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Cartilage-Derived Morphogenetic Proteins Enhance the Osteogenic Protein-1-Induced Osteoblastic Cell Differentiation of C2C12 Cells

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Previous studies have shown that osteogenic protein-1 (OP-1; also known as BMP-7) induces differentiation of the pluripotent mesenchymal cell line C2C12 into osteoblastic cells. OP-1 also alters the steady-state levels of messenger RNA (mRNA) encoding for the cartilage-derived morphogenetic proteins (CDMPs) in C2C12 cells. In the present study, the effects of exogenous CDMPs on bone cell differentiation induced by OP-1 in C2C12 cells were examined. Exogenous CDMP-1, -2, and -3 synergistically and dose-dependently enhanced OP-1 action in stimulating alkaline phosphatase (AP) activity and osteocalcin (OC) mRNA expression. AP staining studies revealed that the combination of OP-1 and CDMP enhanced OP-1 action by stimulating those cells that had responded to OP-1 and not by activating additional cells. The combination did not change the mRNA expression of the BMPs and their receptors. CDMP-1 enhanced the suppression of the OP-1-induced expression of the myogeneic differentiation regulator MyoD. CDMP-1 and OP-1 alone stimulated Smad5 protein expression, but the combination of OP-1 and CDMP-1 stimulated synergistically Smad5 protein expression. Thus, one mechanism of the observed synergy involved enhancement of the induced Smad5 protein expression. At the same protein concentration, CDMP-1 is most potent in enhancing OP-1 activity in inducing osteoblastic cell differentiation of C2C12 cells. CDMP-3 is about 80% as potent as CDMP-1, and CDMP-2 is the least potent (about 50% of CDMP-1). J. Cell. Physiol. 201: 401–408, 2004. © 2004 Wiley-Liss, Inc.

Bone morphogenetic proteins (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 extent of their sequence homology, the BMP proteins are 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, OP-1 (Asahina et al., 1993; Chen et al., 1995; Wu et al., 1997; Klein-Nulend et al., 1998), BMP-2/BMP-4 (Paralkar et al., 1992; Katagiri et al., 1994; Hogan, 1996) induces bone and cartilage formation in vivo and stimulates 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. With varying degrees of potency, CDMP-1, -2, and -3 have been shown to affect several skeletal processes, including joint formation (Francis-West et al., 1999; Merino et al., 1999; Storm and Kingsley, 1999), tendon/ligament repair (Wolfman et al., 1997; Aspenberg and Forslund, 1999, 2000; Lou et al., 2001; Rickert et al., 2001), and endochondral ossification. For example, the mouse with a mutated GDF-5 (CDMP-1) gene shows a limb brachypodism phenotype and disruption of tail formation (Polinkovsky et al., 1997; Clark et al., 2001). Furthermore, GDF-5 deficiency in mice alters the ultrastructure, mechanical properties, and composition of the Achilles tendon and the cortical bone (Mikic et al., 2001, 2002). The patient with a mutated human CDMP-1 gene shows the Hunter–Thompson syndrome with shortened limb bones and abnormal joint development (Thomas et al., 1996, 1997). Studies with transgenic mice of a mis-expressed CDMP-1 gene show that this protein increases commitment of mesenchymal cells into the

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chondrogenic lineage (Tsumaki et al., 1999). CDMP-1 and -2 stimulate osteogenic differentiation of bone marrow cells (Gruber et al., 2000) and of periosteum-derived cells (Gruber et al., 2001). CDMP-1 and -2 are capable of inducing cartilage and bone formation when implanted ectopically in intramuscular sites (Hotten et al., 1996; Erlacher et al., 1998; Spiro et al., 2000).

Previous studies have suggested that the pluripotent mesenchymal precursor cell line C2C12 may be a suitable model to examine the early stage of osteoblast differentiation during bone formation in muscular tissues. Subsequently, the effects of several members of the BMP family and TGF-β on C2C12 cell differentiation have been reported. For example, TGF-β inhibited myotube formation, but failed to induce the osteoblastic phenotype (Katagiri et al., 1994). On the other hand, BMP-2 (300 ng/ml) inhibited myoblast differentiation of C2C12 cells and promoted osteoblastic cell differentiation (Katagiri et al., 1994). Similar results were reported when C2C12 cells were transfected with a human BMP-2 expressing adenoviral vector (Okubo et al., 1999). TGF-β enhanced the inhibitory effect of BMP-2 on myotube formation, but also reduced the BMP-2-induced alkaline phosphatase (AP) activity and osteocalcin (OC) expression (Katagiri et al., 1994). Treatment of C2C12 cells with BMP-6 led to a dose-dependent increase in AP-positive cells (Ebisawa et al., 1999). Treatment of C2C12 cells with OP-1 inhibited myotube formation and induced osteoblastic cell formation (Yeh et al., 2002). In contrast, CDMP-2 (BMP-13) and -3 (BMP-12) inhibited myoblast cell differentiation without the induction of osteoblastic cell differentiation in C2C12 cells (Inada et al., 1996). Both BMP-12 and -13 were much less efficient in inhibiting myotube formation than BMP-2.

