0013-7227/97/$03.00/0
Endocrinology
Copyright © 1997 by The Endocrine Society
Vol. 138, No. 10
Printed in U.S.A.

Osteogenic Protein-1 and Insulin-Like Growth Factor I Synergistically Stimulate Rat Osteoblastic Cell Differentiation and Proliferation*

LEE-CHUAN C. YEH, MARTIN L. ADAMO, MERLE S. OLSON, AND JOHN C. LEE

Department of Biochemistry, University of Texas Health Science Center, San Antonio, Texas 78284-7760

ABSTRACT

Previous studies have shown that osteogenic protein-1 (OP-1; also known as BMP-7) alters the steady state levels of messenger RNA (mRNA) encoding insulin-like growth factor I (IGF-I), IGF-II, and IGF-binding proteins (IGFBPs) in primary cultures of fetal rat calvaria (FRC) cells. In the present study, the effects of exogenous IGF-I on bone cell differentiation and mineralized bone nodule formation induced by OP-1 were examined. Exogenous IGF-I synergistically and dose dependently enhanced OP-1 action in stimulating [^{3}H]thymidine incorporation, alkaline phosphatase activity, PTH-dependent cAMP level, and bone nodule formation. Maximal synergism between OP-1 and IGF-I was observed when both factors were added simultaneously. Synergism was not observed when FRC cells were pretreated with IGF-I for 24 h, followed by OP-1 treatment. These findings suggest that IGF-I acted on OP-1-sensitized FRC cells. To examine the mechanism(s) by which this sensitization may occur, levels of mRNA encoding OP-1 receptor, IGF-I receptor, and IGFBPs were measured. The mRNA levels of both type I and II OP-1 receptors were elevated by OP-1, but were not changed further by combined OP-1 and IGF-I treatment. IGF-I receptor gene expression was not changed by OP-1, IGF-I, or a combination of both factors. OP-1 alone or together with IGF-I increased the steady state IGFBP-3 mRNA level and reduced the steady state mRNA levels of IGFBP-4, -5, and -6. IGF-I alone did not change the steady state mRNA levels of IGFBP-3, -4, and -6, but elevated that of IGFBP-5. Des(1–3)-IGF-I, which has a lower affinity for IGFBPs, was more effective than the full-length IGF-I in enhancing the OP-1-induced alkaline phosphatase activity. Exogenous IGFBP-5 inhibited the OP-1-induced alkaline phosphatase activity and reduced the synergistic stimulatory effect of IGF-I and OP-1. These findings strongly suggest that the OP-1-induced down-regulation of IGFBPs, especially that of IGFBP-5, is an important mechanism by which OP-1 and IGF-I synergize to stimulate FRC cell differentiation. (*Endocrinology* **138:** 4181–4190, 1997)

OSTEOGENIC protein-1 (OP-1; also known as BMP-7) is a member of the bone morphogenetic protein (BMP) subfamily of the transforming growth factor-β (TGFβ) superfamily (1–3). Recombinant human OP-1 induces new bone formation *in vivo* (4–6) and stimulates the synthesis of various biochemical markers characteristic of *in vitro* osteoblast differentiation. These markers include, but are not limited to, alkaline phosphatase (AP), osteocalcin, osteopontin, and PTH receptors (7–13). In primary cultures of fetal rat calvaria (FRC) cells, OP-1 stimulates cell proliferation and promotes differentiation of confluent FRC cells into an osteoblastic phenotype (9). OP-1 not only stimulates the proliferation and differentiation of osteoblasts *in vitro*, but also participates in the recruitment of osteoclasts (7, 14).

