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Osteogenic Protein-1 Stimulates mRNA Levels of BMP-6 and Decreases mRNA Levels of BMP-2 and -4 in Human Osteosarcoma Cells

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Abstract. Bone morphogenetic proteins (BMPs) are novel growth and differentiation factors that act on mesenchymal stem cells to initiate new bone formation *in vivo* and promote the growth and differentiation of cells in the osteoblastic lineage. In the present study, we examined the effects of recombinant human osteogenic protein-1 (also known as BMP-7) on the expression of related members of the BMP family using SaOS-2 and U2-OS, two human osteosarcoma cell strains. Evaluation of BMP-2, -4, and -6 mRNA expression indicates that OP-1 stimulated the mRNA levels of BMP-6 in both SaOS-2 cells (threefold) and U2-OS cells (fivefold) after 24 hours of treatment, while decreasing the mRNA levels of BMP-4 in SaOS-2 cells (80%) and BMP-2 and BMP-4 in U2-OS cells by 50% and 72%, respectively. BMP-2 mRNA expression, as examined by Northern blot analysis, was below detectable limits in SaOS-2 cultures. These results demonstrate that OP-1 modulates the mRNA expression of related members of the BMP family, suggesting a possible mode of action of OP-1 on the growth and differentiation of cells in the osteoblastic lineage *in vitro.*

Key words: Bone morphogenetic protein — Osteogenic protein-1 — Transforming growth factor-β — Osteoblasts — Growth factors.

Extracellular bone matrix contains proteins responsible for mesenchymal cell recruitment, proliferation, and differentiation, a cascade of cellular events involved in the initiation of bone morphogenesis *in vivo* [1–6]. The demonstration that these bone morphogenetic proteins (BMPs) could be isolated using the rat subcutaneous bone induction assay has led to the discovery of novel growth and differentiation factors, also called osteogenic proteins (OP), which are members of the transforming growth factor-β (TGF-β) superfamily. They share a striking amino acid sequence identity within the highly conserved 7 cysteine region of the C-terminal domain [2, 6, 7–10]. Members of the TGF-β superfamily include the activins and inhibins, Drosophila decapentaplegic (dpp) and 60A, and the growth and differentiation factors (GDFs); all are signaling molecules and were shown to play a significant role during embryogenesis, tissue repair, and regeneration in postfetal life [2, 6–10].

It has been shown that recombinant human BMPs are capable of inducing new bone formation *in vivo* and healing large segmental bone defects in various animal models [6, 9–12]. Although a single BMP, in combination with an appropriate carrier, is capable of inducing new bone formation *in vivo*, little is known about the mode of action. It is likely that, in addition to direct action on responding cells, BMPs may promote their effects on osteoblast line of cells via stimulating the local production of paracrine and autocrine factors [13–19]. In this regard, we and others recently showed that treatment of bone cells with BMPs regulates the synthesis of various insulin-like growth factor (IGF) system components, suggesting that BMPs may mediate some of their actions on cells of the osteoblastic lineage by modulating the autocrine/paracrine actions of IGFs [14, 16]. In addition, Cunningham et al. [15] have demonstrated that treatment of human monocytes with BMP-4 increased expression of TGF-β1 mRNA. Assuming that the BMPs may work in concert to achieve bone morphogenesis, we hypothesized that BMPs may regulate the expression of other members of the BMP family differentially. To test this hypothesis, U2 and SaOS-2 human osteosarcoma cells were treated with vehicle or OP-1, also known as BMP-7, for 4 and 24 hours and the expression of various growth factors was studied by Northern blot analysis. U2 and SaOS-2 cells were chosen as model systems because of the previous findings that these cell types express mRNA transcripts for various members of the BMP family and that they respond to exogenously added OP-1 [1, 16]. We chose to study the effects of OP-1 on the expression of BMP-2, BMP-4, and BMP-6 based on previous studies that treatment of pluripotent mesenchymal stem cell lines with retinoic acid increased differentiation, increased expression of BMP-6, but decreased expression of BMP-2 and BMP-4 [20]. We chose to study the effects of OP-1 on OP-1 to determine if OP-1, like TGF-β, autoinduces of its own expression [21]. Our findings demonstrate that OP-1 differentially modulates the expression of other members of the BMP family in human osteosarcoma cells.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM) and trypsin were purchased from GIBCO (Grand Island, NY). Bovine calf serum (CS) was obtained from Flow Research Laboratories. Recombi-

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nant human OP-1 was obtained and purified as described [3]. Guanidine thiocyanate was obtained from Fluka (Ronkkokoma, NY). Other chemicals were at least reagent grade and were obtained from Sigma Chemical Company (St. Louis, MO). The human osteosarcoma cell lines SaOS-2 (subpopulation of American Type Culture Collection (ATCC) HTB85 with low alkaline phosphatase activity) was isolated as described [22] and was kindly provided by Dr. J. Farley (Loma Linda, CA). U2 human osteosarcoma cells (# HTB96) were obtained from the ATCC (Rockville, MD).