Subsequently, we reported that OP-1 differentially altered the mRNA expression of several BMPs and GDFs in C2C12 cells (Yeh et al., 2002). In particular, C2C12 cells treated with OP-1 showed significant increases in CDMP-1 and -2 mRNA expression compared to the control. These data led to the hypothesis that the effects of OP-1 on C2C12 cell differentiation are mediated in part through changes in gene expression of CDMPs. The present study was pursued to assess whether OP-1 and the CDMPs interact to influence osteoblastic cell differentiation of C2C12 cells.

MATERIALS AND METHODS

Materials

Fetal bovine serum (FBS), Hanks' Balanced Salt Solution (HBSS), Dulbecco's modified Eagle medium (DMEM), penicillin/streptomycin, trypsin–EDTA, and collagenase were purchased from Life Technologies (Grand Island, NY). Recombinant growth factors were provided by Stryker Biotech (Hopkinton, MA) and were dissolved in 47.5% ethanol/0.01% trifluoroacetic acid. Radioisotopes were purchased from ICN (Irvine, CA). 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

C2C12 cells (originally purchased from American Type Culture Collection, Rockville, MD) were cultured in DMEM containing 10% FBS and penicillin/streptomycin at 37°C in a humidified 5% CO_2 atmosphere. Media were replenished every 3 days. For measurement of AP activity, cells were grown in 48-well plates in DMEM containing 5% FBS in the absence or presence of various concentrations of the growth factors. For isolation of total RNA, cells were grown in 100-mm culture dishes.

Alkaline phosphatase activity assay

At the end of the indicated culture period, enzymatic activity was measured in cell lysates that were prepared by sonication in 0.1% Triton X-100 in PBS (100 μl/well) for 5 min at room temperature. The total cellular AP activity was measured using a commercial assay kit (Sigma Chemical Co.) as described previously (Yeh et al., 1997). 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.

Alkaline phosphatase staining

C2C12 cells, grown in 12-well plates, were cultured in the absence or presence of OP-1, CDMP-1, -2, and -3 alone and the combination of a fixed concentration of OP-1 and two different concentrations of each CDMP. After 5 days, the cultures were rinsed with PBS and stained for AP activity using a commercial kit (Sigma). Images of stained cells were captured with a CCD camera.

Northern blot analysis

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 Strip-EZ Degradation and Reconstitution buffers following manufacturer's recommendation (Ambion, Austin, TX). The blots were also probed with an 18S rRNA oligonucleotide probe to correct for loading variations.

The cDNA probe for MyoD was a 440-bp fragment isolated from pT7T3D-Pac (ATCC Clone ID 1064620) following PstI digestion. The cDNA probes for OC and scleraxis were obtained as described previously (Yeh et al., 2002; Tsai et al., 2003) and were labeled with ^{32}P-dATP using the Strip-EZ labeling kit from Ambion.

RNase protection assay (RPA)

The RiboQuant RPA kits with the mBMP-1, the mGDF-1, and the mBMPR 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. The mBMPR kit allows detection of ALK-1, ALK-2 (ActR-I), ALK-3 (BMPR-IA), ALK-4, ALK-5, ALK-6 (BMPR-IB), ALK-7, AVR-2 (ActR-II), AVR2B (ActR-IIB), and MIS2R with the protected fragments of 430, 388, 349, 313, 280, 250, 223, 199, 178, and 161 nucleotides in length, respectively. All three kits also detect mRNA for ribosomal protein L32 and GAPDH as controls, allowing for correcting sampling or technique errors. The protected RNA fragments were separated on 8 M urea/5% polyacrylamide gels. After electrophoresis, gels were fixed and dried. Radioactive bands were detected using the PhosphorImager and their intensities were quantified with the ImageQuant Software (Molecular Dynamics).