OP-1 has been shown to change the expression of the different components of the insulin-like growth factor (IGF) system (15–18). For example, OP-1 increased the IGF-II level in the conditioned medium (CM) of SaOS-2 and TE85 human osteosarcoma cell lines, but did not change the very low level of IGF-I secreted into the CM or the level of the cell surface IGF-I receptor (15). OP-1 also increased the levels of IGF-binding protein-3 (IGFBP-3) and IGFBP-5 and decreased that of IGFBP-4 in the CM (15–17). In primary cultures of FRC cells, OP-1 stimulated the steady state messenger RNA (mRNA) levels of IGF-I, IGF-II, and IGFBP-3, and decreased the mRNA levels of IGFBP-4, -5, and -6, without changing the IGF-I receptor mRNA level (18). Inhibition of IGF-I expression by an antisense oligonucleotide in FRC cells partially blocked OP-1-induced AP activity. These data have led to the hypothesis that the effects of OP-1 on FRC cells are mediated at least in part through changes in gene expression of IGF-I, IGF-II, and the IGFBPs. The present study was pursued to assess whether OP-1 and IGF-I act independently to regulate osteoblast development or whether these two growth factors interact to influence osteoblastic cell proliferation and/or differentiation.

Materials and Methods

Materials

All reagents were of molecular biology grade. All buffers were prepared with diethyl pyrocarbonate-treated water. SeaKem ME and GTG agarose were purchased from FMC BioProducts (Rockland, ME). Restriction enzymes were purchased from New England Biolabs (Beverly, MA). FBS, Hanks' Balanced Salt Solution (HBSS), serum-free αMEM medium, penicillin/streptomycin stock, trypsin-EDTA (0.05% trypsin-0.53 mM EDTA), and collagenase were obtained from Life Technologies (Grand Island, NY). Radioisotopes were purchased from ICN Pharmaceuticals (Irvine, CA). Recombinant human OP-1 was provided by Stryker Biotech (Natick, MA) and was dissolved in 50% acetonitrile-0.1% trifluoroacetic acid. Human IGF-I (Life Technologies) was dissolved in 0.1 M acetic acid and stored as aliquots (100 ng/μl) at −20 C. Human TGFβ1 (R&D Systems, Minneapolis, MN) was reconstituted in sterile 4

Received February 24, 1997.

Address all correspondence and requests for reprints to: Dr. Lee-Chuan C. Yeh, Department of Biochemistry, University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, Texas 78284-7760.

* Presented in part at the 10th International Congress of Endocrinology, San Francisco, CA, 1996, p 463 (Abstract P2–233).

Downloaded from https://academic.oup.com/endo/article/138/10/4181/2987658 by guest on 16 August 2022

mM HCl-0.1% BSA and stored as a 1 ng/ml stock solution at −20 C. Human PTH [PTH-(1–34)] and 3-isobutyl-1-methylxanthine were obtained from Sigma Chemical Co. (St. Louis, MO). Human PTH was reconstituted in sterile 0.01% acetic acid-0.1% BSA and stored at −20 C as a 1-mM stock. The cAMP phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine, was dissolved in dimethylsulfoxide at a concentration of 500 mM and stored as aliquots at −20 C. Des(1–3)-IGF-I was purchased from Peninsula Laboratories (Belmont, CA) and was solubilized and stored as described for IGF-I.

Cell culture

Primary osteoblast cell cultures were prepared from calvaria of day 19 or 20 fetal rats using previously described procedures (18–20). Briefly, the calvarium was stripped of the periosteum and digested with a mixture of trypsin and collagenase for five 20-min intervals. The digestion mixture after each 20-min interval was removed, and fresh digestion mixture was added to the calvarium. Digestions 1 and 2 were discarded. FRC cells were harvested from digestions 3–5. Cells were plated in T-75 flasks at a density of $2.5 \times 10^4/cm^2$ in complete αMEM medium containing 10% FBS, vitamin C (100 μg/ml), and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin sulfate). Cells were incubated at 37 C with 5% CO_2 for 3–4 days until they reached confluence. Cells [passage 1 (P1)] were replated at a density of $1–2 \times 10^6$/T-75 flask, allowed to grow 3–4 days until confluent, and then frozen in liquid N_2 at the second passage. For experimentation, frozen cells (P2) were thawed and cultured in T-75 flasks for 4 days until confluent, then subcultured into 48-well plates (Corning Glass Works, Corning, NY) or T-150 flasks (Corning) at a density of $2.5 \times 10^4/cm^2$. Cells (P3) were allowed to grow for 4–5 days until confluent. Confluent FRC cells were rinsed once with HBSS to remove CM and then incubated in serum-free αMEM medium (with 0.1% BSA) in the presence (100–500 ng/ml) or absence of OP-1 and with (10–100 ng/ml) or without exogenous IGF-I as described in the legend of each figure.