RNA Extraction and mRNA Isolation

For RNA studies, U2 and SaOS-2 cells were plated in 100 mm Petri dishes in 10 ml DMEM containing 10% CS and grown to 50–60% confluence [16]. The medium was then changed to 10 ml fresh serum-free DMEM containing 0.1% bovine serum albumin (BSA). After 24 hours of incubation, the medium was changed to 4 ml fresh DMEM containing 0.1% BSA prior to the addition of the effectors. OP-1 was dissolved in acetonitrile (AcN) and was tested at a final concentration of 100 ng/ml based on the previous findings that OP-1 at 100 ng/ml produced maximal effects on cell proliferation and on the expression of various IGF system components in human bone cells [16]. The final concentration of AcN in the culture medium was 0.3% which was added to the cultures as the vehicle control. In previous studies, we found that acetonitrile at this concentration had no significant effect on growth factor production compared with medium control [16]. After 4 and 24 hours of treatment with the effector, total RNA was extracted from cell cultures by a single-step acid guanidinium isothiocyanate-phenol chloroform method [23]. To enrich BMP mRNA in SaOS-2 cells, poly(A) RNA was isolated from total RNA by oligo (dT)-cellulose chromatography according to the manufacturer's instructions (Qiagen Inc., Chatsworth, CA).

Northern Blot Analysis

Twenty micrograms of total RNA or 4 μg of poly(A) RNA was size fractionated on a 1.5% agarose 3-(N-Morpholino)propanesulfonic acid (MOPS)-formaldehyde gel and transferred to nylon membranes (MagnaGraph, Micron Separations Inc., Westboro, MA) for Northern blot preparations. The membranes were hybridized with the following gel purified [^{32}P]-labeled cDNA probes: BMP-2 (#40345, ATCC, Rockville, MD) [24]; BMP-4 (#40342, ATCC)[24], BMP-6 (#68021, ATCC) [1], OP-1 (#68020, ATCC) [1]; and GAPDH (#57090, ATCC) cDNA probes were labeled with [^{32}P]dCTP by the random priming method [25]. Unincorporated [^{32}P]dCTP was separated from the labeled probe by NACS column chromatography (Gibco/BRL Life Technologies Research Laboratories, Gaitherburg, MD). The RNA blots were prehybridized in 50% formamide, 5× SSPE, 5× Denhardt's, 0.1% SDS, and 200 μg/ml denatured herring sperm DNA for 2 hours at 42°C and hybridized in the prehybridization solution containing 10% dextran sulphate and 1-5 × 10^6 dpm/ml of the radiolabeled cDNA probes as described [26, 27]. Posthybridization washes were performed stringently [16] and filters were exposed to X-ray film (Fuji Photo Film Co., Kanagawa, Japan) for 1–7 days at −70°C.

The relative densities of the different transcript bands were quantitated by laser densitometry (Biomedical Instruments, Fullerton, CA) and standardized to methylene blue stained 28S rRNA or GAPDH mRNA. Northern blots were performed using RNA extracted from two to three independent experiments. The experimental results obtained in this study have been confirmed in one or more independent experiments, depending on the growth factor studied.

Results

Effect of OP-1 on mRNA Levels of Various BMPs

The BMP-2 cDNA probe recognized two transcripts of approximately 4.0 and 3.8 kb in U2 cells, and OP-1 treatment had no significant effect on these BMP-2 transcripts at 4 hours but decreased BMP-2 mRNA transcripts to 50% of vehicle-treated control at 24 hours. The expression of BMP-2 was increased with time in control cultures of U2 cells (Fig. 1). In SaOS-2 cells, BMP-2 mRNA transcripts were below detectable limits in both vehicle-treated control and OP-1-treated cultures (data not shown).

