Journal of Cellular Biochemistry 97:494–500 (2006)

ARTICLES

C-Type Natriuretic Peptide Enhances Osteogenic Protein-1-Induced Osteoblastic Cell Differentiation via Smad5 Phosphorylation

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Abstract In the present study, we examined the hypothesis that the C-type natriuretic peptide (CNP) enhances osteogenic protein-1 (OP-1) action in stimulating osteoblastic cell differentiation in primary cultures of fetal rat calvaria cell (FRC). CNP enhanced synergistically the OP-1-induced Alkaline Phosphatase (AP) activity and mineralized bone nodule formation in a dose- and time-dependent manner. To examine possible mechanism of the synergy between OP-1 and CNP, the expression levels of key BMP receptors and signaling molecules were examined. Western blot analysis showed that BMPR-IB and -II receptor protein expression was not affected by CNP alone, but was stimulated by OP-1 alone. The combination of OP-1 and CNP did not further increase their protein levels. The Runx2 protein expression level was not altered by CNP alone, but was elevated by OP-1 alone, and was slightly reduced by the combination. The Smad5 protein expression level was slightly decreased by CNP alone, but was stimulated by OP-1 alone, and was not further stimulated by the combination. Smad5 phosphorylation was not stimulated by CNP alone, but was stimulated significantly by OP-1 alone. The combination of OP-1 and CNP further stimulated the OP-1-induced Smad5 phosphorylation. Thus, one mechanism of the observed synergy between OP-1 and CNP involves enhancement of the Smad5 phosphorylation. J. Cell. Biochem. 97: 494–500, 2006.

Key words: C-natriuretic peptide; osteogenic protein-1; bone morphogenetic protein-7; osteoblast differentiation; synergy; smads

Natriuretic peptides (NPs) comprise at least three structurally related peptides: atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP) [Rosenzweig and Seidman, 1991]. CNP has been detected in a wide variety of tissues where it acts as a local regulator [Komatsu et al., 1991; Rosenzweig and Seidman, 1991; Suga et al., 1992; Vollmar et al., 1993]. CNP acts on the differentiation and proliferation of osteoblasts, chondrocytes, and osteoclasts [Holliday et al., 1995; Inoue et al., 1996b, 1999; Suda et al., 1996; Yasoda et al., 1998; Komatsu et al., 2002; Miyazawa et al., 2002]. For example, CNP promotes the differentiation of osteoblast-like cells from newborn rat calvaria and clonal osteoblastic MC3T3-E1 cells [Inoue et al., 1996a, 1999]. Mice deficient in the specific protein kinase involved in CNP signaling or the NP receptor show defects in endochondral ossification at the growth plate [Jaubert et al., 1999].

Osteogenic Protein-1 (OP-1, BMP-7) is a member of the bone morphogenetic protein (BMP) family that belongs to the transforming growth factor-β (TGF-β) superfamily [Kingsley, 1994]. OP-1 has been shown to induce formation of new bone and cartilage in vivo [Sampath et al., 1992; Cook, 1999]. BMPs exert their cellular effects *via* binding to specific heteromeric complexes of two related serine/threonine kinase receptors, type I and type II receptors, followed by activation of Smads, leading eventually to transcription of BMP target genes (for a recent review see [Korchynskyi et al., 2004] and references therein).

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Received 1 August 2005; Accepted 2 August 2005

DOI 10.1002/jcb.20657

The present study was pursued to assess whether OP-1 and CNP act independently or interact with each other to stimulate osteoblastic cell differentiation. We hypothesized that CNP enhances OP-1 action in stimulating osteoblastic cell differentiation or development. To test the hypothesis, we examined the effects of exogenous CNP on OP-1-stimulated osteoblastic cell differentiation in cultured FRC cells. We found that CNP enhanced synergistically the OP-1-induced Alkaline Phosphatase (AP) activity and bone nodule formation in a dose- and time-dependent manner. Smad5 phosphorylation was stimulated by OP-1 alone and was further enhanced by the combination of CNP and OP-1.