Thymidine incorporation

The extent of thymidine incorporation into DNA was determined essentially as previously described (11). Briefly, confluent FRC cells grown in 48-well plates were treated with varying concentrations (100–500 ng/ml) of OP-1 with (10–100 ng/ml) or without IGF-I in serum-free αMEM medium for 18 h. Cells were incubated with [^{3}H]thymidine (5 μCi/ml) for an additional 6 h. After removal of the medium containing the unincorporated thymidine, cells were rinsed with cold 1 × PBS. The radiolabeled DNA was precipitated by cold 10% trichloroacetic acid for 15 min, solubilized in 0.1 N NaOH at 37 C for 10 min, and neutralized with 0.1 N HCl. The amount of radioactivity was determined by scintillation spectrometry in the presence of UniverSol cocktail (ICN, Costa Mesa, CA).

AP activity assay

For measurements of AP activity, confluent FRC cells were grown and treated in 48-well plates as described above. The medium was replenished with fresh medium after 24 h. After 48 h of treatment, cells were rinsed with PBS and lysed by sonication in 0.05% Triton X-100 in PBS (100 μl/well) for 60 sec at room temperature. Total cellular AP activity was measured with *p*-nitrophenyl phosphate as a substrate in 2-amino-2-methyl-1-propanol buffer, pH 10.3, at 37 C using a commercial assay kit (Sigma Chemical Co.). Reactions were terminated by the addition of 0.5 N NaOH. Absorbance of the reaction mixture was measured at 405 nm using a Genenchem automatic plate reader (Hewlett-Packard, Palo Alto, CA). Protein was measured according to the method of Bradford (21), using BSA as a standard. AP activity was expressed as nanomoles of *p*-nitrophenol liberated per μg total cellular protein.

cAMP assay

Confluent FRC cells grown in 48-well plates were treated with varying concentrations of OP-1 with or without IGF-I in serum-free αMEM. After 24 h, the medium was replenished with fresh medium. After 48 h of treatment, cells were rinsed with HBSS and incubated in fresh serum-free medium containing 3-isobutyl-1-methylxanthine (1 mM) for 15 min. Cells were treated with 0.01% acetic acid (HAc)-0.1% BSA or 100 nM PTH for 10 min. The level of cAMP in the cell lysate was determined using a Biotrak cAMP enzyme immunoassay (Amersham, Arlington Heights, IL) following the manufacturer's instructions. cAMP levels were normalized to total cellular protein.

Bone nodule formation

Confluent FRC cells in six-well plates were treated in serum-free αMEM containing ascorbic acid (100 μg/ml) with solvent vehicle or OP-1 (200 ng/ml) in the absence or presence of varying concentrations of IGF-I (10, 25, and 50 ng/ml). Treatments were refreshed every other day. From day 11 on, 10 mM β-glycerol phosphate was included in each treatment to stimulate mineralization (22). On day 15, cells were rinsed with 1 × PBS, fixed in 10% neutral buffered formalin, and stained using a modified von Kossa method (23). The total nodule area in each culture well was quantified using Visage 110 (BioImage, Ann Arbor, MI).

RNA isolation

Confluent FRC cells in T-150 flasks were treated with solvent vehicle or OP-1 (200 ng/ml) in the absence or presence of IGF-I (25 ng/ml) for 48 h. At the end of treatment intervals, cells in T-150 flasks were rinsed with ice-cold 1 × PBS solution to remove serum-free αMEM. Total RNA was isolated using the RNeasy kit from Qiagen (Chatsworth, CA) following the manufacturer's instructions. RNA was dissolved in diethyl pyrocarbonate-treated H_2O, and the concentration of RNA was measured by its absorbance at 260 nm. The intactness of the RNA sample was examined by gel electrophoresis on 1% agarose after ethidium bromide staining. Only RNA preparations showing intact species were used for subsequent analyses.