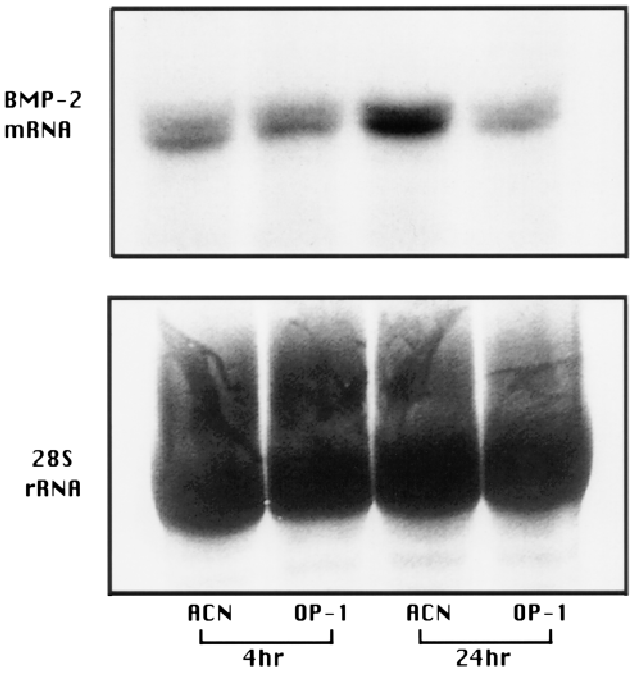

Fig. 1. Effect of OP-1 treatment on the mRNA level for BMP-2 in U2 cells. The cells were plated in 100 mm Petri dishes in 10 ml DMEM containing 10% calf serum and grown to 50–60% confluency. The medium was then changed to 10 ml of fresh DMEM containing 0.1% BSA and the effectors were added. Serum-free cultures of U2 cells were treated for 4 and 24 hours with vehicle or 100 ng/ml OP-1 after which RNA was extracted. Total RNA was subjected to Northern analysis and hybridized with the BMP-2 cDNA probe as described in the methods section. The blot shown is a representative of two independent experiments

In both U2 and SaOS-2 cells, BMP-4 cDNA recognized two transcripts of approximately 2.0 kb and 1.6 kb. In U2 cells, OP-1 at 100 ng/ml decreased BMP-4 mRNA to 56% and 28% after 4 hours and 24 hours of treatment, respectively, of vehicle-treated control (Fig. 2A). In SaOS-2 cells, the relative level of both 2.0 and 1.6 kb BMP-4 transcripts increased with time in culture. One hundred ng/ml of OP-1 decreased BMP-4 mRNA to 66% and 20% after 4 hours and 24 hours of treatment, respectively, of vehicle-treated SaOS-2 control cultures. (Fig. 2B).

The BMP-6 cDNA probe hybridized with a single transcript of 4.0 kb in both U2 and SaOS-2 cells. In U2 cells, treatment with OP-1 at 100 ng/ml increased BMP-6 mRNA level 2.3-and 5-fold after 4 hours and 24 hours of treatment, respectively, of vehicle-treated control (Fig. 3A). In SaOS-2 cells, 100 ng/ml OP-1 increased the BMP-6 mRNA level 2-and 3-fold after 4 hours and 24 hours, respectively, of vehicle-treated control (Fig. 3B).

The OP-1 probe hybridized with a more abundant 2.4 kb transcript and a less abundant 4.1 kb transcript in both cell