Western blot analysis

Cells were treated with solvent, OP-1 (100 ng/ml), CDMP-1 (200 ng/ml), or the combination of OP-1 (100 ng/ml) and CDMP-1 (200 ng/ml). After 5 days, the conditioned media was collected for measurement of CDMP-1 protein levels. Cells were washed thoroughly with PBS and lysed. Proteins in the conditioned media and total cell lysates were analyzed by SDS–PAGE, and transferred to nitrocellulose membranes using a semi-dry Trans-Blot transfer system (Bio-Rad, Hercules, CA). Membranes were blocked at 4°C in 20 mM Tris, pH 7.6, 137 mM NaCl, 0.1% Tween-20, and 5% nonfat dry milk. To detect CDMP-1 in the conditioned media, the membranes were probed with anti-mouse CDMP-1 (R&D Systems, Minneapolis, MN) as the primary antibody followed by anti-goat IgG HRP-conjugated antibody as the secondary antibody. To detect Smad5 in the total cell lysates, the membranes were probed as described above, except anti-human Smad5 antibody (Cell Signaling Technology, Inc., Beverly, MA) and anti-rabbit IgG HRP-conjugated antibody were used as the primary and secondary antibodies, respectively. Immunoreactive bands were detected using the SuperSignal West Femto Maximum Sensitivity Substrate detection system (Pierce, Rockford, IL), according to the manufacturer's instructions.

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).

RESULTS

Effects of exogenous CDMPs on OP-1-stimulated AP activity in C2C12 cells

Published studies showed that OP-1 alone stimulated a dose-dependent increase in AP activity in C2C12 cells (Yeh et al., 2002). The effects of exogenous CDMP-1 on the OP-1-stimulated AP activity in C2C12 cells were examined as a function of time and protein concentration (Fig. 1A). After 5 and 7 days, OP-1 (100 ng/ml) alone stimulated AP activity by about fourfold ($P < 0.01$) and 23 fold ($P < 0.01$), respectively. After 5 and 7 days, exogenous CDMP-1 (200 ng/ml) alone stimulated a two and fourfold ($P < 0.05$) increase in AP activity, respectively. Exogenous CDMP-1 enhanced the OP-1-stimulated AP activity in a dose-dependent manner. Maximum enhancements of about 16- and 85-fold ($P < 0.01$) compared with the solvent-treated control value were observed at 100 ng/ml OP-1 and 200 ng/ml CDMP-1 after 5 and 7 days, respectively. Under these conditions, the AP activity was enhanced by about fourfold ($P < 0.01$) compared with that induced by OP-1 alone for both treatment periods.

The effects of exogenous CDMP-2 on the OP-1-stimulated AP activity in C2C12 cells were also examined as a function of time and protein concentration (Fig. 1B). After 5 days of treatment, exogenous CDMP-2 (up to 200 ng/ml) alone did not stimulate AP activity significantly. After 7 days of treatment, CDMP-2 at 200 ng/ml alone stimulated AP activity by about threefold ($P < 0.05$), and enhanced the OP-1-stimulated AP activity in a dose-dependent manner. A maximum enhancement was observed at 100 ng/ml OP-1 and 100 ng/ml CDMP-2. Maximum enhancements of about 9- and 40- to 45-fold ($P < 0.01$) compared with the solvent-treated control value were observed after 5 and 7 days, respectively. Under these conditions, the AP activity was stimulated by approximately twofold ($P < 0.05$) compared with that induced by OP-1 alone for both treatment durations.

The effects of exogenous CDMP-3 on the OP-1-stimulated AP activity in C2C12 cells were also examined as a function of time and protein concentration (Fig. 1C). After 5 and 7 days of treatment, exogenous CDMP-3 (200 ng/ml) alone stimulated AP activity by about twofold ($P < 0.05$) and about fivefold ($P < 0.02$), respectively. After 7 days, CDMP-3 alone further enhanced the OP-1-stimulated AP activity in a dose-dependent manner. A maximum enhancement was observed at 100 ng/ml OP-1 and 200 ng/ml CDMP-3. Maximum enhancements of about 16- and 69-fold ($P < 0.01$) compared with the solvent-treated control value were observed after 5 and 7 days, respectively. Under these conditions, the AP activity was elevated by approximately fourfold ($P < 0.01$) above the levels produced by OP-1 alone for both treatment periods.