Labeling of complementary DNA (cDNA)

All cDNA fragments used for Northern analyses were produced by digestion of the parent plasmids with the appropriate pairs of restriction endonucleases as previously described (18). The resultant DNA fragments were purified by agarose gel electrophoresis and Geneclean II (BIO 101, La Jolla, CA). The 400-bp IGFBP-2 probe was obtained by digestion of pRBP2–501 with *Eco*RI/*Hin*dIII. The 700-bp IGFBP-3 probe was obtained by digestion of pRBP3-AR with *Kpn*I/*Bam*HI. The 444-bp IGFBP-4 probe was obtained by digestion of pRBP4-SH with *Sma*I/*Hin*dIII. The 270-bp IGFBP-5 probe was obtained by digestion of pRBP5-SH with *Sac*I/*Hin*dIII. The 246-bp IGFBP-6 probe was obtained by digestion of pRBP6-PP with *Pst*I/*Pst*I. The 265-bp IGF receptor probe was obtained by *Eco*RI/*Bam*HI digestion of the IGF-I receptor gene sequence cloned in pGEM-3. The cDNA probes for ActR-I (Activin receptor I; ALK-2), BMP receptor IA (BMPR-IA; ALK-3), and BMPR-IB (ALK-6) were generated by reverse transcription-PCR using the specific primer sets described below: for ActR-I: sense primer, 5′-ACG CCT CTT GAA TTC TCC GAG-3′; antisense primer, 5′-CTC CAC GTC TCG GGG ATT GAG-3′; for BMPR-IA: sense primer, 5′-CAG TAC ACA GGA AAG CTT ACA-3′; antisense primer, 5′-GTA ACA AAA GCA GCT **G**GA GAA-3′; and for BMPR-IB: sense primer, 5′-AAG CGG CGG CGG GTT AAC TTC-3′; antisense primer, 5′-GAT CCA CTT CCC GAG CTC TGA-3′. The PCR-generated fragments were subcloned and propagated in *Escherichia coli*. The 580-bp ActR-I insert was obtained by digestion with *Eco*RI/*Ava*II. The 530-bp BMPR-IA insert was obtained by digestion with *Hin*dIII/*Pvu*II. The 660-bp BMPR-IB insert was obtained by digestion with *Hpa*I/*Sac*I. The 800-bp BMPR-II insert was obtained by *Pst*I digestion of human BMPR-II cloned in pCMV5. Purified cDNA fragments were labeled with [α-^{32}P]deoxy-CTP using the DECAprime II DNA labeling system (Ambion Co., Austin, TX). The labeled cDNA probes were purified through a Midi-SELECT G-25 spin column (5 Prime-3 Prime, Boulder, CO) to remove the unincorporated nucleotides. The 18S ribosomal RNA (rRNA) was probed with a ^{32}P-labeled 18S-specific oligonucleotide (5′-GCCGTGCGTACTTAGACATGCATG-3′).

Northern blot analysis

The mRNA levels for IGF-I receptor, type I and II OP-1 receptor, and IGFBPs were determined by Northern analysis as previously described (18). Denatured total RNAs (20 μg) were analyzed on 2.2 M formalde-

Downloaded from https://academic.oup.com/endo/article/138/10/4181/2987658 by guest on 16 August 2022

hyde-1% Seakem Genetic Technology Grade agarose gels. RNA standards (0.24–9.5 kilobases) from Life Technologies were used as size markers. The fractionated RNA was transferred onto a Nytran Plus membrane using a Turboblot apparatus (Schleicher and Schuell, Keene, NH). After cutting the lane containing the standards from the blot, the RNA was covalently linked to the membrane using the UV cross-linker (Stratagene, La Jolla, CA). The membranes were incubated overnight at 42 C with cDNA probes. The blots were washed and exposed to a screen for the PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and analyzed as previously described (18). Results represent the average from probing three or four blots with different RNA preparations for each cDNA probe. Blots were also probed with an 18S rRNA oligonucleotide probe to correct for loading variations.

Statistical analysis

Multiple means were compared with one-way ANOVA, followed by Student's t test for paired comparisons with the control. The ANOVA and Student's t test programs in the PSI-Plot (Poly Software International, Salt Lake City, UT) for personal computers were used for the analyses.

