mediated osteoblast maturation and differentiation. These findings are novel and may be important in the treatment and prevention of osteoporosis.

Key words fraxetin; osteoblast; cell differentiation; alkaline phosphatase (ALP); osteocalcin; bone morphogenetic protein-2 (BMP-2)

Osteoporosis is a reduction in skeletal mass due to an imbalance between bone resorption and bone formation, whereas bone homeostasis requires balanced interactions between osteoblast and osteoclast.1–3 Current drugs used to treat osteoporosis include bisphosphonates, calcitonin, estrogen, vitamin D analogues, and ipriflavone. These are all bone agents which regulate bone formation act by either increasing or recovering bone mass is relatively small, certainly no more than 2% per year.4 It is desirable, therefore, to have satisfactory bone-building (anabolic) agents, such as teriparatide, that would stimulate new bone formation and correct the imbalance of trabecular microarchitecture which is characteristic of established osteoporosis.1,2 Since new bone formation is primarily a function of the osteoblast, agents which regulate bone formation act by either increasing the proliferation of cells of the osteoblastic lineage or inducing differentiation of the osteoblasts.1,6

Bone morphogenetic proteins (BMPs) form a unique group of proteins within the transforming growth factor beta (TGF-β) superfamily and have pivotal roles in the regulation of bone induction, maintenance and repair, as well as being important determinants of mammalian embryological development.7,8 Fifteen BMPs have currently been identified, and are further divided into subfamilies according to their amino acid sequences.9,10 In vitro, BMPs induce osteoblast differentiation of various types of cells including undifferentiated mesenchymal cells, bone marrow stromal cells, and pre-osteoblasts. BMPs not only inhibit myogenic differentiation, but also convert the differentiation pathway of some myogenic cells into an osteoblast lineage.10–12 In the BMP subfamily, BMP-2 was the earliest BMP detected in condensing prechondrocytic mesenchyme of developing limb buds.9,10,13 BMP-2 has also demonstrated a strong osteo-inductive capacity in vivo and in vitro.10,14 Also, BMP-4 has been reported to increase fibronectin synthesis and fibrillogenesis in rat osteoblast and stimulate cell differentiation in murine pre-osteoblastic KS483 cells.15,16

Fraxetin (7,8-dihydroxy-6-methoxy coumarin), a coumarin derivative, has been reported to possess antioxidative, anti-inflammatory and neuroprotective effects.17–22 Fraxetin exhibits its anti-inflammatory effect through inhibiting the formation of the 5-lipoxygenase product, 5-HETE, and the cyclooxygenase product, HHT.18 In addition, a previous studies report that some coumarin derivatives include fraxetin, is able to protect neuroblastoma cells against toxic effects induced by rotenone.20 However, to our knowledge, the effects of fraxetin in the bone metabolism have not been reported. In the present study, we examined the effect of fraxetin on the proliferation and differentiation of osteoblast cells.

MATERIALS AND METHODS

Chemicals and Reagents Fetal bovine serum (FBS), minimal essential medium (MEM), penicillin G, and streptomycin were purchased from GIBCO-BRL (Gaithersburg, MD, U.S.A.). BMP-2 ELISA kit, BMP-4 ELISA kit, human BMP-2 protein, human BMP-4 protein, and noggin were obtained from R&D Systems (Minneapolis, MN, U.S.A.). Sodium 3’-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzensulfonic acid hydrate (XTT) kit was purchased from Roche Diagnostics GmbH (Mannheim, Germany). The osteocalcin ELISA kit was supplied from Biosource Technology (Nivelles, Belgium). Fraxetin and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). The stock solution of fraxetin was prepared at a concentration of 2 mg/ml of DMSO. It was then stored at −20 °C until use. For all experiments, the final concentrations of the test compound were prepared by diluting the stock with cultural medium. Control

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cultures received the carrier solvent (0.1% DMSO).