EXPERIMENTAL PROCEDURES

Materials

Fetal bovine serum (FBS), MEM, penicillin/streptomycin, trypsin-EDTA, and collagenase were purchased from Life Technologies (Grand Island, NY). Recombinant human OP-1 and anti OP-1 antibodies were provided by Stryker Biotech (Hopkinton, MA). CNP and Alizarin Red S stain were purchased from Calbiochem (La Jolla, CA) and Sigma (St. Louis, MO), respectively. Primary antibodies against BMPR-IB, BMPR-II, and Runx2 were obtained from R&D Systems (Minneapolis, MN). Antibodies against Smad5 and Phospho-Smad1(ser463/465)/5(ser463/465)/8(ser426/428) were obtained from Cell Signaling Technology (Beverly, MA). Secondary antibody-HRP conjugates were purchased from Southern Biotechnology (Birmingham, AL). All other reagents were of molecular biology grade. All buffers were prepared with diethylpyrocarbonate-treated water.

Fetal Rat Calvaria Cell Culture

Primary osteoblast cultures were prepared from fetal rat calvarial bones as described previously [Yeh et al., 1997]. Cells were grown in complete αMEM containing 10% FBS, penicillin and streptomycin, and vitamin C (100 μg/ml), and were treated with OP-1 in serum-free αMEM. Control cultures contained an equal amount of vehicle. Cultures were incubated in a humidified atmosphere containing 95% air and 5% CO_2 at 37°C.

Alkaline Phosphatase Activity Assay

Total cellular AP activity was measured using a commercial assay kit (Sigma Chemical Co., St. Louis, MO). Protein was measured according to the method of Bradford using BSA as a standard. AP activity was expressed as nanomoles of *p*-nitrophenol liberated per microgram of total cellular protein.

Alizarin Red S Staining for Mineralized Bone Nodules

Confluent FRC cells in 12-well plates were incubated in αMEM plus 5% FBS with vehicle, CNP alone (10^{-8} and 10^{-7}M), OP-1 alone (200 ng/ml), or OP-1 (200 ng/ml) plus two concentrations of CNP (10^{-8} and 10^{-7}M) for 15 days. The mineralized bone nodules were stained with 40 mM Alizarin Red S solution at room temperature for 10 min, rinsed with water and 1X PBS. The dye was extracted with 0.5 N HCl/5% SDS at room temperature for 30 min. The absorbance at 405 nm of the extracted dye was determined with a microplate reader MRXII (Thermo Labsystems, Chantilly, VA).

Western Blot Analysis

Confluent FRC cells in 6-well plates were incubated in serum-free αMEM containing vehicle, CNP alone (10^{-8} and 10^{-7}M), OP-1 alone (200 ng/ml), or OP-1 (200 ng/ml) plus varying concentrations of CNP (10^{-8} and 10^{-7}M) for 48 h. The cell lysate was collected, analyzed by SDS-PAGE, and transferred to Nitrocellulose membrane with the Semi-Dry Transfer Unit from Bio-Rad (Hercules, CA). The membrane was blocked overnight with 5% milk, incubated with the primary antibody, washed and incubated with the secondary antibody-HRP conjugate. Immunoreactive signals were detected using the SuperSignal West Femto Chemiluminescent Substrate detection system from Pierce (Rockford, IL), according to the manufacturer's instructions.

Statistics

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 were considered significant at $P < 0.05$.

RESULTS

Effects of Exogenous CNP on OP-1-Stimulated AP Activity in FRC Cells

The effects of exogenous CNP on OP-1 action were examined in FRC cells as a function of time and protein concentration (Fig. 1). Previous studies showed that OP-1 alone induced a dose-dependent elevation in AP activity in FRC cells [Yeh et al., 1997]. The present results confirmed the previous finding: OP-1 alone stimulated AP activity by about fourfold compared to the control (Fig. 1A, column 2 vs. column 1). CNP alone stimulated slightly the basal level of AP activity (columns 3–6). CNP enhanced the OP-1-induced AP activity in a CNP dose-dependent manner (columns 7–10). At low CNP concentration (10^{-9} M), the OP-1-induced AP activity was not further stimulated compared to the OP-1 alone treated value (column 7 *vs.* column 2). At the higher CNP concentrations (10^{-6} to 10^{-8}M), CNP synergistically stimulated the OP-1 effect. At the highest CNP concentration tested (10^{-6} M), a twofold stimulation compared to OP-1 alone was detected (column 10 *vs.* column 2) and about 10-fold compared to the solvent-treated control (column 10 *vs.* column 1).