The enhancement of the OP-1-induced AP activity by CDMPs might be the consequence of an increase in either the number of AP-positive cells or the AP level in the OP-1-responsive cells, without increasing the number of responsive cells. To distinguish these two possibilities, equal number of FRC cells were plated and treated with two concentrations of CDMP-1, -2, and -3 in the absence or presence of OP-1 (100 ng/ml). Cells were stained for AP activity after 7 days (Fig. 1D). AP-positive cells were not detected in cultures treated with solvent or any one of the three CDMPs in the absence of OP-1. However, OP-1 alone increased the number of AP-positive cells. The number of AP-positive cells in cultures treated with the combination of CDMP + OP-1 appeared to be similar to that treated with OP-1 alone (183 ± 15 vs.

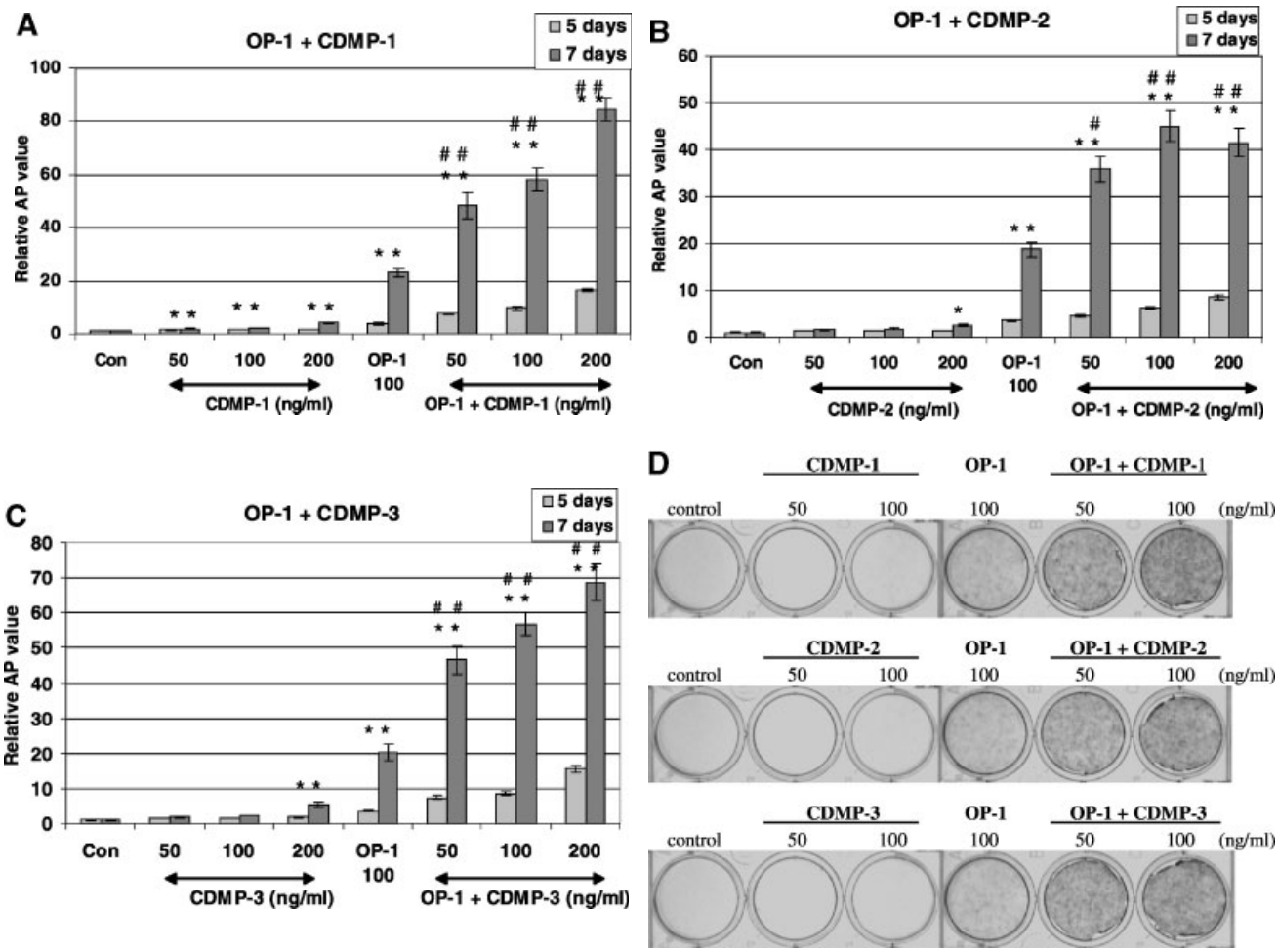


Fig. 1. Synergistic effects of exogenous CDMP-1, -2, and -3 on OP-1-induced AP activity in C2C12 cultures as a function of time of treatment. **A**: Cells were grown in 48-well plates in the presence of solvent, CDMP-1 alone (50, 100, and 200 ng/ml), OP-1 alone (100 ng/ml), and the combination of a fixed concentration of OP-1 (100 ng/ml) and varying concentrations of CDMP-1 (50, 100, and 200 ng/ml). At 5 and 7 days, cells were lysed with 0.1% Triton X-100/PBS as described in Materials and Methods. Total cellular AP activity was measured and expressed as nanomoles of ρ-nitrophenol per μg total protein. The relative AP activity was normalized to that of the solvent-treated control (as 1). Values represent