**Cell Cultures**

The human osteoblast-like cell line MG-63 (CRL-1427) was purchased from American Type Culture Collection (ATCC). Cells were cultured in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 IU/ml of penicillin G and 100 µg/ml of streptomycin). The conditionally immortalized human fetal osteoblastic cell line (hFOB, CRL-11372), was maintained in a 1:1 mixture of phenol-free DMEM/Ham's F12 medium (GIBCO-BRL, Gaithersburg, MD, U.S.A.) containing 10% FBS supplemented with genetin (300 µg/ml) and antibiotics at 33.5 °C, the permissive temperature for the expression of the large T antigen. All experiments of hFOB cells were carried out at the permissive temperature of 33.5 °C.

**Cell Proliferation Assay (XTT)**

Inhibition of cell proliferation by fraxetin was measured by XTT assay. Briefly, cells were seeded in 96 well culture plates (5×10^4 cells/well). After 24 h incubation, the cells were treated with fraxetin (0, 1, 5, 10, 20 µM), BMP-2 (0, 25, 50, 100 ng/ml) or BMP-4 (0, 25, 50, 100 ng/ml) for 48 h. Fifty microliters of XTT test solution, which was prepared by mixing 5 ml of XTT-labeling reagent with 100 µl of electron coupling reagent, was then added to each well. After 4 h of incubation, absorbance was measured on an ELISA reader (Multiskan EX, Labsystems) at a test wavelength of 492 nm and a reference wavelength of 690 nm.

**Alkaline Phosphatase (ALP) Activity**

Cells were seeded into 96 well plates at a density of 5×10^4 cells/well and cultured for 24 h. The agent to be tested was added to the wells, and incubation continued for 2 d. The cells were then washed with three times with physiological saline, and cellular protein concentration was determined by incubation in BCA (bicinchoninic acid) protein assay reagent containing 0.1% Triton X-100 for 1 h at 37 °C. The reaction was stopped by adding 1 M NaOH, and absorbance measured at 560 nm.

ALP activity in the cells was assayed after appropriate treatment periods by washing the cells three times with physiological saline. ALP activity in the cells was then measured by incubation for 1 h at 37 °C in 0.1 M NaHCO₃–Na₂CO₃ buffer, pH 10, containing 0.1% Triton X-100, 2 mM MgSO₄, and 6 mM PNPP (4-nitrophenyl phosphate). The reaction was stopped by adding 1 M NaOH, and absorbance measured at 405 nm. The percentages of changes of ALP activity with respect to the value found in the control were calculated according to this formula: $M = \frac{\text{value of absorbance at 405 nm/value of absorbance at 560 nm}}{\text{percentage of change}} = \left(\frac{M_{\text{test}} - M_{\text{control}}}{M_{\text{control}}}\right) \times 100$.

**Assaying the Levels of Osteocalcin and BMP-2**

Osteocalcin, BMP-2, and BMP-4 ELISA kits were used to detect osteocalcin, BMP-2, and BMP-4 levels, respectively. Briefly, cells were treated with various concentrations of fraxetin, BMP-2, and BMP-4 for the indicated times. The culture medium was collected and measured for osteocalcin, BMP-2, and BMP-4 respectively. These samples were seeded in 96 well microtiter plates coated with monoclonal detective antibodies and incubated for 2 h at room temperature. After removing unbound material by washing with washing buffer (50 mM Tris, 200 mM NaCl, and 0.2% Tween 20), horseradish peroxidase conjugated streptavidin was added to bind to the antibodies. Horseradish peroxidase catalyzed the conversion of a chromogenic substrate (tetramethylbenzidine) to a colored solution, with color intensity proportional to the amount of protein present in the sample. The absorbance of each well was measured at 450 nm. Results are presented as the percentage of change of the activity compared to the untreated control.

**Statistical Analysis**

Data were expressed as means±S.D. Statistical comparisons of the results were made using analysis of variance (ANOVA). Significant differences ($p<0.05$) between the means of control and test group were analyzed by Dunnett’s test.

