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Osteogenic Protein-1 (OP-1, BMP-7) Induces Osteoblastic Cell Differentiation of the Pluripotent Mesenchymal Cell Line C2C12

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Abstract The effects of Osteogenic Protein-1 (OP-1, BMP-7) on the differentiation of the pluripotent mesenchymal cell line, C2C12, were examined. OP-1 at 50 ng/ml partially inhibited myotube formation in C2C12 cells, while OP-1 at 200 ng/ml completely inhibited myotube formation and induced the formation of cells displaying osteoblastic morphology. High concentrations of OP-1 elevated the alkaline phosphatase (AP) activity dramatically, both as a function of time and OP-1 concentration. Osteocalcin (OC) mRNA expression was detected as early as 8 days in OP-1-treated cultures and subsequently increased considerably. Expression of bone sialoprotein (BSP) mRNA was low in control cultures and stimulated by OP-1. Collagen type I mRNA expression was enhanced by OP-1 during the early days in culture, but gradually decreased thereafter. MyoD mRNA expression, high in control cultures, was suppressed by OP-1 in a dose- and time-dependent manner. OP-1 enhanced ActR-I mRNA expression and significantly elevated the mRNA expressions of BMP-1, BMP-4, BMP-5, GDF-6, and GDF-8. The present results indicate that OP-1 is a potent inducer of C2C12 differentiation into osteoblastic cells. J. Cell. Biochem. 87: 292–304, 2002. © 2002 Wiley-Liss, Inc.

Key words: osteogenic protein-1; bone morphogenetic protein-7; osteoblastic cell differentiation; C2C12; gene expression; BMP expression; BMP receptor expression

Urist [1965] first reported that demineralized bone matrix could induce ectopic bone formation when implanted into muscular tissues and later attributed the activity to a factor(s) named bone morphogenetic protein (BMP) [Urist and Strates, 1971]. Subsequently, numerous BMPs have been discovered, purified, and their genes cloned. BMPs belong to the transforming growth factor-β (TGF-β) superfamily [Ozkaynak et al., 1990; Sampath et al., 1990; Kingsley, 1994; Wozney and Rosen, 1998; Reddi, 2000]. Based on the degree of their sequence homology, they can be further classified into several subfamilies: the BMP-2/BMP-4 subfamily, the BMP-3 subfamily, the BMP-5/BMP-6/BMP-7(OP-1)/BMP-8 subfamily, the BMP-9 subfamily, and the BMP-12(GDF-7, CDMP-3)/BMP-13(GDF-6, CDMP-2)/BMP-14 (GDF-5, CDMP-1) subfamily. Several BMPs exhibit multiple biological activities on different cell types [Dudley et al., 1995; Luo et al., 1995]. For example, Osteogenic Protein-1 (OP-1) [Asahina et al., 1993; Chen et al., 1995; Wu et al., 1997; Klein-Nulend et al., 1998], BMP-2, and BMP-4 [Paralkar et al., 1992; Katagiri et al., 1994; Hogan, 1996] induce bone and cartilage formation in vivo and stimulate expression of the osteoblast phenotype in osteoprogenitor cells in vitro [Thies et al., 1992; Kawasaki et al., 1998]. OP-1 also appears to be involved in the development/differentiation of different organs, such as the neural system, the heart, the kidney, the eye, and the oral tissues.

BMPs transduce their effects through the binding to two types of transmembrane serine/threonine kinase receptors: type I and type II. They are distinguishable by their amino acid sequences and functional features. Both type I and type II receptors bind ligands independently,

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but the binding affinity is increased in the presence of both receptor types [Liu et al., 1995; Nohno et al., 1995; Rosenzweig et al., 1995]. Down-stream signaling also requires both type I and type II receptors. Three type I receptors have been shown to bind BMPs, i.e., activin receptor-like kinase (ALK-2, ActR-I), BMP type IA receptor (BMPR-IA, ALK-3), and BMPR-IB (ALK-6) [Koenig et al., 1994; ten Dijke et al., 1994; Macias-Silva et al., 1998]. Three type II receptors have also been identified, i.e., activin type II receptor (ActR-II), ActR-IIB, and BMPR-II [Liu et al., 1995; Nohno et al., 1995; Rosenzweig et al., 1995; Yamashita et al., 1995].

Previous studies have suggested that the pluripotent mesenchymal precursor cell line C2C12 may be a model to examine the early stage of osteoblast differentiation during bone formation in muscular tissues. For example, BMP-2 (300 ng/ml) inhibited myoblast differentiation of the C2C12 cells and promoted osteoblastic cell differentiation [Katagiri et al., 1994]. Similar results were obtained when C2C12 cells were transfected with a replication-deficient adenoviral vector expressing human BMP-2 [Okubo et al., 1999]. Subsequent studies showed that BMP-6 inhibited growth of C2C12 cells, reaching a maximum inhibition of about 40% at 1 μg/ml. The number of AP-positive cells in C2C12 cells increased in a BMP-6 dose-dependent manner, and BMP-6 appeared to be tenfold more potent than OP-1 in stimulating formation of AP-positive cells at the similar dosage [Ebisawa et al., 1999]. On the contrary, Inada et al. [1996] showed that BMP-12 and -13 inhibited myoblast cell differentiation without the induction of osteoblastic cell differentiation in C2C12 cells. These two BMPs were much less efficient in inhibiting myotube formation than BMP-2. TGF-β alone also inhibited myotube formation, but failed to induce the osteoblastic phenotype. TGF-β potentiated the inhibitory effect of BMP-2 on myotube formation, but also reduced the BMP-2-induced alkaline phosphatase (AP) activity and osteocalcin expression.

Here we report the effects of OP-1 on cell differentiation and gene expression in the pluripotent mesenchymal precursor C2C12 cells. Continuous exposure of C2C12 cells to OP-1 inhibited myotube formation and induced the formation of osteoblasts. Concomitantly, MyoD mRNA expression was suppressed, but the AP activity and the mRNA expression of osteocalcin (OC) as well as bone sialoprotein (BSP) were stimulated by OP-1. Northern blot analysis also showed detectable mRNA levels coding for ActR-I, BMPR-IA, BMPR-IB, and BMPR-II in control cells. OP-1 stimulated ActR-I mRNA expression, but did not appear to alter the expression of the others.

MATERIALS AND METHODS

Materials

Recombinant human OP-1 was provided by Stryker Biotech (Hopkinton, MA) and was dissolved in 47.5% ethanol/0.01% trifluoroacetic acid. Radioisotopes were purchased from ICN (Irvine, CA). Fetal bovine serum (FBS), Hank's Balanced Salt Solution (HBSS), Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin, trypsin-EDTA, and collagenase were purchased from Life Technologies (Grand Island, NY). TRI Reagent was from Sigma (St. Louis, MO). All reagents were of molecular biology grade. All buffers were prepared with diethylpyrocarbonate-treated water.