Effects of adding CNP to the culture at different times after OP-1 treatment on the synergy between OP-1 and CNP were studied. An average of 1.6-fold enhancement was observed when CNP was added simultaneously with OP-1 or within 2 h after OP-1 treatment (Fig. 1B). When CNP was added 4 h or later after OP-1 treatment, the degree of synergy was reduced significantly. The extent of enhancement was statistically insignificant when CNP was added 6 h or more after OP-1 treatment.

Effects of Exogenous CNP on Mineralized Bone Nodule Formation

The effects of exogenous CNP on OP-1-induced mineralized bone nodule formation were also examined. Figure 2A shows representative images of Alizarin Red S stained cultures that were treated with OP-1 alone, CNP alone, and the combination of CNP and OP-1. Figure 2B shows the quantitative data. Neither of the two concentrations of CNP tested induced bone nodule formation (Fig. 2A,B, columns 2 and 3 *vs.* column 1). OP-1 stimulated staining intensity by about 10-fold compared to the control (column 4 *vs.* column 1). The combination of OP-1 and CNP further stimulated the OP-1-induced staining by 40% (column 5 or 6 *vs.* column 4). The Alizarin Red S staining data and the microscopic observation are in agreement that the OP-1-induced nodule formation in FRC cultures is enhanced by CNP.

Effects of Exogenous CNP on the Protein Level of OP-1 and BMP Receptors

To determine whether the combination of OP-1 and CNP altered the expression level of OP-1, the OP-1 protein level was measured by Western blot analysis. The OP-1 protein levels in cells treated with solvent or CNP alone were below detection (Fig. 3A, lanes 1 and 2). The OP-1 protein levels in FRC cultures treated with OP-1 alone and the combination of OP-1 and CNP were detectably higher than the controls (lanes 3 and 4 *vs.* lane 1) and were similar. The observations indicate that the CNP and OP-1 combination did not change the intracellular level of OP-1, compared to that in cells treated with OP-1 alone.

The protein levels of BMPR-IB and -II were measured by Western blot analysis. These two BMP receptors have been shown to bind OP-1. The BMPR-IB and -II protein levels were detectable in control cells (Fig. 3B, lane 1), and CNP alone did not change their protein levels (lanes 2 and 3). OP-1 alone stimulated both BMPR-IB and -II protein levels significantly, compared to the control (lane 4 vs. lane 1). The combination of CNP and OP-1 did not affect the protein level of either receptor, compared to the OP-1 value (lanes 5 and 6 *vs.* lane 4).

Effects of Exogenous CNP on Protein Expression Levels of Transcriptional Factor Runx2 and Signaling Molecule Smad5

Figure 3C shows representative Western blots indicating the protein expression level of the osteoblastic cell specific transcription factor Runx2, which was not altered in cells treated with CNP alone. Treatment with OP-1 alone elevated Runx2 protein level significantly (1.5-fold compared to the control, $P < 0.01$). Treatment with the OP-1 + CNP combination did not significantly change the protein expression level.