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**Fig. 1.** Fraxetin, BMP-2, and BMP-4 Did Not Increase the Proliferation of MG-63 and hFOB Cells

Adherent cells that proliferated in 96-well plates (10^4 cells/well) were incubated with different concentrations of fraxetin, BMP-2, and BMP-4 for 48 h. Cell proliferation was determined by XTT assay. Each value is the mean±S.D. of three independent experiments.
RESULTS

No Effect of Fraxetin, BMP-2, and BMP-4 on the Proliferation of MG-63 and hFOB Cells We first determined the effect of fraxetin, BMP-2, and BMP-4 on the cell proliferation of MG-63 and hFOB cells by XTT assay. As shown in Figs. 1A, B, and C, fraxetin, BMP-2, and BMP-4 did not exhibit significant effects on cell proliferation at the concentrations used 1—20 µM (for fraxetin) and 25—100 ng/ml (for BMP-2 and BMP-4) respectively after 48 h of treatment in either cell line.

Effect of Fraxetin, BMP-2, and BMP-4 on Maturation and Differentiation Markers, ALP Activity and Osteocalcin Expression in MG-63 and hFOB cells During differentiation in vitro, osteoblast phenotypic markers appear in the following order: accumulation of collagenous matrix, expression of ALP, secretion of osteocalcin, and finally mineralization of bone nodules.[10,25—27]

The effect of fraxetin, BMP-2, and BMP-4 on the maturation of osteoblasts was studied by determining ALP activity in MG-63 an hFOB cells. The results showed that fraxetin, BMP-2, and BMP-4 increased ALP activity in a dose-dependent manner after 48 h of treatment in both cell lines (Figs. 2A, B, C). The effect of fraxetin, BMP-2, and BMP-4 on the terminal differentiation of osteoblast cells was also assessed by determining the production of osteocalcin. As shown in Figs. 2D, E, and F, treatment of MG-63 and hFOB cells with fraxetin, BMP-2, and BMP-4 increased the level of osteocalcin in a dose-dependent manner after 72 h of treatment.

Fig. 2. Fraxetin, BMP-2, and BMP-4 Induced the Differentiation of MG-63 and hFOB Cells
(A, B, C) Fraxetin, BMP-2, and BMP-4 increased the activity of ALP at 48 h. (D, E, F) Fraxetin, BMP-2 and BMP-4 enhanced the production of osteocalcin at 72 h. For (A) to (C), cells were treated with various concentrations of fraxetin, BMP-2, and BMP-4 for 48 h. ALP activity was assessed by the conversion of PNPP in 0.1 M NaHCO₃–Na₂CO₃ buffer, pH 10, containing 2 mM MgSO₄ and 0.1% Triton. For (D) to (F), cells were treated with various concentrations of fraxetin, BMP-2, and BMP-4 for 72 h. The amount of osteocalcin in culture medium was assessed by osteocalcin ELISA kit. The asterisk indicates a significant difference between control and fraxetin, BMP2 or BMP-4-treated groups, as analyzed by Dunnett’s test ($p<0.05$).
BMP-2 and BMP-4 Mediates Fraxetin-Induced Maturation and Differentiation in MG-63 and hFOB Cells

The upregulation of BMP-2 by 10 μM fraxetin started to increase 6 h after treatment with fraxetin, and maximum expression was observed at 24 h (Fig. 3A). After 6 h of treatment, fraxetin increased production of BMP-2 in a dose-dependent manner (Fig. 3B). A similar result was observed for BMP-4 (Figs. 3C, D). However, both of purified BMP-2 and BMP-4 proteins did not increase the activity and secretion of ALP and osteocalcin induced by fraxetin, supporting the hypothesis that BMP-2 and BMP-4 may mediate fraxetin-induced maturation and differentiation (Figs. 4A, B). To further examine the role of BMP-2 and BMP-4 in cell differentiation by fraxetin, osteoblast cells were pretreated with a BMPs inhibitor, 100 ng/ml noggin protein, for 1 h and then co-treated with 10 μM fraxetin and the inhibitor for the indicated times. Noggin directly binds to BMPs, thereby preventing its interaction with BMP receptor. Addition of purified noggin protein did not change ALP activity and osteocalcin secretion, but abrogated fraxetin, BMP-2, and BMP-4 induced cell differentiation (Figs. 5A, B). Therefore, fraxetin-induced cell differentiation may operate by BMP-2 and BMP-4-dependent pathway.