Cell Culture and Microscopic Examination

The mouse pluripotent mesenchymal precursor cell line C2C12 was purchased from American Type Culture Collection (Rockville, MD). C2C12 cells were cultured in DMEM containing 10% FBS and penicillin/streptomycin at 37°C in a humidified 5% CO_2 atmosphere. For experimentation, C2C12 cells were subcultured in DMEM containing 5% FBS and in the absence or presence of various concentrations of OP-1. For the AP activity assay, cells were grown in 48-well plates. For isolation of total RNA, cells were grown in 100-mm culture dishes. Media were replenished every 3 days. Cell morphological changes were monitored with a phase contrast microscope, and the images were captured after 2, 4, 8, 12, and 16 days of treatment, using an Olympus CK2 inverted microscope equipped with a CCD camera.

Alkaline Phosphatase Activity Assay

After 2, 5, 8, 12, and 15 days of culturing in the presence of OP-1, cells were rinsed with PBS and lysed by sonication in 0.1% Triton X-100 in PBS (100 μl/well) for 5 min at room temperature. The total cellular AP activity in C2C12 cells was measured using a commercial assay

kit (Sigma Chemical Co.) as described previously [Yeh et al., 1996]. Reactions were terminated by the addition of 0.5N NaOH. Absorbance of the reaction mixture was measured at 405 nm using a MRX-II microplate reader (Dynex Technologies, Chantilly, VA). Protein was measured according to the method of Bradford [1976] using BSA as a standard. AP activity was expressed as nanomoles of p-nitrophenol liberated per microgram of total cellular protein.

Northern Blot Analysis

After 2, 4, 8, 12, and 16 days of culturing in the presence of 0, 50, or 200 ng/ml of OP-1, total RNA was isolated using the TRI reagent following the manufacturer's recommendation. The intactness of the RNA preparation was examined by agarose (1%) gel electrophoresis followed by ethidium bromide staining. Only RNA preparations showing intact species were used for subsequent analyses. The cDNA probe for the rat Cbfa1 gene was a 680-bp fragment isolated from the plasmid with EcoRI digestion. The plasmid contained the 680-bp fragment of the rat Cbfa1 gene cloned in the pCRII-TOPO vector (Invitrogen, Carlsbad, CA). The Cbfa1 sequence was generated by RT-PCR. The forward primer was (5′) ATG CTT CAT TCG CCT CAC AAA CAA CCA (3′); the reverse primer: (5′) GAA GGC CAC GGG CAG GGT CTT GTT GCA (3′). The cDNA probe for MyoD was a 440-bp fragment isolated from the plasmid with PstI digestion. The plasmid contained a 2.25 kb mouse MyoD cDNA cloned in the pT7T3D-Pac vector was obtained from ATCC (clone ID 1064620). The cDNA probes for OC, BSP, TIC, ActR-I, BMPR-IA, BMPR-IB, and BMPR-II were obtained by digestion of the corresponding plasmids with the appropriate restriction endonucleases as reported previously [Yeh et al., 2000]. The cDNA probes were labeled with ^{32}P α-dATP using the StripEZ DNA labeling kit from Ambion (Austin, TX).

Northern analyses were conducted as previously described [Yeh et al., 1997]. Briefly, total RNAs (20 μg) were denatured and analyzed on 2.2 M formaldehyde/1% GTG agarose gels. RNA standards (0.24–9.5 kb) from Life technologies were used as size markers. The fractionated RNA was transferred onto a "Nytran Plus" membrane using a Turboblot apparatus (Schleicher & Schuell, Inc., Keene, NH). The lane containing the standards was removed from the blot, and the RNA was covalently linked to the membrane using a UV Crosslinker (Stratagene, La Jolla, CA). The membranes were incubated overnight at 42°C with the cDNA probes. The radioactive signal was detected using the PhosphorImager, and the intensity of the signal was quantified using the ImageQuant Software from Molecular Dynamics (Sunnyvale, CA). Before probing with another DNA probe, the signal from the previous probe was stripped from the blot using Ambion's StripEZ Degradation and Reconstitution buffers following manufacturer's recommendation. The blots were also probed with an 18S rRNA oligonucleotide probe to correct for loading variations.

RNase Protection Assay

Twenty micrograms of total RNA were used to determine the mRNA levels for other BMPs and GDFs by RNase protection assay (RPA). The RiboQuant RPA kits with the mBMP-1 and the mGDF-1 Multi-Probe Template Sets were purchased from BD PharMingen (San Diego, CA) and used according to the manufacturer's instruction. The mBMP-1 kit allows detection of mRNAs for BMP-1, -2, -3, -4, -5, -6, -7, -8A and -8B with the protected fragment of 148, 160, 181, 226, 253, 283, 316, 353, and 133 nucleotides in length, respectively. The mGDF-1 kit allows detection of mRNAs for GDF-1, -3, -5, -6, -8, and -9 with the protected fragment of 148, 160, 181, 226, 283, and 316 nucleotides in length, respectively. Both kits also allow detection of mRNA for ribosomal protein L32 and GAPDH. Their mRNA levels were used for correcting sampling or technique errors. The protected RNA fragments were fractionated on 5% polyacrylamide gel containing 8 M urea. After electrophoresis, the gel was fixed in 10% acetic acid/10% methanol for 10 min, dried, and exposed to a Phosphorscreen. Radioactive bands were detected using the PhosphorImager and their intensities were quantified with the ImageQuant Software (Molecular Dynamics, Sunnyvale, CA).

Statistical Analysis

Data are presented as the mean ± SEM. Statistical differences between means were determined by one-way ANOVA, followed by post-hoc Least Significant Difference Multiple Comparisons in the SIMSTAT3 software package (Normand Peladeau, Provalis Research, Montreal, Canada). Differences at $P < 0.05$ were considered significant.

RESULTS

Conversion of C2C12 to Cells With Osteoblastic Morphology When Cultured in the Presence of OP-1

Figure 1 shows a time study of the morphology of cultures treated with solvent, 50 or 200 ng/ml of OP-1. In the control cultures, C2C12 cells were elongated in shape, resembling myoblastic cells. In the presence of low concentrations of OP-1, the cells began to change morphology around day 4 after treatment, but still maintained the elongated morphology. When incubated with OP-1 at 200 ng/ml, cells assumed a morphology that is more akin to that of osteoblastic cells, beginning about day 4. The osteoblastic cell morphology persisted thereafter and the cultures became quite confluent.