Results

Effects of exogenous IGF-I on DNA synthesis in OP-1-treated FRC cells

Figure 1A shows that OP-1 treatment of confluent FRC cells resulted in a dose-dependent stimulation of [^{3}H]thymidine incorporation, with a maximum 2-fold stimulation. Half-maximal and maximal stimulation of [^{3}H]thymidine incorporation occurred at OP-1 concentrations of approximately 150 and 500 ng/ml, respectively. The effect of IGF-I on DNA synthesis in OP-1-treated FRC cells was also examined. IGF-I alone stimulated DNA synthesis slightly (1.3-fold), but significantly ($P < 0.04$; Fig. 1B). This observation is in agreement with the previous finding that IGF-I has a weak mitogenic activity in FRC cells (24). The combined OP-1 and IGF-I treatment of FRC cells produced a statistically significant ($P < 0.05$) enhancement of DNA synthesis beyond that caused by OP-1 or IGF-I alone (Fig. 1B). Maximum enhancement was observed at 200 ng/ml OP-1 and 25 ng/ml IGF-I (1.3-fold stimulation compared with OP-1 alone and 1.8-fold compared with IGF-I alone).

Effects of exogenous IGF-I on OP-1-stimulated AP activity in FRC cells

The effects of exogenous IGF-I on OP-1-stimulated AP activity in FRC cells were examined (Fig. 2). OP-1 alone stimulated a dose-dependent increase in AP activity of 2.5- to 5-fold ($P < 0.001$; Fig. 2A). The increase in AP activity was accompanied by an increase in AP mRNA level, as measured by Northern analysis (data not shown). Exogenous IGF-I alone (up to 100 ng/ml) did not stimulate AP activity in FRC cells, but enhanced OP-1-stimulated AP activity in a dose-dependent manner (Fig. 2B). A maximum enhancement was observed at 25 ng/ml IGF-I and 200 ng/ml OP-1. Under these conditions, enhancements of AP activity were about 2-fold ($P < 0.008$) compared with that produced by OP-1 alone, and approximately 8-fold ($P < 0.001$) compared with the solvent-treated control value.