The above results suggest that the synergy between CNP and OP-1 might act at the post-receptor level of the signaling pathway. Accordingly, effects of CNP on the protein expression level of Smad5 and its phosphorylation levels were examined. CNP alone did not stimulate

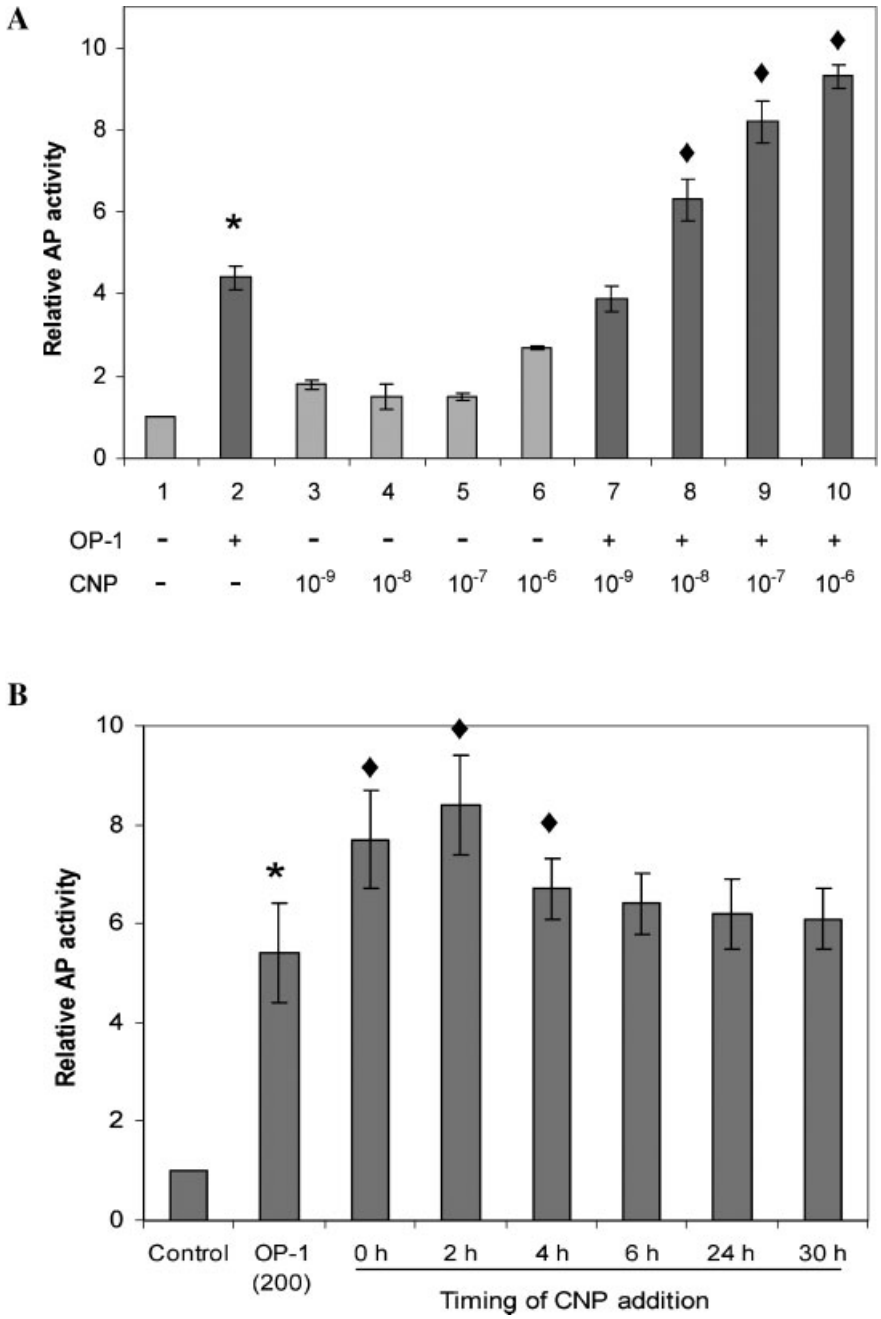

Fig. 1. **A**: Effects of exogenous CNP on the OP-1-induced AP activity in FRC cells. Confluent FRC cells were treated with vehicle alone, OP-1 (200 ng/ml) alone, varying concentrations of CNP (10^{-9} to 10^{-6} M), or OP-1 (200 ng/ml) + CNP (10^{-9} to 10^{-6} M). Total AP activity was determined after 48 h. Values represent the mean ± SEM of 10 independent determinations with 2 different FRC cell preparations and normalized to the vehicle control as 1. *, $P < 0.005$, compared to the control. ◆, $P < 0.01$, compared to OP-1 alone. **B**: Enhancement of the OP-1-induced AP activity in FRC cells by CNP is dependent on the time of addition of CNP after OP-1. Confluent FRC cells were treated with OP-1 (200 ng/ml). CNP (10^{-7} M) was added simultaneously or at 2, 4, 6, 24, or 30 h after OP-1. Total AP activity was measured 48 h later. Values represent mean ± SEM of six independent determinations using two separate FRC cell preparations and normalized to the vehicle control as 1. *, $P < 0.01$, compared to the control. ◆, $P < 0.01$, compared to OP-1 alone.