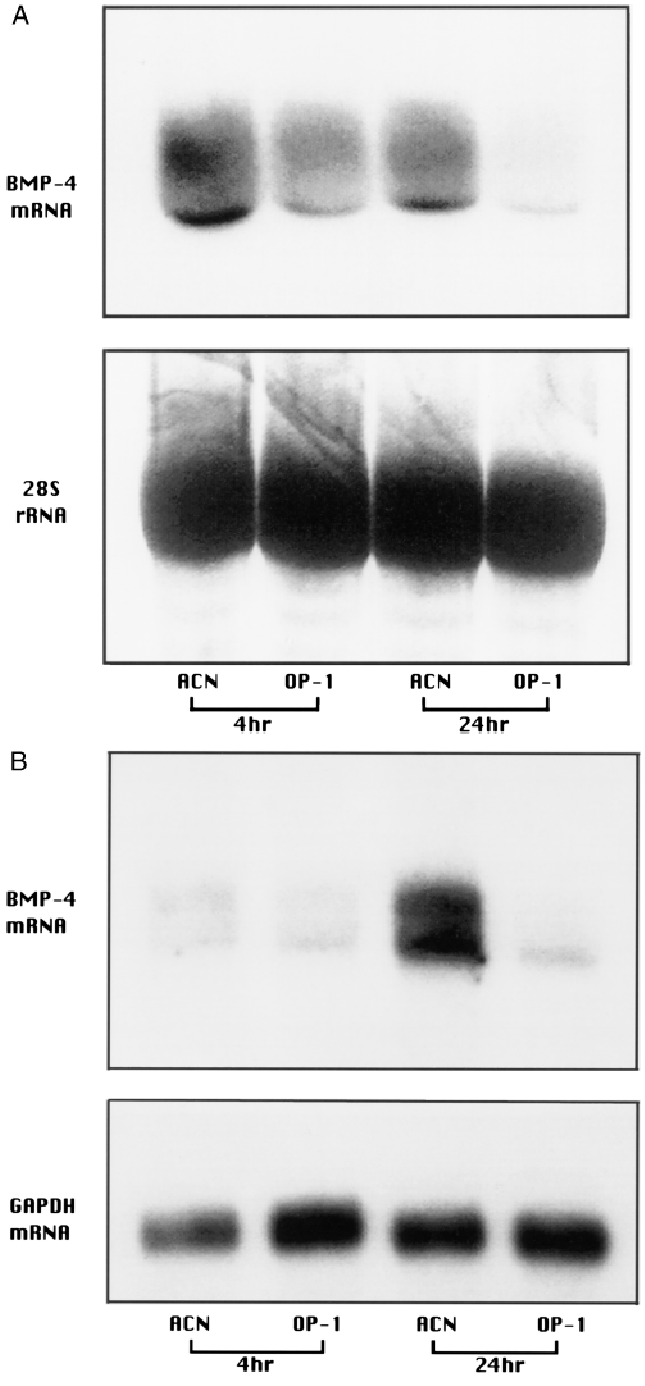

Fig. 2. Effect of OP-1 treatment on the mRNA level for BMP-4 in U2 (**A**) and SaOS-2 (**B**) cells. The cells were plated and treated as described in Figure 1, after which RNA was extracted. Total RNA (U2 cells) or poly(A)+ RNA (SaOS-2 cells) was subjected to Northern analysis and hybridized with the BMP-4 cDNA probe or GAPDH probe as described in Methods. The blot shown is a representative of two independent experiments