Total AP Activity was Stimulated in Cultures Grown in the Presence of OP-1

In the absence of OP-1, a very low level of AP activity was detected in the C2C12 cultures, for as long as 15 days. When C2C12 cells were cultured in the presence of various concentrations of OP-1, the total AP activity in the cell lysates changed as a function of both the time in culture (Fig. 2A) and the OP-1 concentration (Fig. 2B). In the presence of low concentrations of OP-1 ($<$100 ng/ml), the AP activity increased only slightly beyond the basal level, for as long as 15 days in culture. This observation is in agreement with the observed morphology (Fig. 1), i.e., most of the cells remained myoblastic. However, in the presence of higher concentrations of OP-1 ($\geq$200 ng/ml), the AP activity was elevated significantly beyond the control, and increased dramatically as a function of time. Specifically, the total AP activity was not notably elevated prior to day 8 of treatment in all the cultures treated with the range of OP-1 concentrations tested (Fig. 2A). After 8 days, cells cultured in OP-1 at 200 ng/ml or higher concentrations showed significant elevation in AP activity. It is noteworthy that the magnitude of the response to OP-1 was more dramatic on day 12 than those on day 8 and 15. On day 8 and 15, the AP activity in cells treated with 400 ng/ml of OP-1 was about 27- and 40-fold higher than the control, respectively. On day 12, the AP activity was about 74-fold higher.

Effects of OP-1 on mRNA Expression of Selected Osteoblastic Cell Markers

To confirm further that the cells cultured in the presence of OP-1 became osteoblastic in nature, the mRNA level of several biochemical markers characteristic of osteoblastic cells was measured by Northern blot analysis. These included OC, BSP, and type I collagen (TIC). Figure 3 shows a representative PhosphorImage of the Northern blots and Figure 4 shows the quantitative data. No OC mRNA was detected in cultures treated with vehicle or OP-1 at 50 ng/ml for up to 16 days in culture (Fig. 4A). A significant increase in the OC mRNA level was detected at 8 days in OP-1-treated cultures (200 ng/ml) and its level continued to increase, reaching a 4- to 5-fold stimulation at 16 days.

A very low level of BSP mRNA was detected in cultures treated with vehicle or OP-1 at 50 ng/ml. The level remained low throughout the 16 days of culture (Fig. 3). In cultures treated with OP-1 at 200 ng/ml, a considerable increase in BSP mRNA was observed at 8 days and its level continued to increase, reaching a fivefold stimulation at 12 days, and remained elevated at 16 days in culture (Fig. 4B).

Figure 4C shows the quantitative data on type I collagen mRNA expression. The level was low but detectable in control cultures up to about 12 days and became undetectable afterwards (Fig. 3). In cultures treated with either the low or the high concentration of OP-1, the mRNA level increased by about 40% during the early stages (2–8 days), but dropped dramatically to the control level thereafter.

Effects of OP-1 on mRNA Expression of Selected Transcription Regulatory Factors

To assess whether the expression of regulator factors of myogeneic differentiation is inhibited by OP-1, MyoD mRNA expression was examined in C2C12 cells cultured in the absence and presence of two concentrations of OP-1 for varying time periods. Figure 3 shows that MyoD mRNA expression decreased both as a function of time of treatment and OP-1 concentration. At the low OP-1 concentration, MyoD mRNA expression was stimulated transiently from 4 to 8 days, reaching a peak at 8 days, but decreased thereafter to an almost non-detectable level on day 16 (Figs. 3 and 5A). At 200 ng/ml of OP-1, the MyoD mRNA level decreased as early as 4 days and became completely undetectable at 12 days