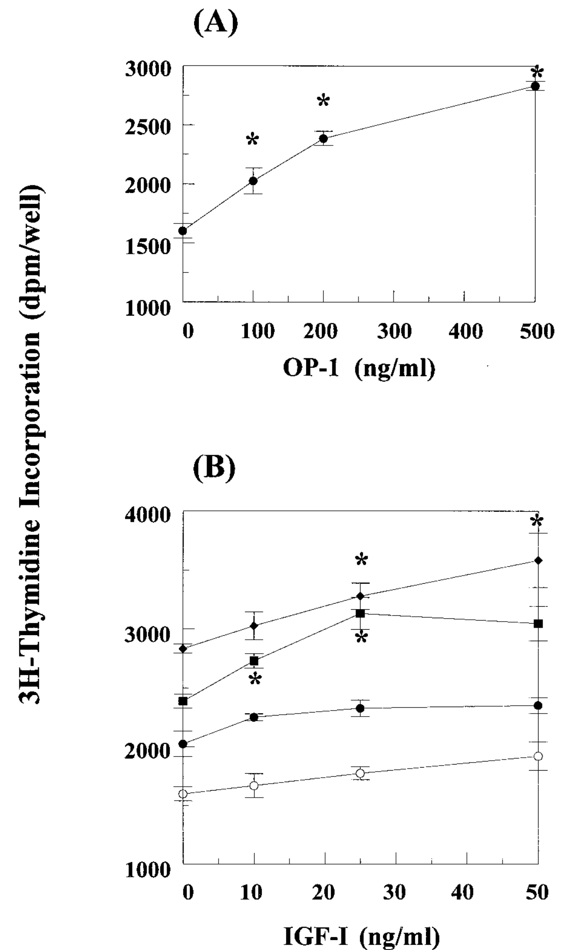

FIG. 1. A, Dose response of OP-1 on [^{3}H]thymidine incorporation in FRC cells. Confluent FRC cells in 48-well plates were incubated in serum-free αMEM containing vehicle or varying concentrations of OP-1 (100, 200, or 500 ng/ml) for 18 h. The treatments (6 wells/treatment) were then pulsed with [^{3}H]thymidine (5 μCi/ml) for 6 h, as described in *Materials and Methods*. After a total of 24 h of incubation, the extent of [^{3}H]thymidine incorporation into DNA was determined and expressed as disintegrations per min/well. Values are the mean ± SE of four independent experiments using different preparations of FRC cells. B, The synergistic effect of exogenous IGF-I on OP-1-induced [^{3}H]thymidine incorporation in FRC cells. Confluent FRC cells in 48-well plates were incubated with OP-1 in the presence of exogenous IGF-I (10, 25, and 50 ng/ml) for 18 h and pulsed with [^{3}H]thymidine for an additional 6 h, as described above. ○, Control (no OP-1), only vehicle was added in serum-free medium; ●, 100 ng/ml OP-1; ■, 200 ng/ml OP-1; ♦, 500 ng/ml OP-1. Values are the mean ± SE of four independent experiments using different preparations of FRC cells. *, $P < 0.01$ compared with vehicle control (A) and with OP-1 alone control (B).

Effects of exogenous IGF-I and OP-1 on PTH-dependent cAMP level in FRC cells

The extent to which OP-1 modulates expression of the osteoblast phenotype in the presence of IGF-I was assessed further by measuring the production of cAMP in response to PTH, another marker of osteoblastic differentiation. IGF-I alone (10–50 ng/ml) did not increase PTH-stimulated cAMP levels. OP-1 alone (100 or 200 ng/ml) for 48 h increased

Downloaded from https://academic.oup.com/endo/article/138/10/4181/2987658 by guest on 16 August 2022