the Smad5 protein expression compared to the control (Fig. 3D, bottom panel, lanes 2 and 3). However, OP-1 alone stimulated slightly (about 25% compared to the control) Smad5 protein expression (Fig. 3D, bottom panel, lane 4); the increase is statistically insignificant. The combination of CNP (10^{-8} M) and OP-1 did not further stimulate Smad5 expression (Fig. 3D,

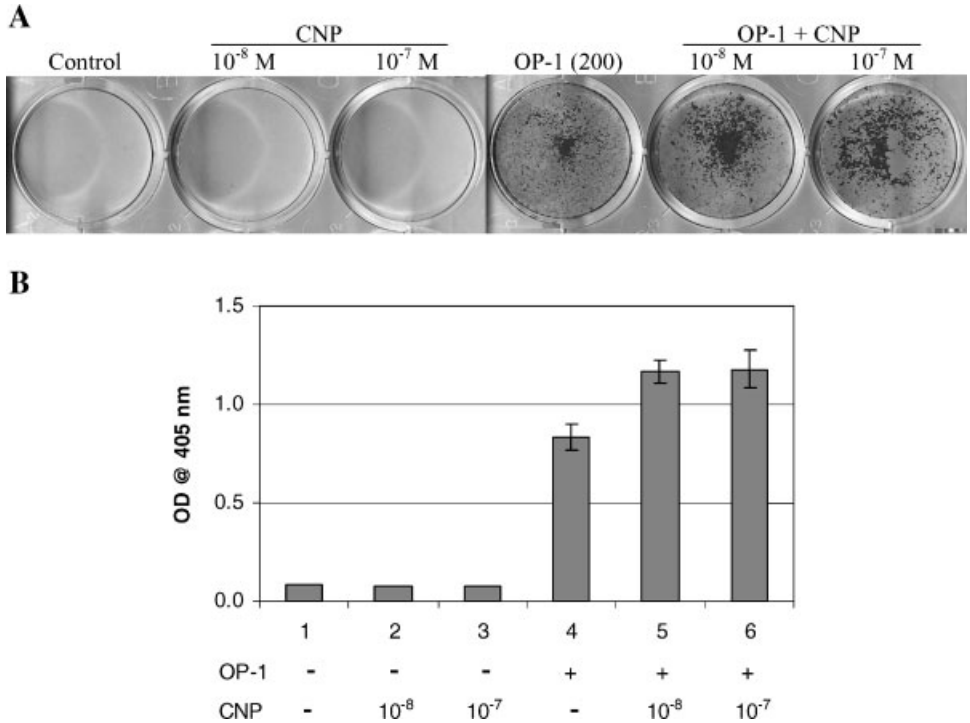

Fig. 2. **A**: Exogenous CNP enhanced the OP-1-induced mineralized bone nodule formation in FRC cells. Confluent FRC cells were treated with solvent control, 10^{-8} or 10^{-7} M CNP, 200 ng/ml of OP-1 in the absence or presence of 10^{-8} or 10^{-7}M CNP. Cells were grown in complete αMEM containing 5% FBS, ascorbic acid, and β-glycerol phosphate. Media were changed every 3 days. After 15 days, the mineralized bone nodules were stained with 40 mM Alizarin Red S solution and photographed. **B**: Quantitation of Alizarin Red S staining of FRC cells after long-term culture. The stained cultures shown in (A) were extracted with 0.5N HCl/5% SDS. The absorbance at 405 nm of the extracted dye was measured. Values represent the mean ± SEM of two independent measurements using two separate FRC cell preparations.