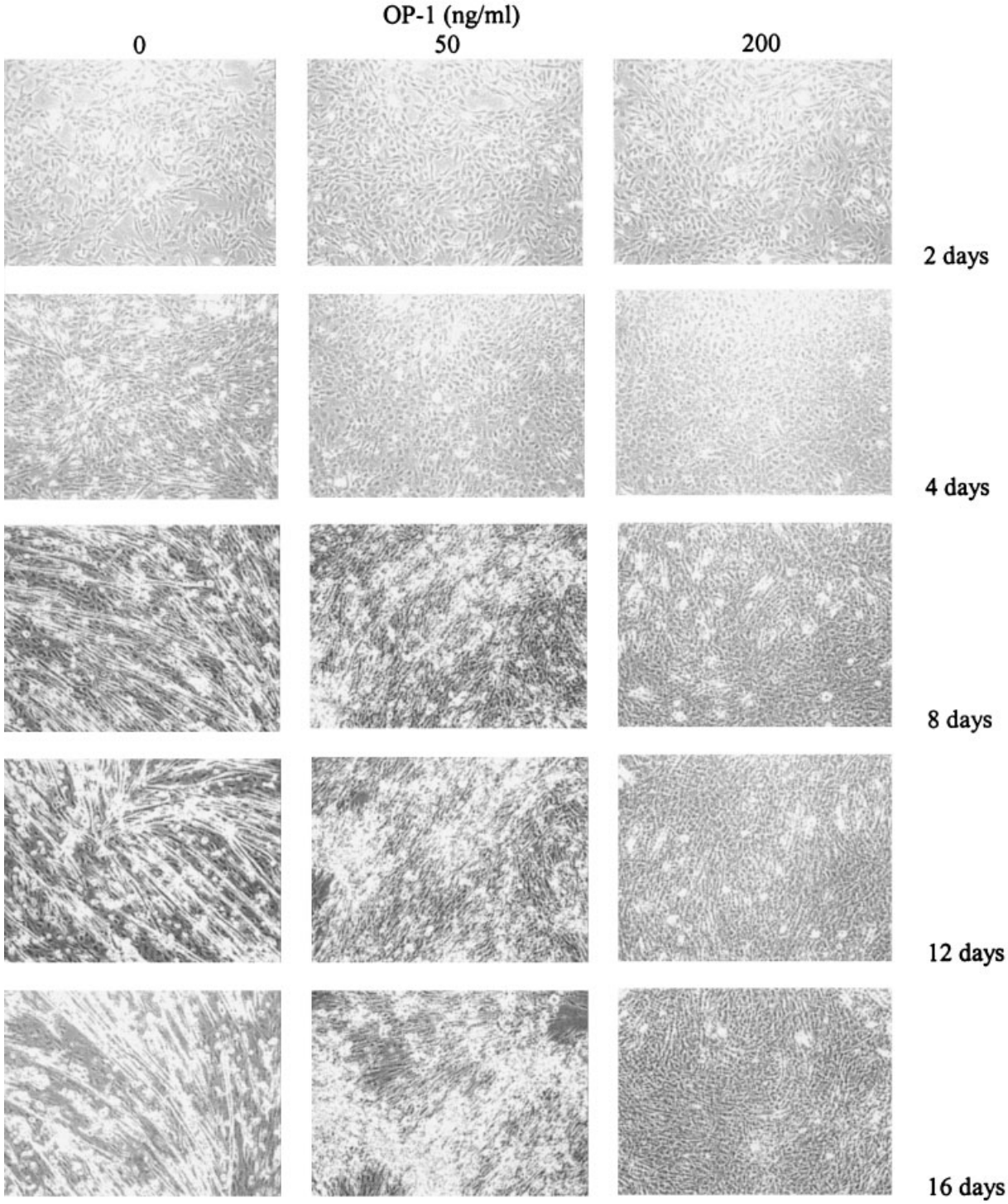

Fig. 1. Morphological changes in C2C12 cells cultured in the presence of OP-1. C2C12 cells were cultured in the absence or presence of 50 or 200 ng/ml of OP-1. Media were changed every 3 days. Cell morphology was monitored with a phase contrast microscope, and the images were captured with a CCD camera. Representative images (phase contrast with 100× magnification) are presented.

(Fig. 3). The expression of an osteoblast specific transcription factor, Runx2/Cbfa1, was also studied by Northern blot analysis. Figure 3 shows that a relatively high level of Runx2/Cbfa1 mRNA level was detected in control cultures. In cells cultured in the presence of a low concentration of OP-1, the Runx2/Cbfa1 mRNA level increased slightly (by about 20%

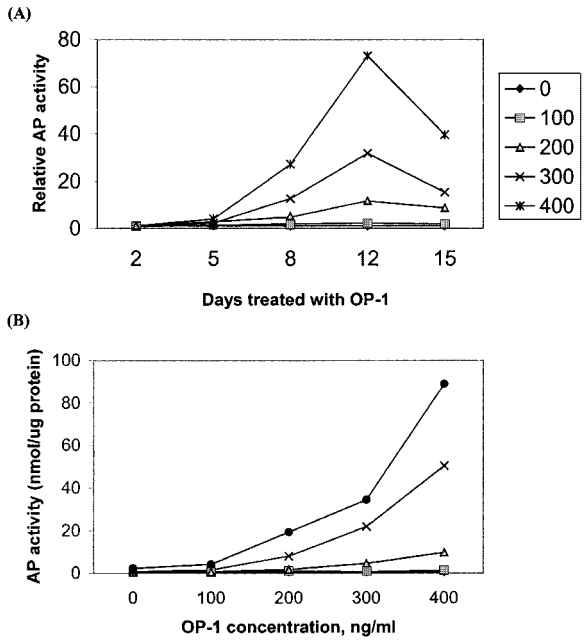

Fig. 2. Alkaline phosphatase activity in C2C12 cultures as a function of time of OP-1 treatment (**A**) and OP-1 concentration (**B**). Cells were grown in 48-well plates in the presence of OP-1. Control cultures were treated with equal amount of vehicle. At the indicated time, total cellular AP activity was measured and expressed as nanomoles of p-nitrophenol liberated per microgram of total cellular protein. Values represented mean ± SEM of three independent determinations. (A) ◆, 0; ■, 100; ▲, 200; ✖, 300; ✱, 400 ng/ml of OP-1. (B) ●, 15; X, 12; ▲, 8; ■, 5; ✱, 2 days of treatment.

compared to the same day control) at day 4 and then dropped to the control level thereafter. In cells cultured in 200 ng/ml of OP-1, the Runx2/Cbfa1 mRNA level raised slightly by about 16–20% compared to the same day control throughout the remaining time in culture (Fig. 5B).

Effects of OP-1 on OP-1 Receptor Gene Expression

In light of the results described above and previous findings that OP-1 differentially regulated BMPR mRNA expression in osteoblastic cells derived from fetal rat calvaria [Yeh et al., 2000], we examined the expression of ActR-I, BMPR-IA, BMPR-IB, and BMPR-II by Northern blot analysis. Figure 6 shows a representative PhosphorImage and the quantitative data are shown in Figure 7. In control cultures, the steady-state mRNA levels for all four receptor types were detectable and remained unchanged throughout the 16 days of culture. Of the four receptor types studied, the ActR-I mRNA level increased most significantly (about 40% higher than the same day control) in cultures treated

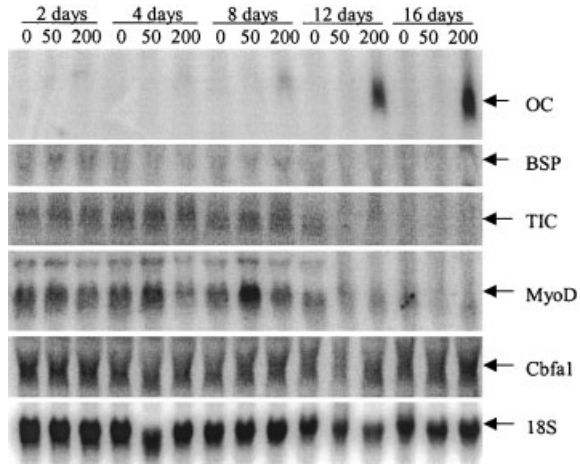

Fig. 3. Effects of continuous OP-1 treatment on bone sialoprotein (BSP), osteocalcin (OC), type I collagen (TIC), MyoD, Runx1/Cbfa1 mRNA expression in C2C12 cultures. C2C12 cells were grown in 100-mm dishes in the presence of vehicle or OP-1 (50 or 200 ng/ml) for 2, 4, 8, 12, and 16 days. Total RNA was isolated using the TRI reagent. Twenty micrograms of total RNA was fractionated on an agarose gel containing formaldehyde, and subsequently transferred to a Nytran Plus membrane. The mRNA expressions of OC and BSP, type I collagen (TIC), MyoD and Cbfa1 were measured by Northern analysis using ^{32}P-labeled cDNA probes. The blots were also hybridized with the oligonucleotide probe for 18S rRNA. Representative PhosphorImages are presented.

with the high OP-1 concentration (Fig. 7A). The increase began at day 4 and remained at the elevated level thereafter. The steady-state mRNA levels for BMPR-IA, BMPR-IB, and BMPR-II remained relatively unchanged throughout the 16 days in culture for both the control and the OP-1-treated cells (Fig. 7B–D).

Effects of OP-1 on BMP-1, -4, -5, -6, and -8A mRNA Expression

Effects of continuous OP-1 treatment on the mRNA expression of several BMPs as a function of culture time were examined by RPA. Figure 8 is a representative PhosphorImage showing the protected fragments for BMP-1, -4, -5, -6, and -8A in control and C2C12 cells treated with two concentrations of OP-1 up to 16 days. The mRNA expression level for each BMP was quantified and normalized to that of ribosomal protein L32. The relative levels of mRNA for the different BMPs as a function of time and OP-1 concentration are shown in Figure 9.