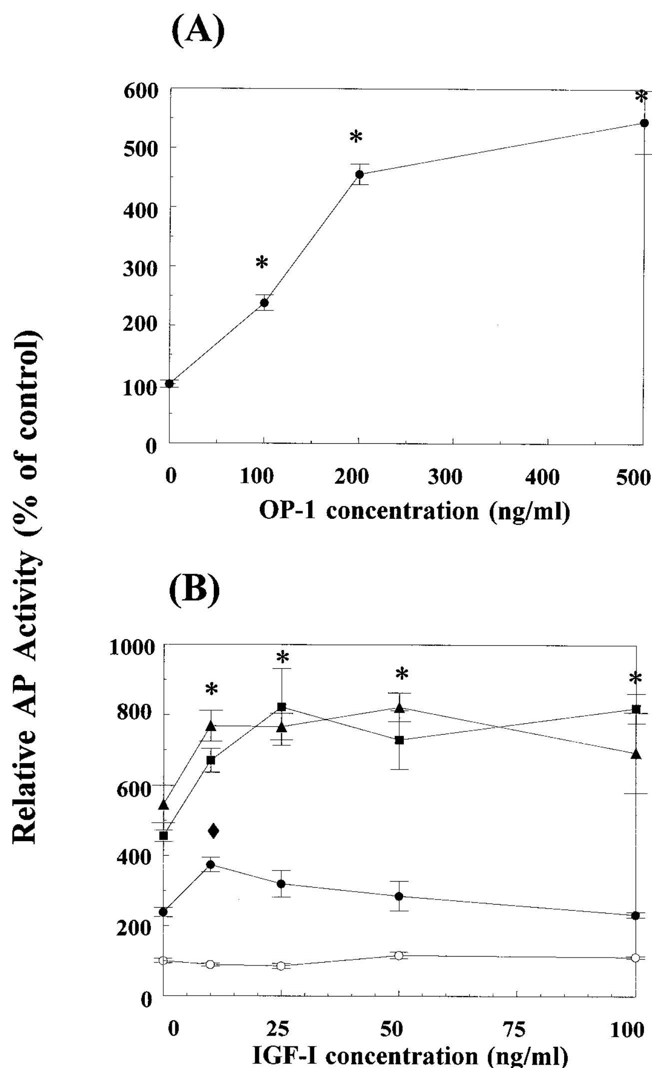

FIG. 2. A, Dose response of OP-1 on AP activity in FRC cells. Confluent FRC cells in 48-well plates were incubated in serum-free αMEM containing vehicle or varying concentrations of OP-1 (100, 200, or 500 ng/ml). After 24 h of incubation, medium was replaced with fresh medium containing the appropriate additives. After a total of 48-h incubation, cells were lysed with 0.05% Triton X-100/PBS as described in *Materials and Methods*. The activity of AP was expressed as nanomoles of *p*-nitrophenol per μg total protein. The relative activity of AP was normalized to that of the solvent vehicle-treated control (100%). Values are the means of four independent experiments (with 6 wells/treatment condition) of different preparations of FRC cells. *, $P < 0.01$ compared with the control. B, The synergistic effect of exogenous IGF-I on OP-1-induced AP activity in FRC cells. Confluent FRC cells in 48-well plates were incubated with OP-1 in the presence of exogenous IGF-I (10, 25, 50, and 100 ng/ml) for 48 h as described above. ○, Control (no OP-1), only vehicle was added in serum-free medium; ●, 100 ng/ml OP-1; ■, 200 ng/ml OP-1; ▲, 500 ng/ml OP-1. *, $P < 0.01$; ♦, $P < 0.05$ (compared with each OP-1 concentration alone).

PTH-stimulated cAMP level by 3- to 4-fold relative to those in solvent-treated and PTH-treated control cells (Table 1). OP-1 (100 or 200 ng/ml) and IGF-I (10–50 ng/ml) treatment for 48 h caused a dose-dependent stimulation of cAMP levels, with a maximum increase of about 6.8-fold over that produced by PTH alone.

TABLE 1. PTH-stimulated cAMP accumulation in OP-1-treated and OP-1- plus IGF-I-treated FRC cells

OP-1 (ng/ml)	IGF-I (ng/ml)	Fold stimulation by PTH (+PTH/−PTH)	Fold stimulation (compared with control)
0	0	4 ± 1	1.0
0	10	4 ± 2	1.0
0	25	5 ± 2	1.2
0	50	4 ± 1	1.0
100	0	12 ± 2	3.0
100	10	13 ± 2	3.3
100	25	17 ± 3[a]	4.3
100	50	19 ± 2[a]	4.8
200	0	17 ± 3	4.3
200	10	19 ± 3[b]	4.8
200	50	27 ± 3[a]	6.8

Confluent FRC cells in 48-well plates were treated (6 wells/treatment) with solvent vehicle, OP-1 (100 or 200 ng/ml) alone, IGF-I (10, 25 or 50 ng/ml) alone, or OP-1 (100 or 200 ng/ml) plus IGF-I (10, 25, or 50 ng/ml) in serum-free medium. The treatments were refreshed with medium containing the appropriate additives after 24 h. After a total of 48 h, cells were washed with HBSS, and stimulated with 100 nM PTH or 0.01% HAc-0.1% BSA in the presence of 3-isobutyl-1-methylxanthine (1 mM) as described in *Materials and Methods*. The cAMP level was determined and normalized to that total protein level. The ratio of cAMP level in cultures treated with PTH to that in cultures without PTH was calculated. The fold stimulation under each experimental condition was calculated and expressed as a ratio of the control (*i.e.* incubation with PTH, but without OP-1 or IGF-I). Values represent triplicate determinations in two independent experiments.

[a] $P < 0.01$ *vs.* OP-1 alone.

[b] $P < 0.05$ *vs.* OP-1 alone.

Effects of exogenous IGF-I and OP-1 on nodule formation in FRC cells

Formation of mineralizing nodules, a hallmark of bone formation in long term cultures of bone-derived cells, was examined in FRC cells treated with OP-1 and IGF-I. Figure 3 shows a photograph of bone nodule formation in FRC cells treated with OP-1 in the absence or presence of varying concentrations of IGF-I. Table 2 shows the quantitation of these images. These data indicate that OP-1 stimulated nodule formation in FRC cells by about 5-fold, an observation consistent with the previously published data (25). Additionally, the stimulation of nodule formation was enhanced synergistically by IGF-I, resulting in a 2- to 3-fold stimulation over that produced by OP-1 alone and a 12- to 15-fold stimulation over the vehicle control value.