bottom panel, lane 5 *vs*. lane 4). A higher CNP concentration (10^{-7} M) decreased the Smad5 protein expression. CNP alone did not stimulate Smad5 phosphorylation (Fig. 3D, top panel, lanes 2 and 3), compared to the control. In contrast, OP-1 alone stimulated Smad5 phosphorylation by an average of fourfold compared to the control (Fig. 3D, top panel, lane 4). The combination of OP-1 and CNP (10^{-8} M) further stimulated Smad5 phosphorylation by sixfold compared to the control and 1.8-fold compared to OP-1 alone ($P < 0.01$) (Fig. 3D, top panel, lane 5). At the higher concentration of CNP (10^{-7} M), the level of phosphorylated Smad5 was reduced compared to the control (Fig. 3D, top panel, lane 6). Although the reason(s) for the decline is not clear at present, the finding was reproducible in experiments with two different FRC cell preparations.

DISCUSSION

In the present study, we demonstrated that exogenous CNP alone was moderately effective in increasing the expression of AP activity in FRC cells. This observation is consistent with published reports that CNP stimulated osteoblastic cell differentiation as indicated by increases in the levels of mRNA coding for AP and osteocalcin, and mineralization of nodules in clonal osteoblastic MC3T3-E1 cells and osteoblast-like cells from newborn rat calvaria [Hagiwara et al., 1996; Suda et al., 1996].

Furthermore, we demonstrated that treatment of FRC cells with OP-1 and CNP generated a greater stimulation of AP activity than did OP-1 or CNP alone. CNP also enhanced the OP-1 action in stimulating mineralized bone nodule formation, a hallmark of bone formation in osteoblastic cell cultures. Taken together, the present studies provide biochemical and morphological data supporting the idea that CNP synergistically enhanced the differentiation activity of OP-1 in primary cultures of osteoblastic cells. The observation that the synergistic effect could be observed when FRC cells were treated with OP-1 for up to 2 h before CNP addition suggests that "sensitization" by OP-1