DISCUSSION

Coumarins comprised a group of phenolic compounds widely distributed in natural plants, and they have recently attracted much attention because of their pharmacological activities. They exhibit a variety of biological activities, such as anti-inflammatory, anti-oxidant, anti-viral, and anti-tumor effects. Many coumarins, such as fraxetin, showed scavenging activity against reactive oxygen species and inhibit lipid peroxidation in rat brain. Previous studies have shown that fraxetin possesses significant neuroprotective effects against apoptosis induced by rotenone, suppressed caspase-3 activation and poly (ADP-ribose) polymerase cleavage in rotenone-treated SH-SY5Y cells, increases the total glutathione levels in vitro, and inhibits lipid peroxidation induced by rotenone.

The present study is the first to demonstrate that fraxetin induced maturation and differentiation in two human osteoblast-like cell lines, MG-63 and hFOB, without exhibiting a significant effect on cell growth. Our results indicated that the presence of fraxetin caused a significant increase in ALP and osteocalcin synthesis. As the appearance of ALP activity is an early phenotypic marker for mature osteoblasts, our results suggested that the presence of fraxetin stimulated an early stage of osteoblast differentiation. In contrast, osteocalcin is a phenotypic marker for a later stage of osteoblast differentiation, one that coincides with mineralization, and it is one of the major noncollagenous proteins specific to mineralized connective tissues of vertebrates. In summary, these results indicate that fraxetin stimulated maturation and differentiation of osteoblast cells could be affected at various levels, from early to terminal stages of the cell differentiation process.

BMPs play an important role in the process of bone for-
tion and remodeling. It has been well documented that stimulation of osteoblast cell differentiation is characterized mainly by increased expression of ALP, type I collagen, and osteocalcin. BMPs are of tremendous interest as therapeutic agents for healing bone fracture, preventing osteoporosis, and enhancing bone response around alloplastic materials implanted in bone. RhBMP-2 delivered with an absorbable collagen sponge (ACS) has been used for the augmentation of the maxillary sinus floor in humans. BMP-2 regenerated bone in irradiated tissues also provides the clinical potential to treat patients who have undergone radiation therapy and need bone reconstruction. The bone inductive ability of BMP-2 is diminished in older organisms and higher doses are required to induce the bone formation effect. BMP-4 also has been demonstrated that induce the differentiation of osteoblast cell.

Our study indicates that the productions of BMP-2 and BMP-4 increases in fraxetin-treated MG-63 and hFOB cells. Treatment of cells with purified BMP-2 and BMP-4 not only increased ALP activity, but also enhanced the expression of osteocalcin. Furthermore, additions of purified BMP-2 and BMP-4 did not increase fraxetin’s effect on cell maturation and differentiation, whereas BMPs antagonist noggin inhibited fraxetin’s effect on cell maturation and differentiation. Thus, our results have demonstrated that the BMP-2 and BMP-4 signaling system plays an important role in fraxetin-mediated cell maturation and differentiation in osteoblast cells.

Taken together, these observations indicate that fraxetin stimulates osteoblast differentiation at various stages (from osteoprogenitors to terminally differentiated osteoblasts) in MG-63 and hFOB cells. Fraxetin’s effect on cell maturation and differentiation is strongly associated with BMP-2 and BMP-4 production. It would, therefore, suggest that fraxetin may be beneficial in stimulating the osteoblastic activity resulting in bone formation.

REFERENCES