the mean ± SEM of three independent experiments (with six wells/treatment condition). *, $P < 0.01$ compared with vehicle control. #, $P < 0.01$ compared with the OP-1 treated value. **B**: Experimental conditions were similar to those described in A, except CDMP-2 was used. **C**: Experimental conditions were similar to those described in A, except CDMP-3 was used. **D**: Alkaline phosphatase staining of C2C12 cells. Top part: Cells were grown in the presence of solvent, CDMP-1 alone (50 and 100 ng/ml), OP-1 alone (100 ng/ml), and the combination of OP-1 (100 ng/ml) and CDMP-1 (50 and 100 ng/ml). Cells were stained for AP activity using the cytochemical kit (Sigma) following the manufacturer's instruction. The intensity of the blue stain is proportional to the AP activity. Middle part: Cells were grown in the presence of solvent, CDMP-2 alone (50 and 100 ng/ml), OP-1 alone (100 ng/ml), and the combination of OP-1 (100 ng/ml) and CDMP-2 (50 and 100 ng/ml). Bottom part: Cells were grown in the presence of solvent, CDMP-3 alone (50 and 100 ng/ml), OP-1 alone (100 ng/ml), and the combination of OP-1 (100 ng/ml) and CDMP-3 (50 and 100 ng/ml). Representative images are shown.

192 ± 14 AP-positive cells/field). However, cultures treated with the combination of OP-1 and CDMPs showed dramatically stronger AP staining intensity than those treated with OP-1 or CDMP alone. The increase in staining intensity was also dependent on the CDMP concentration.

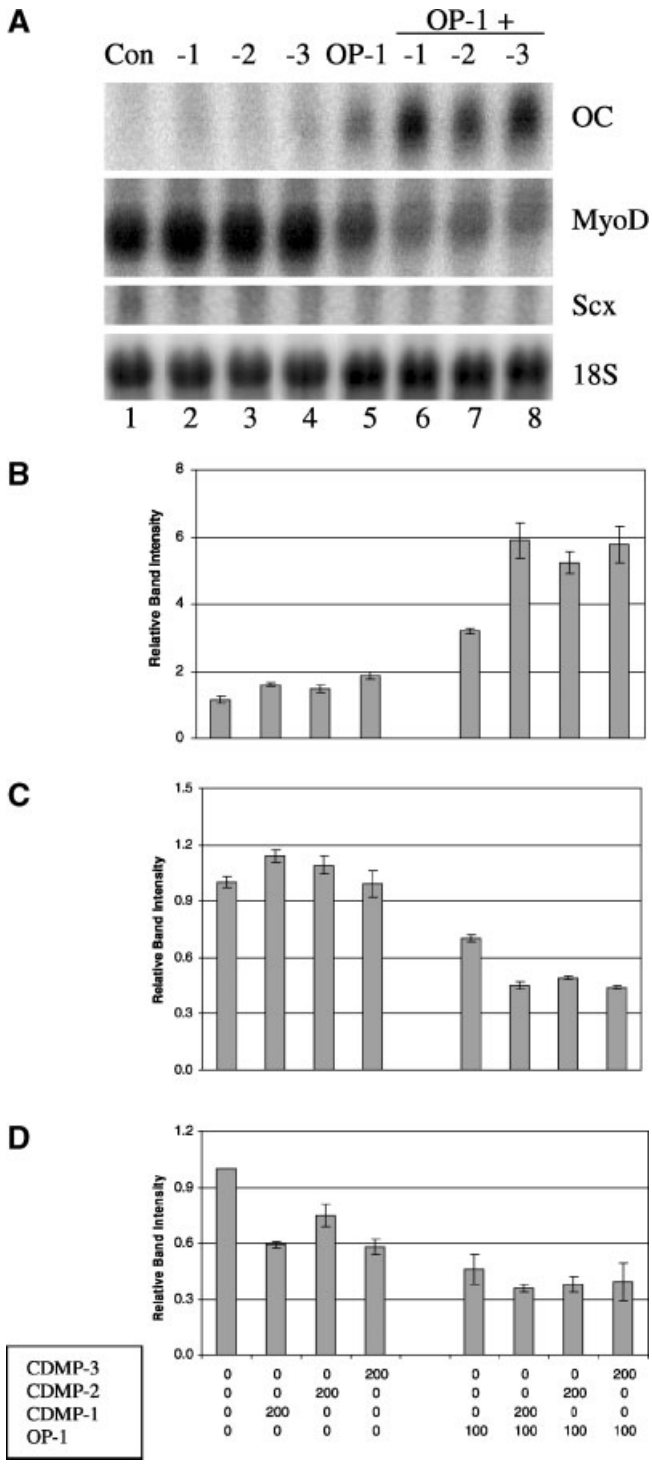
Effects of exogenous CDMPs on osteocalcin mRNA expression in C2C12 cells