BMP mRNA Expression in Control Cultures

In control cultures, the BMP-1 mRNA level was detectable and remained unchanged throughout the 16 days in culture (Fig. 8). The BMP-4

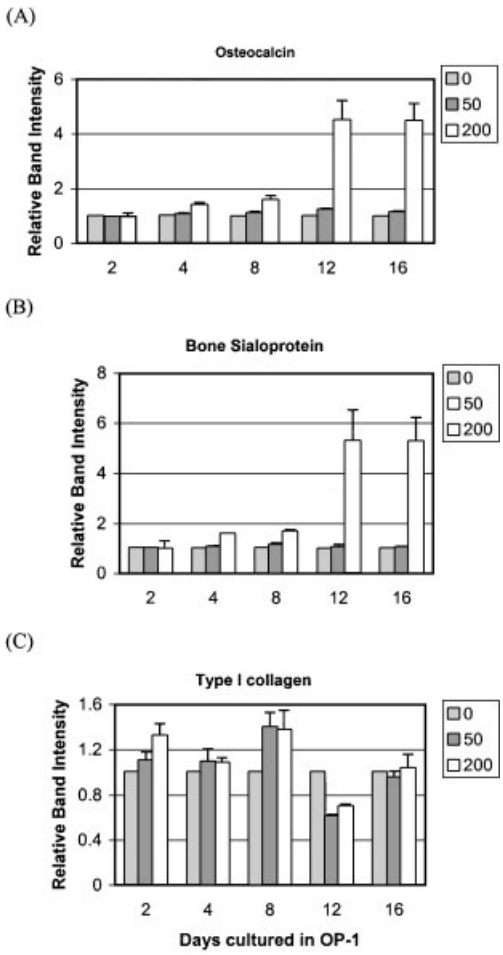

Fig. 4. Quantitative analysis of (**A**) OC, (**B**) BSP, and (**C**) TIC mRNA levels in long-term cultures of C2C12 cells in the presence of OP-1. The intensity of the hybridized RNA species on Northern blots, as shown in Figure 3, was analyzed by the ImageQuant software. The mRNA level was normalized to the 18S rRNA level. The normalized mRNA level was then compared to that in the same day control (as 1). Values represent mean ± SEM from 2 to 4 independent determinations.

mRNA expression was significantly lower than those of BMP-1 initially and changed during culture, reaching a maximum of threefold after 16 days. The BMP-5 and -6 mRNA expressions were very low initially, but increased gradually beginning at 12 days, reaching a maximum of about threefold at 16 days. BMP-8A mRNAs were not detectable initially, but became detectable about 12 days, reaching a maximum of about twofold at 16 days.

BMP mRNA Expression in OP-1-Treated Cultures

Figures 8 and 9 also show the effects of OP-1 on the mRNA expression of several BMP members. At both concentrations, OP-1 increased

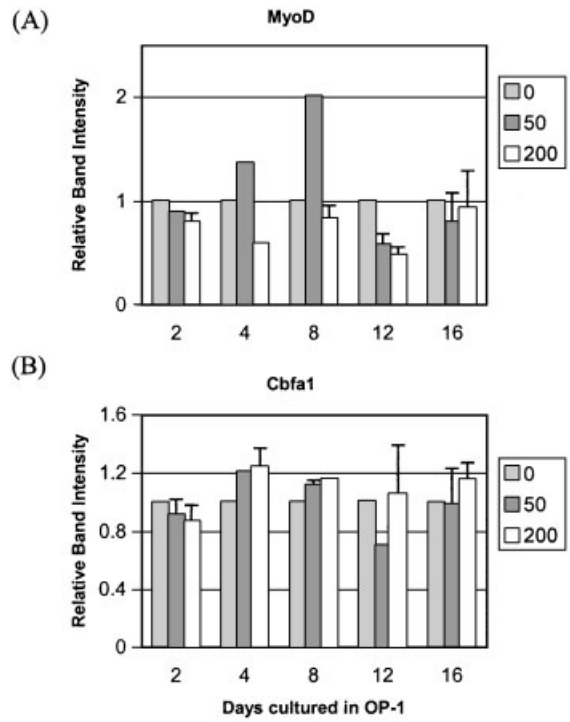

Fig. 5. Quantitative analysis of (**A**) MyoD, and (**B**) Runx1/Cbfa1 mRNA levels in long-term cultures of C2C12 cells grown in the presence of OP-1. See legend of Figure 4.

BMP-1 mRNA expression initially in an OP-1-dose-dependent manner, but reduced it to the initial control level after 12–16 days. The BMP-4 mRNA levels also increased in an OP-1-dose-dependent manner. In cells cultured in the

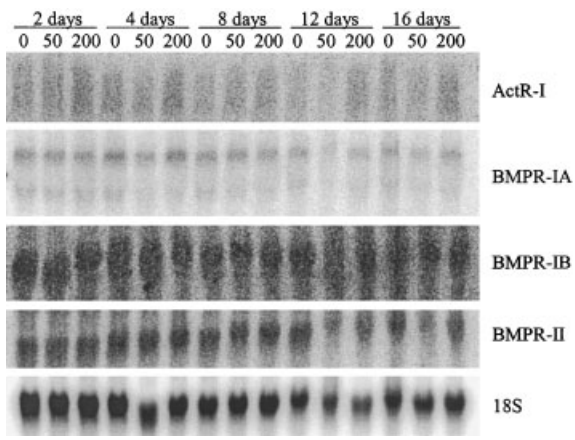

Fig. 6. Northern analysis of the effect of OP-1 on BMP receptor mRNA expression in long-term cultures of C2C12 cells grown in the presence of OP-1. C2C12 cells were treated for 2, 4, 8, 12, and 16 days with different concentrations of OP-1 (0, 50, or 200 ng/ml). Total RNA was isolated as described in Figure 3. The blots were hybridized with the cDNA probes for ActR-I, BMPR-IA, BMPR-IB, and BMPR-II sequentially, and finally the oligonucleotide probe for 18S rRNA. After washings, the blots were exposed to a PhosphorImage screen.