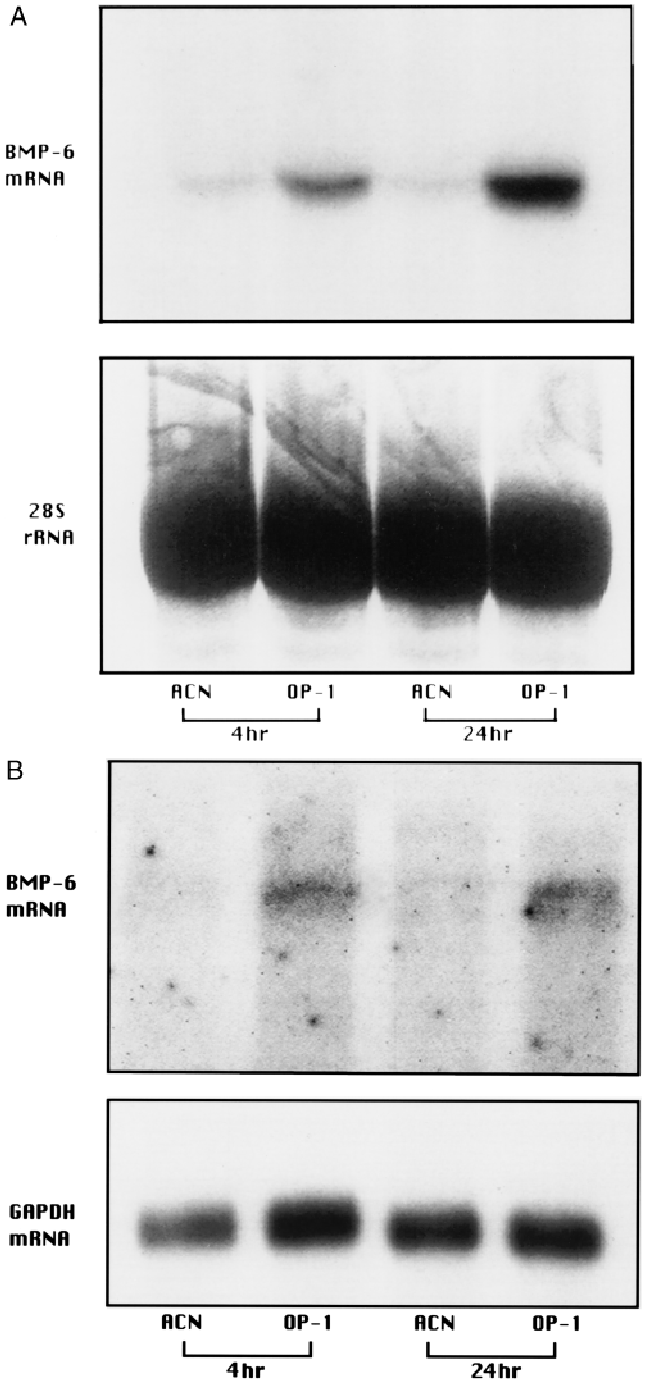

Fig. 3. Effect of OP-1 treatment on the mRNA level for BMP-6 in U2 (**A**) and SaOS-2 (**B**) cells. The cells were plated and treated as described (Fig. 1), and RNA was extracted. Total RNA (U2 cells) or poly(A)+ RNA (SaOS-2 cells) was subjected to Northern analysis and hybridized with the BMP-6 cDNA probe or GAPDH probe as described in the methods section. The blot shown is a representative of two independent experiments

types. OP-1 treatment, however, had no significant effect on the expression of either of these transcripts in U2 or SaOS-2 cells (data not shown). In addition, acetonitrile at the concentration used in this study had no significant effect on the expression of various BMPs compared with control cultures (data not shown).

Discussion

The present study demonstrates for the first time that OP-1 exerts a direct effect on the expression of mRNA levels of the BMP family members, as examined in two cultured human osteosarcoma cell lines, U2 and SaOS-2. OP-1 in-

creased mRNA levels of BMP-6, with a concomitant decrease in mRNA levels of BMP-2 and of BMP-4, thus supporting the hypothesis that OP-1 may exert some of its effects on human osteosarcoma cells by locally regulating the autocrine/paracrine actions of these growth factors. Although BMPs have been shown to exhibit significant biological effects on bone cells *in vitro* and *in vivo*, very little is known about the regulation of expression of various BMPs in any cell type. In this regard, Gazit et al. [20] have recently shown that retinoic acid, which increases alkaline phosphatase expression and responsiveness to PTH in two pluripotent mesenchymal stem cell lines, also modulates the expression of TGF-β and BMP family members. Retinoic acid treatment increased mRNA levels of TGF-β1, TGF-β2, and BMP-6 and decreased mRNA levels of BMP-2 and BMP-4 in C3H-10T 1/2 and C26 stem cell lines. Furthermore, native BMP-3 preparations and rhBMP-4 have been shown to stimulate TGF-β1 mRNA expression in human blood monocytes *in vitro* [15]. These findings indicate that TGF-βs and BMPs may act in concert to initiate osteogenic differentiation and that their local actions may be influenced by both local and systemic modulators.

Several recombinant human BMPs including OP-1 have been shown to independently induce *de novo* bone formation [1–6, 9, 12]. Studies on the mechanisms by which BMPs exert their effects on bone formation suggest that several BMPs exhibit significant biological effects on proliferation and differentiation of osteoblast-line cells *in vitro* [28–31]. Because mixed populations of cells with various degrees of osteoblast differentiation were used for studies on biological effects of BMPs, it is not known at this time whether different BMPs affect the same target cell or exhibit a specificity among different target cells. In addition, it is not known why OP-1 treatment caused a marked increase in BMP-6 expression and a reduction in the expression of BMP-2 and BMP-4 mRNA since the various BMPs tested appear to exhibit similar biological effects on bone cells *in vitro*.

The BMPs form a subset of growth and differentiation factors within the TGF-β superfamily. It is interesting to note that OP-1 treatment increases the mRNA level of BMP-6, which is a member of the same subgroup, and decreases mRNA level of BMP-2 and BMP-4, members of a different TGF-β superfamily subgroup. Consistent with the findings of this study, retinoic acid which initiates differentiation of pluripotent stem cells towards the osteoblast lineage also increased mRNA level of BMP-6 while producing a corresponding decrease in the mRNA levels of BMP-2 and BMP-4 [20]. Although it has not been established whether changes in mRNA levels for various BMPs correlate with changes in protein levels due to lack of availability of valid assays, these data raise the possibility that each subgroup of the BMP family may have a specific role in the initiation of mesenchymal cell migration, proliferation, and differentiation activities involved in bone morphogenesis.

In conclusion, the present study provides evidence that OP-1 influences the mRNA levels of BMP-2, -4, and -6 in human osteosarcoma cells, suggesting that OP-1 may regulate synthesis of one or more polypeptide growth factors besides IGF-II [16] to mediate some of its effects on proliferation and differentiation of osteoblast cell lineage. These data also suggest that BMP actions on osteoblast line of cells may involve complex regulation of gene expression including both increases and decreases in the mRNA expression of related members of the BMP family. However, further studies are needed to establish the relationship between OP-1 induced regulation of the BMP family and initiation of osteoblast cell proliferation/differentiation.

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