The degree to which OP-1 modulates expression of the osteoblast phenotype in the presence of CDMP was assessed further by measuring the expression of OC, another biochemical marker of osteoblastic differentiation. Representative Northern blots for OC mRNA are shown in Figure 2A and the quantitative data are shown in Figure 2B. OC mRNA was not detectable in control cultures after 5 days of treatment, and its level was not changed significantly in cultures treated with CDMP-1, -2, and -3 compared to that in control cells. By comparison, the OC mRNA level in OP-1-treated cultures increased by about threefold ($P < 0.01$). In cells treated with the combination of 100 ng/ml OP-1 and 200 ng/ml CDMP-1, -2, or -3, the mRNA levels increased further by approximately 6-, 5-, and 6-fold, respectively,

compared with the control (Fig. 2B). Under these conditions, the enhancements were about twofold above the levels produced by OP-1 alone.

Effects of exogenous CDMPs on MyoD mRNA expression

Previous studies indicated that OP-1 inhibited the mRNA expression of regulator factors of myogenic differentiation (Yeh et al., 2002). In the present study, MyoD mRNA expression was examined in C2C12 cells cultured in the absence and presence of the CDMPs after 5 days of treatment. Representative Northern blots for MyoD mRNA are shown in Figure 2A and the quantitative data are shown in Figure 2C. MyoD mRNA expression was not altered in cultures treated with CDMPs alone, but decreased to about 70 % of the control in cultures treated with OP-1 alone. The mRNA level was further decreased by treatment with the combination of OP-1 and CDMP-1, -2, and -3. The combination treatments decreased the MyoD mRNA level to about 40–50% of the control.



Effects of exogenous CDMPs on scleraxis mRNA expression

Published studies showed that CDMPs could influence several skeletal processes. In the present study, we examined the mRNA expression of scleraxis in C2C12 cell cultures that were treated with OP-1 in the presence of varying concentrations of CDMPs. Figure 2A shows a representative Northern blot analysis and the quantitative data (Fig. 2D). OP-1 and all three CDMPs tested decreased the mRNA expression level of scleraxis in C2C12 cells. The combination of OP-1 and CDMP-1, -2, or -3 did not change significantly the effects exerted by the individual protein factor.

Effects of exogenous CDMPs on mRNA expression of members of the BMP family and their receptors

To determine whether the combination of OP-1 and CDMP altered the expression levels of other BMPs, the mRNA expression levels of BMPs were measured by RPA. The expression levels of BMP-1, -4, -5, -6, and -8 as well as GDF-1, -5, -6, -8, and -9 in cultures treated with the combination were not significantly changed compared to those treated with OP-1, CDMP-1, -2, or -3 alone (data not shown).

RPA was also used to measure the mRNA expression levels of 10 different BMP receptors. ActR-I, BMPR-IA, BMPR-IB, and Alk-7 mRNAs were detected by the kit and their levels of expression were not significantly changed compared to those treated with OP-1, CDMP-1, -2, or -3 alone (data not shown).