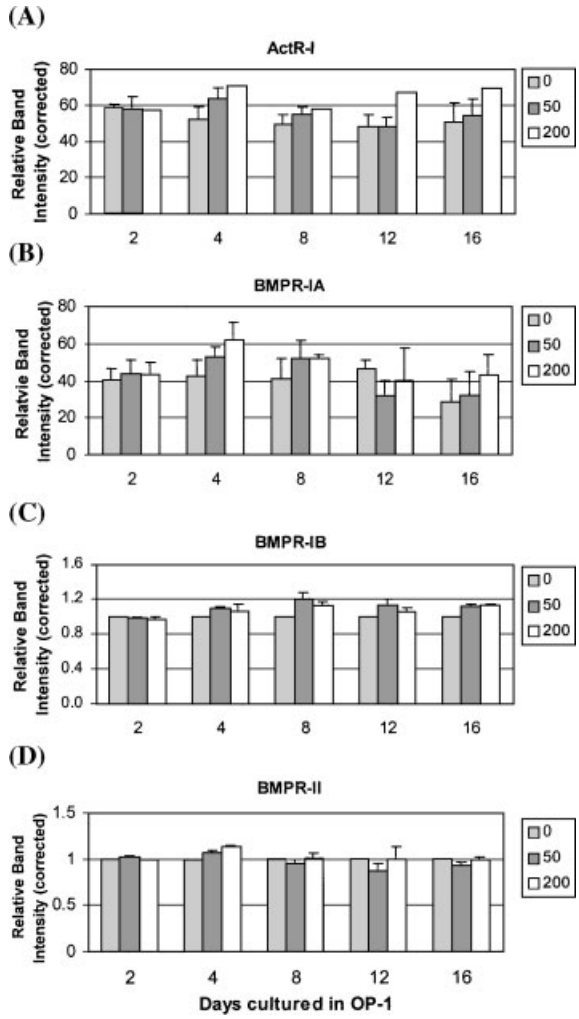

Fig. 7. Quantitative analysis of the BMP receptor mRNA level in long-term cultures of C2C12 cells grown in the presence of OP-1. The intensity of the hybridized RNA species on Northern blots, as shown in Figure 6, was analyzed by the ImageQuant software. The mRNA level was normalized to the 18S rRNA level. The normalized mRNA level was then compared to that in the same day control (as 1). Values represent mean ± SEM from 2 to 3 independent determinations. (**A**) ActR-I, (**B**) BMPR-IA, (**C**) BMPR-IB, and (**D**) BMPR-II.

presence of OP-1 at 50 ng/ml increased by about fivefold at day 4 but began to decline thereafter to the control level. The BMP-4 mRNA level increased by about 10-fold at 4 days and an additional 10-fold at 8 days in cells cultured in the presence of OP-1 at 200 ng/ml. However, the level dropped thereafter. The BMP-5 mRNA levels also increased in an OP-1-dose-dependent manner, reaching a maximum of about fourfold at 8 days. Both the BMP-6 and -8A mRNA levels were not changed significantly.

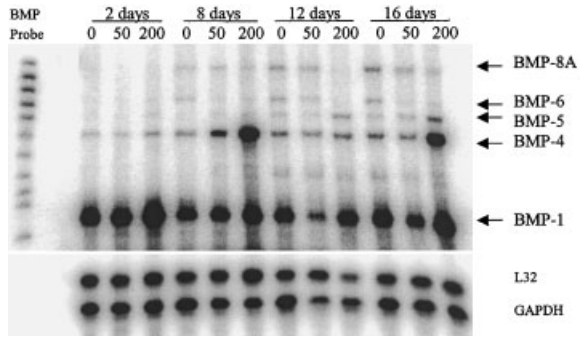

Fig. 8. RNase protection analysis of the effect of OP-1 on BMP mRNA expression in long-term cultures of C2C12 cells. Confluent cultures were treated with vehicle or OP-1 (50 or 200 ng/ml) for 2, 8, 12, and 16 days. Total RNA was isolated using the TRI reagent. Twenty micrograms of total RNA was used for the measurement of BMP mRNA expression by the RNase protection assay. The protected RNA fragments were fractionated on 5% polyacrylamide gels containing 8 M urea and detected by PhosphorImaging. Positions of labeled probes for the different BMPs and the two housekeeping gene controls (ribosomal protein L32 and GAPDH) are marked on the left of the image. The protected fragments are indicated on the right with arrows.

Effects of OP-1 on GDF mRNA Expression

Effects of continuous OP-1 treatment on the mRNA expression of several GDFs as a function of time in culture were examined by RPA.

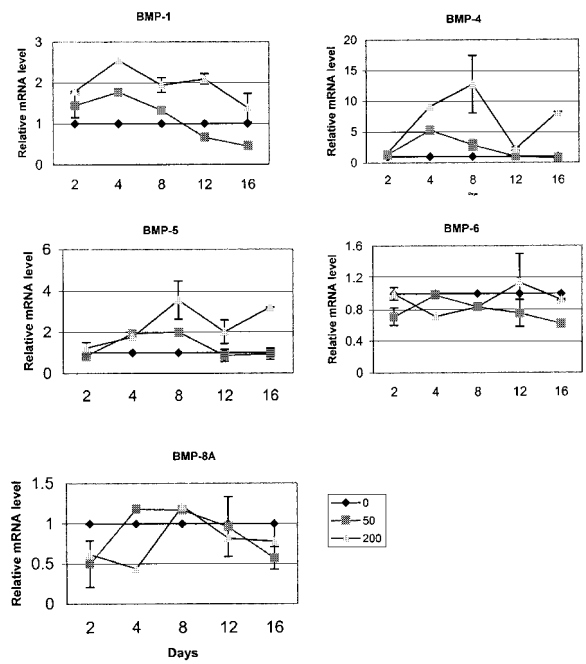

Fig. 9. Quantitative analysis of the BMP mRNA levels in C2C12 cells. The intensity of the protected fragments as shown in Figure 8 was analyzed, quantified using the PhospImageQuant Software, and normalized to the L32 expression level. Values represent mean ± SEM from two different determinations.

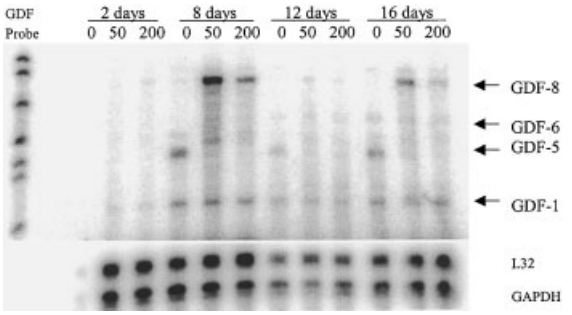

Fig. 10. RNase protection analysis of the effect of OP-1 on GDF mRNA expression in long-term cultures of C2C12 cells. Same as in Figure 8.

Figure 10 is a representative PhosphorImage showing the protected fragments for GDF-1, -5, -6, and -8A in C2C12 cells cultured in the presence of vehicle or two concentrations of OP-1 up to 16 days. The mRNA expression level for each GDF was quantified and normalized to the mRNA level of ribosomal protein L32. The relative levels of mRNA for the different GDFs as a function of time and OP-1 concentration are shown in Figure 11.