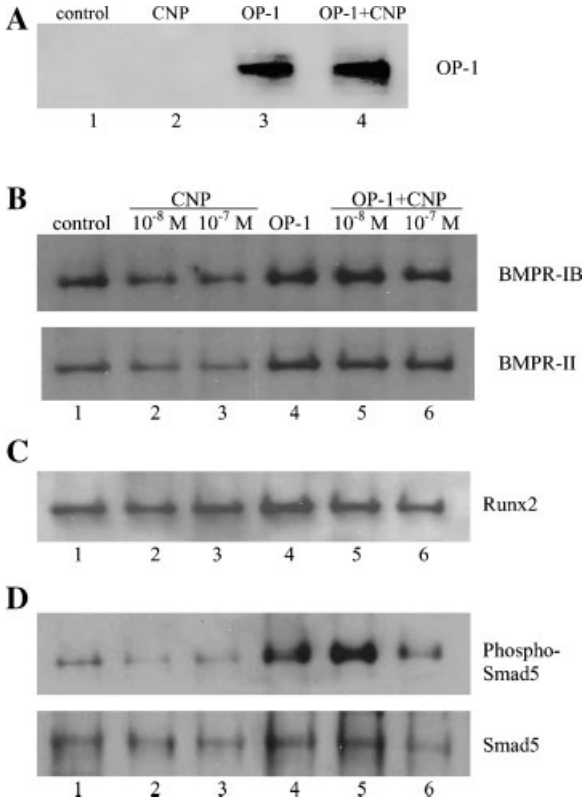

Fig. 3. Western blot analysis. FRC cultures were treated with solvent control, CNP alone, OP-1 alone, and OP-1 plus 10^{-8} or 10^{-7}M CNP for 48 h. Total cell lysates were resolved by PAGE; the proteins were transferred onto Nylon membrane and probed with (**A**) anti OP-1 antibody, (**B**) anti BMPR-IB or anti BMPR-II, (**C**) anti Runx2, and (**D**) anti Smad5 or anti phosphor-Smad5 (Ser463/465). The antibody-antigen complexes were detected using anti-IgG HRP-conjugated antibody. Representative results of two independent experiments using two different FRC cell preparations are shown. For (A), **lane 1**, control; **lane 2**, CNP (10^{-8} M); **lane 3**, OP-1; **lane 4**, OP-1 + CNP (10^{-8} M). For (B), (C), and (D), Lane 1, control; lane 2, CNP (10^{-8} M); lane 3, CNP (10^{-7} M); lane 4, OP-1; **lane 5**, OP-1 + CNP (10^{-8} M); **lane 6**, OP-1 + CNP (10^{-7} M).

of FRC cells is a prerequisite for the synergism between OP-1 and CNP. The present finding is consistent with our previous observation with IGF-I [Yeh et al., 1997]. In that study, maximal synergism between OP-1 and IGF-I was observed when both factors were added simultaneously. When IGF-I was added 6–24 h after OP-1 treatment, the synergistic effect was no longer statistically significant. Moreover, synergism was not observed when FRC cells were treated with IGF-I for 24 h, followed by OP-1 treatment.

Our results on Runx2 protein expression is interesting, in view of the previous report that CNP induces the expression of genes whose products might be involved in mineralization [Inoue et al., 2002]. We are tempted to speculate that the combination of OP-1 and CNP enhances osteoblastic cell differentiation by stimulating genes involved, at least in part, in bone nodule formation. Our data that CNP enhances not only the OP-1-induced AP activity which is an active participant in the mineralization stage of osteoblastic cell differentiation, but also the number of mineralized bone nodules would support the speculation.

Our findings that the OP-1 protein level in FRC cells treated with the combination of OP-1 and CNP was similar to that in cells treated with OP-1 alone suggest that the enhanced effects of OP-1 with exogenous CNP are not likely the consequence of increased intracellular OP-1 level. Our observation that the BMP receptor protein levels were not changed in FRC cultures treated with the OP-1/CNP combination is consistent. These observations, taken together, suggest that the synergy between OP-1 and CNP is caused by the action of CNP on the signaling pathways.

The major intracellular signals of BMPs are the Smads [Massague, 2000; ten Dijke et al., 2003], of which Smad5 is a BMP pathway-specific Receptor Activated Smad. Smad5 interacts transiently with the activated type I receptor and becomes phosphorylated. The activated Smad5 then interacts with Smad4. The heteromeric Smad complex is translocated into the nucleus where it acts on BMP target genes. OP-1 has been shown to induce the phosphorylation of the C-terminal of Smad5 [Tamaki et al., 1998]. Thus, our current observations that the level of phosphorylated Smad5 is elevated in FRC cultures treated with OP-1 alone are in agreement with the published results. Furthermore, our observation that the level of the phosphorylated Smad5 in FRC cultures treated with the combination of OP-1 and CNP was significantly elevated would support the supposition that the synergy between OP-1 and CNP is directed at the Smad signaling pathway. It is noteworthy that a previous study revealed a synergy between OP-1 and CDMP-1 and that the Smad5 protein level was dramatically increased in cells treated with OP-1 and CDMP-1 [Yeh et al., 2004]. These findings not only substantiate the significance of Smad5 in OP-1 action but also reveal the potential of Smad5 as a focus of cross talk among different signal pathways. Such cross talk can lead to an altered outcome of a growth factor.

In summary, we have demonstrated a synergism between OP-1 and CNP in the stimulation of osteoblastic cell differentiation of FRC cells. One mechanism of the synergy between CNP and OP-1 involves increased Smad5 phosphorylation.

ACKNOWLEDGMENTS

We thank the support provided by Stryker Biotech and the technical assistance of Vicky Yeh and Sangita Paul.

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