Fig. 2. **A**: Northern blot analysis of the synergistic effects of CDMP-1, -2, and -3 on OP-1-induced osteocalcin (OC), MyoD, and scleraxis mRNA expression in C2C12 cells. Cells were grown in the presence of solvent (control), CDMP-1, -2, and -3 alone (200 ng/ml), OP-1 alone (100 ng/ml), and the combination of 100 ng/ml OP-1 and 200 ng/ml CDMPs. After 5 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, MyoD, and scleraxis were measured by Northern blot analysis using ^{32}P-labeled cDNA probes. The blots were also hybridized with the oligonucleotide probe for 18S rRNA. Representative phosphorImages are presented. Results for the 5 days treatment are presented in lanes 1–8: vehicle control, CDMP-1 (200 ng/ml), CDMP-2 (200 ng/ml), CDMP-3 (200 ng/ml), OP-1 (100 ng/ml), OP-1 (100 ng/ml) + CDMP-1 (200 ng/ml), OP-1 (100 ng/ml) + CDMP-2 (200 ng/ml), OP-1 (100 ng/ml) + CDMP-3 (200 ng/ml). Quantitative analysis of OC (**B**), MyoD (**C**), and scleraxis (**D**) mRNA levels in C2C12 cultures. The intensity of the hydridized RNA species on Northern blots, as shown in A, was analyzed using 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 three to four independent determinations.

Effects of exogenous OP-1 on the CDMP-1 protein level in the conditioned media

Published results showed that OP-1 treatment resulted in up-regulation of CDMP-1 mRNA in C2C12 cells (Yeh et al., 2002). The present study, using Western blot analysis showed that the level of CDMP-1 protein in the conditioned media of control cultures (Fig. 3A, lane 1) and OP-1 treated cultures was low and below the sensitivity of detection (Fig. 3A, lane 3). The lack of detectable CDMP-1 in the conditioned media was not likely due to protein degradation, since a significant level of CDMP-1 was still detected in the CDMP-1 treated cultures after 5 days (Fig. 3A, lane 2).

Effects of exogenous OP-1 and CDMP-1 on Smad5 protein expression

The above results suggest that the synergy between OP-1 and CDMP-1 might act at the level of the signaling pathway. Mainly because CDMP-1 produced the most dramatic synergy (compared to CDMP-2 and -3), the following experiments focused on OP-1 and CDMP-1. The Smad5 protein level in control C2C12 cells was hardly detectable by Western blot analysis (Fig. 3B, lane 1). CDMP-1 alone stimulated Smad5 protein expression in C2C12 cells (Fig. 3B, lane 2). OP-1 alone stimulated Smad5 protein expression to a higher level (Fig. 3B, lane 3). The combination of CDMP-1 and OP-1 significantly stimulated Smad5 protein expression even more (Fig. 3B, lane 4).

DISCUSSION

The results presented in this report confirm the previous finding (Yeh et al., 2002) that OP-1 alone is capable of inducing osteoblastic cell differentiation of the pluripotent mesenchymal cell line C2C12. Our observations that exogenous CDMP-1, -2, and -3 individually changed minimally the expression of AP activity and OC mRNA level also agree with published reports on the inductive activity of the CDMPs (Hotten et al., 1996; Erlacher et al., 1998; Spiro et al., 2000). Moreover, the present results show that the induction by OP-1 was significantly and synergistically stimulated by CDMP-1, -2, and -3. The combined OP-1 and CDMP treatment of C2C12 cells produced a greater stimulation of AP activity and OC mRNA expression than did OP-1 alone. All three CDMPs enhanced significantly the OP-1-stimulated AP activity in C2C12 cells. At 200 ng/ml, CDMP-1 is most potent in enhancing AP activity as measured by AP activity, CDMP-3 is about 80% as potent as CDMP-1, and CDMP-2 is the least potent (50%). The AP staining data on C2C12 cells treated with the combination of OP-1 and CDMP further suggest that the combination enhanced OP-1 action by stimulating the same cells that responded to OP-1 and not by activating additional cells. All three CDMPs also enhanced the OP-1-stimulated OC mRNA level with CDMP-1 being the most potent followed by CDMP-3 and -2.