GDF mRNA Expression in Control Cultures

In control cultures, GDF-1 mRNA appeared to be the most abundant among the GDFs examined. The GDF-1 mRNA level increased gradually, reaching a maximum of about fivefold at 16 days compared to the value at day 2. GDF-5 mRNA expression also increased gradually, reaching a maximum of about eightfold at 16 days. GDF-6 and -8 mRNA expressions were relatively low and remained unchanged throughout the culture period examined.

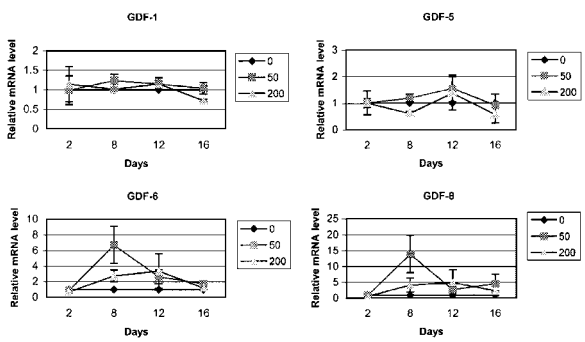

Fig. 11. Quantitative analysis of the GDF mRNA levels in C2C12 cells. The intensity of the protected fragments as shown in Figure 10 was analyzed and quantified as in Figure 9. Values represent mean ± SEM from two different determinations.

GDF mRNA Expression in OP-1-Treated Cultures

Figures 10 and 11 show the consequences of OP-1 treatment on the expression of several GDF members. In the presence of either OP-1 concentrations (50 or 200 ng/ml), the GDF-1 and -5 mRNA expressions remained unchanged compared to the control. OP-1 treatment (at 50 ng/ml) increased GDF-6 mRNA expressions significantly (about sixfold compared to the same-day control) at 8 days and declined thereafter to the control levels. At a higher OP-1 concentration (200 ng/ml), GDF-6 mRNA expression reached a peak at 12 days with an increase of about threefold (compared to the same-day control), but returned to the same-day control at 16 days. At 50 ng/ml, OP-1 treatment elevated the GDF-8 mRNA level by about 15-fold at 8 days and the level dropped subsequently. Similar to GDF-6, at a higher OP-1 concentration, GDF-8 mRNA expression reached a peak later at 12 days with an increase of about fivefold (compared to the same-day control).

DISCUSSION

In the present study, we demonstrated that continuous exposure of C2C12 cells to a moderate concentration (200 ng/ml) of OP-1 inhibits C2C12 cells from differentiating into myoblasts and induces these cells to differentiate into the osteoblast lineage. Previous studies indicated that several members of the BMP family also could inhibit myoblast differentiation but only a limited number could induce osteoblastic cell differentiation. Those that could induce osteoblastic cell differentiation also appeared to display different potencies.

The present data showed that a noticeable morphological change was observed when cells were treated for 4 days in the presence of 50 ng/ml of OP-1. In the presence of a higher OP-1 concentration (200 ng/ml), a complete conversion to osteoblastic cells occurred. Significant increases in total cellular AP were also detected in these cultures. Previous studies reported that incubation of C2C12 cells for 6 days with BMP-2 (300 ng/ml) inhibited myotube formation, and induced osteoblastic cell formation with >90% of the cells stained positive for AP [Katagiri et al., 1994]. Treatment of C2C12 cells with BMP-6 resulted in a dose-dependent increase in the number of AP-positive cells [Ebisawa et al., 1999].

Three other protein factors were reported to fail to induce osteoblastic cell formation. TGF-β1 (5 ng/ml) almost completely inhibited myotubes formation in C2C12 cells, but did not induce expression of AP activity and OC [Katagiri et al., 1994]. Incubation of C2C12 cells with 300 and 1000 ng/ml of BMP-12 (CDMP-3, GDF-7) or BMP-13 (CDMP-2, GDF-6) for 6 days inhibited myotube formation by about 25 and 30%, respectively, but did not stimulate AP activity in C2C12 cells [Inada et al., 1996]. Thus, BMP-12 and -13 could not induce C2C12 cells into the osteoblastic cell differentiation pathway.

The temporal sequence of gene expression in long-term, OP-1-treated cultures of C2C12 cells appears to be similar to that observed in the primary culture of osteoblastic cells. For example, previous reports showed that the primary culture of FRC cells undergoes distinct stages of cell differentiation and a temporal sequence of gene expression can be observed within each stage of differentiation [Owen et al., 1990; Lian and Stein, 1992; Yao et al., 1994; Yeh et al., 2000]. The AP activity and both BSP and TIC mRNA expressions begin to increase in cultures entering the matrix formation stage. OC mRNA expression occurs later in osteoblastic cell differentiation and coincides with the bone nodule mineralization stage. In C2C12 cells cultured in OP-1, OC and BSP mRNA expression as well as AP activity began to increase around day 8. Both the AP activity and BSP expression peaked at about day 12, whereas OC expression did not peak until about day 16. It is interesting to note that the increase in TIC mRNA expression occurred before that in OC and BSP mRNA, and then returned to the control level thereafter. Since TIC participates in mineralization, it is not clear at present why TIC expression returned to the control level after the transient increase. By comparison, in C2C12 cells treated with BMP-2, an increase in AP and OC mRNA was also observed but it occurred around day 2, much earlier than that observed for OP-1 [Katagiri et al., 1997]. The reason(s) for the difference between the two observations is not clear at present. Although BMP-6 also could induce AP activity in C2C12 cells [Ebisawa et al., 1999], the temporal sequence of the expression of these genes is not known.

The current study also showed that OP-1 at 200 ng/ml suppressed MyoD mRNA expression in C2C12 cells early during cell differentiation. However, at the low OP-1 concentration, MyoD mRNA expression was transiently stimulated at 4–8 days and then returned to the control level. MyoD mRNA expression was also transiently stimulated at 1–3 h by BMP-2 (300 ng/ml), but decreased thereafter [Katagiri et al., 1997]. On the other hand, TGF-β1 (5 ng/ml) inhibited MyoD mRNA expression as early as 3–6 h after treatment. Thus, even though all three protein factors inhibited MyoD mRNA expression in C2C12 cells, the timing of the event appeared to be different. The physiological significance, if any, of the difference in timing must await further experimentation.

The present study showed that Cbfa1 mRNA expression in C2C12 cultures was not significantly stimulated by OP-1 at 200 ng/ml. However, several published reports indicated that treatment of C2C12 cells with BMP-2 and TGF-β resulted in an induction of Runx2/Cbfa1 [Lee et al., 1999, 2000]. In particular, the type II/p57 isoform of Runx2/Cbfa1 was induced by BMP-2 [Banerjee et al., 2001]. In addition, treatment with a BMP4/7 heterodimer (100 ng/ml) enhanced Cbfa1 mRNA expression [Tsuji et al., 1998]. However, these authors also observed that overexpression of Cbfa1 resulted in a suppression of TIC and OC mRNA expression.

The current data indicate that significant levels of mRNA for ActR-I, BMPR-IA, BMPR-IB, and BMPR-II are present in control C2C12 cells. By Northern blot analysis, Akiyama et al. [1997] reported detection of BMPR-IA, and BMPR-II mRNA in C2C12 cells but not BMPR-IB mRNA. Namiki et al. [1997] also reported detection of BMPR-IA and TGF-β type I receptor but not BMPR-IB mRNA in C2C12 cells. However, the same authors demonstrated the presence of BMPR-IB protein on the cell surface of C2C12 cells by affinity binding assay followed by immunoprecipitation.

Previous studies suggested that BMPs may regulate their actions by affecting expression of individual BMP receptors. The present study revealed that the ActR-I mRNA expression was elevated in C2C12 cells cultured in the presence of OP-1. BMPR-IA, BMPR-IB, and BMPR-II mRNA expression levels were not significantly affected. Whether these observed changes in the steady-state mRNA levels are translated to the level of surface receptor proteins remains to be established experimentally. Relevant to the current data is the finding of Namiki et al. [1997] who, using a kinase-domain truncated

BMPR-IA, showed that the inductive effect of BMP-2 on converting the C2C12 cells into the osteoblastic cells is mediated via BMPR-IA. By comparison, OP-1 induces an increase in the mRNA expression of ActR-IA, BMPR-IA, and BMPR-II, but has little effect on the BMPR-IB mRNA expression in FRC cells [Yeh et al., 1998, 2000]. The same study also showed that, in the human SaOS-2 osteosarcoma cells, the ActR-I mRNA expression was increased by OP-1, whereas the BMPR-IA and BMPR-IB mRNA levels remained unchanged. The BMPR-II mRNA levels dropped after 24 h of treatment with OP-1. In contrast, in human TE85 osteosarcoma cells, the mRNA levels for ActR-I, BMPR-IA, BMPR-IB, and BMPR-II were significantly elevated following OP-1 treatment for 24 h. Taken together, it appears that the effects of OP-1 on BMP receptor expression in osteoblastic cells vary widely according to the physiological and differentiation stage of the cell.

It is noteworthy that control C2C12 cells expressed a high level of BMP-1 mRNA which is technically not a BMP. BMP-1 is a cysteine-rich zinc-peptidase that has been suggested to activate latent TGF-β [Sarras, 1996]. In C2C12 cells, cultured in the presence of the high concentration of OP-1, BMP-1 mRNA expression was elevated significantly during the entire culture period, suggesting that BMP-1 might play an important role in the conversion and differentiation of the C2C12 cells to osteoblastic cells.

The BMP-2 mRNA level in C2C12 cells was below detection and OP-1 did not stimulate its expression. On the other hand, OP-1 stimulated significantly the expression of the other member of this subgroup, BMP-4. By comparison, OP-1 did not alter the BMP-2 mRNA expression, but suppressed the BMP-4 mRNA expression in FRC cells [Yeh et al., 2000]. On the other hand, BMP-2 stimulated the BMP-2 mRNA expression but inhibited BMP-4 mRNA initially and stimulated BMP-4 expression during the mineralization phase [Chen et al., 1997].

We previously reported that OP-1 down-regulated the mRNA expressions of two (BMP-5, and -6) out of the three members of the subgroup consisting of BMP-5, -6, and -7, but did not change BMP-7 mRNA expression in primary cultures of FRC cells [Yeh et al., 2000]. In contrast to these observations, OP-1 treatment of C2C12 cells stimulated BMP-5 mRNA expression without affecting BMP-6 mRNA expression. BMP-7 mRNA level was below detection. In the U2 human osteosarcoma cell line, OP-1 treatment increased the BMP-6 mRNA level [Honda et al., 1997]. In another human osteosarcoma cell line, SaOS-2, OP-1 treatment resulted in a slightly different effect, that is, OP-1 decreased BMP-4 mRNA and increased BMP-6 mRNA, but had no effect on the BMP-2 mRNA level. Taken together, these observed differences in the response of the various osteoblastic cells to OP-1 suggest that the state of differentiation and perhaps the physiological state of the osteoblastic cells might play an important role in their responsiveness to the BMPs. The differential expression of the different BMP receptors might also contribute to the variation in responsiveness to the different BMPs.

The present study is the first to report detectable, but varying levels of mRNA coding for GDF-1, -5, -6, and -8 in C2C12 cells. Upon treatment of C2C12 cells with a high concentration of OP-1 (200 ng/ml) to induce osteoblastic cell formation and differentiation, the GDF-1 and -5 mRNA levels were not detectably changed, but the GDF-6 and -8 mRNA levels were elevated significantly. During distractal osteogenesis induced by mechanical-tension stress in rats, BMP-2, -4, but not BMP-6, BMP-7, and GDF-5 mRNA levels were elevated [Sato et al., 1999]. In vivo ectopic implantation studies with GDF-5 and -6 showed that both proteins induced de novo cartilage and bone formation [Erlacher et al., 1998]. They also stimulated osteogenesis in bone marrow-derived progenitor cells, although they were less potent than BMP-6 and OP-1 [Gruber et al., 2000]. Previously, GDF-8, also known as myostatin, was detected in cardiac muscle and its level was up-regulated in cardiomyocytes after infarct [Sharma et al., 1999]. Our observation that OP-1 inhibited myoblast differentiation in C2C12 cells agrees with the previous finding that GDF-8 (myostatin) is a negative regulator of skeletal muscle growth [Hamrick et al., 2000]. Whether GDF-8 plays a positive role in osteogenesis is not clear at present.

In conclusion, we demonstrate that OP-1 is a potent inducer of osteoblastic differentiation of the pluripotent mesenchymal cell C2C12. We have further indicated several molecules, such as ActR-I, as potential mediators via which OP-1 blocks myogenic differentiation and induces osteoblast differentiation in C2C12 cells. Future studies on these molecules and

other downstream signaling molecules, such as the Smads, will be needed to more fully determine the mechanism of action of OP-1 in this cell model. The current findings also reveal a complex interplay of several BMPs and GDFs as well as the BMP receptors. Thus, the current results should provide a molecular basis for future studies to further elucidate the actions of the different BMP members and the receptors.

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