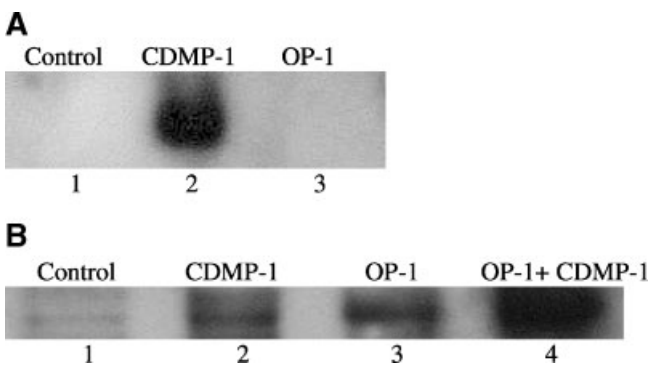


Fig. 3. **A**: Western blot analysis for CDMP-1 protein levels in conditioned media of C2C12 cells. Conditioned media of C2C12 cells treated with solvent control (lane 1), CDMP-1 (200 ng/ml), or OP-1 (100 ng/ml) were collected after 5 days. Proteins in the conditioned media were resolved by SDS–PAGE, and CDMP-1 was detected using an anti-mouse CDMP-1 antibody as the primary antibody and the anti-goat IgG HRP-conjugated antibody as the secondary antibody. Immunoreactive bands were detected using the SuperSignal chemiluminescent detection system (Pierce, Rockford, IL), according to the manufacturer's instructions. **B**: Western blot analysis for Smad5 protein levels in total cell lysates. Experimental conditions were similar to those described for A, except total cell lysates were collected and analyzed using the anti-human Smad5 antibody as the primary antibody and anti-rabbit IgG HRP-conjugated antibody was used as the secondary antibody. Representative results of two independent experiments are shown.

Our observations that the mRNA expression levels of BMP and BMPR were not altered further in cultures treated by the combination of CDMPs and OP-1 compared to those treated by the individual proteins alone indicate the synergistic action is not caused by stimulating the expression of BMPs and BMPRs. Our studies further show that OP-1, but not the CDMPs, down-regulated mRNA expression of the muscle-specific regulatory factor MyoD and that the CDMPs enhanced the OP-1-induced down-regulation of MyoD mRNA expression. The data are consistent with the view that OP-1 inhibits myogenic differentiation and converts the differentiation pathway of C2C12 cells into that of the osteoblast lineage and that the CDMPs synergistically enhanced the OP-1 action.

Previous reports showed that either BMP-2 or TGF-β1 alone also inhibited MyoD mRNA expression in C2C12 cells. However, only BMP-2 promoted osteoblastic cell differentiation, and TGF-β1 enhanced the inhibitory action of BMP-2 on myotube formation (Katagiri et al., 1994). Contrary to the present data on OP-1 and CDMPs, previous studies showed that TGF-β1 reduced the BMP-2-induced AP activity and OC level. Taken together, these results also suggest that these TGF-β superfamily members might inhibit myogenic differentiation via different mechanisms. Furthermore, these results offer an explanation for the observations that BMP-2 and OP-1 induce in vivo bone formation by implantation at a muscular site, but TGF-β did not (Sampath et al., 1987).

The current study also showed that the gene coding for scleraxis, a novel class II helix–loop–helix transcription factor (McLellan et al., 2002) was constitutively expressed in C2C12 cell culture. Scleraxis was shown to be essential for mesodermal development and was found to be a specific marker for developing tendons and ligaments (Schweitzer et al., 2001). In vitro studies also showed that scleraxis mRNA was expressed in C2C12 myoblasts and its expression was decreased by BMP-2 (Liu et al., 1997). Our present findings that OP-1, CDMP-1, -2, and -3 individually or in combinations

suppressed the scleraxis mRNA expression in C2C12 cells are in agreement with the idea that, under these experimental conditions, these protein factors do not stimulate this cell line to form tendon/ligament.

Previously published data showed that OP-1 stimulated CDMP-1, -2, and -3 mRNA expression in C2C12 cells (Yeh et al., 2002). The current study extended the data by showing that the level of CDMP-1 protein secreted by the OP-1 treated cells was very low. Thus, the inductive effects of OP-1 alone and the enhanced effects of OP-1 and exogenous CDMPs observed on AP activity were not likely the consequence of a combination of OP-1 and the secreted CDMPs. Taken together with the CDMP dose-response data, these findings suggest that the synergy is caused by the action of these proteins on their signaling pathways.

The major intracellular signals of TGF-β superfamily, including the BMPs are the Smads (Massague, 2000; ten Dijke et al., 2003). Smad5 is a BMP pathway-specific Receptor Activated Smad. The current observation that the protein level of Smad5 was dramatically increased in cells treated with the combination of OP-1 and CDMP-1 would support the supposition that the synergy between OP-1 and CDMP-1 is likely directed at the Smad signaling pathway. The current finding thus provides the foundation for future experimental studies to examine the mechanism of increased Smad5 protein expression related to the synergy.

In summary, we have demonstrated a synergism between OP-1 and CDMP-1, -2, and -3 in the inhibition of myogenic differentiation and stimulation osteoblastic cell differentiation of C2C12 cells.

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