Effects of order of treatment on the synergistic effect

To evaluate the effect of pretreatment of FRC cells with OP-1 on the synergistic effect of IGF-I, confluent cells were incubated with a fixed concentration of OP-1 followed by the addition of a fixed concentration of IGF-I at different times after the addition of OP-1. The AP activity in culture was determined after 48 h of incubation. Figure 4 shows that the maximum synergistic effect was observed when FRC cells were treated with OP-1 and IGF-I simultaneously ($P < 0.0006$ compared with OP-1 alone) or were treated with IGF-I within 2–4 h after OP-1 ($P < 0.0003$ compared with OP-1 alone). When IGF-I was added 6–24 h after OP-1 treatment, the synergistic effect was no longer statistically significant. In

Downloaded from https://academic.oup.com/endo/article/138/10/4181/2987658 by guest on 16 August 2022

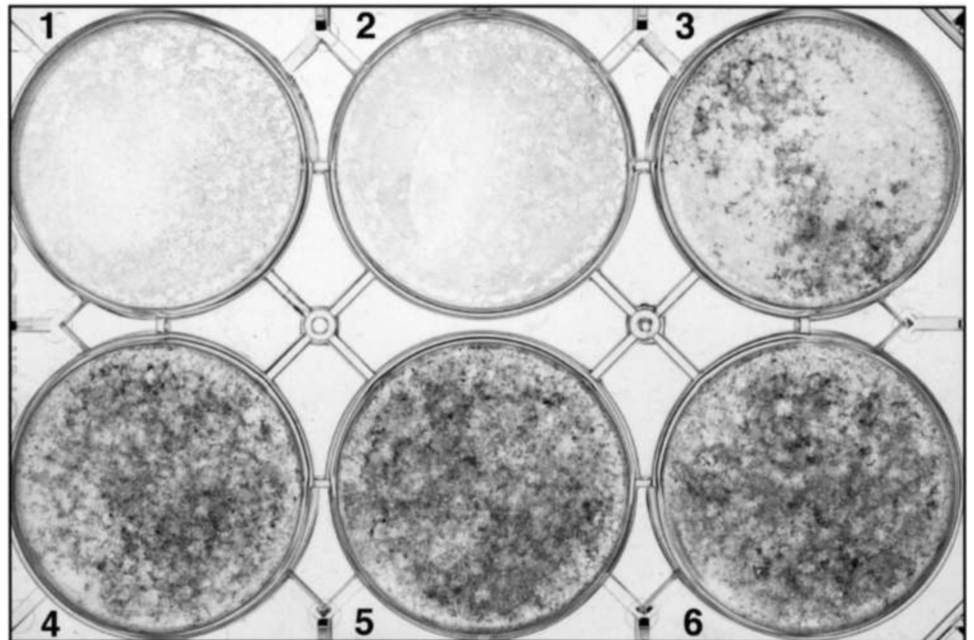

FIG. 3. Formation of bone nodules in FRC cells as a function of OP-1 and IGF-I/OP-1. Confluent FRC cells in six-well plates were treated with vehicle (well 1), IGF-I (25 ng/ml; well 2), OP-1 (200 ng/ml; well 3), and OP-1 (200 ng/ml) in the presence of increasing concentrations of IGF-I (10, 25, and 50 ng/ml; wells 4, 5, and 6, respectively). Media were changed every 2 days. On days 11 and 13, β-glycerol phosphate (10 mM) was added to all cultures. On day 15, the cells were processed for visualization and quantitation of nodule formation. Nodules were visible under the phase contrast microscope after staining by the von Kossa technique.

TABLE 2. Effects of OP-1 and IGF-I on nodule formation in FRC cells

OP-1 (ng/ml)	IGF-I (ng/ml)	Relative total nodule area
0	0	1.0
0	25	0.68 ± 0.03
200	0	4.85 ± 2.09
200	10	11.95 ± 0.48[a]
200	25	11.71 ± 0.93[a]
200	50	14.73 ± 4.51[a]

Confluent FRC cells in 6-well plates were treated with solvent vehicle, IGF-I, OP-1, and OP-1 plus IGF-I. The treatments were refreshed with corresponding mixtures every 48 h. On days 11 and 13 of treatment, 10 mM β-glycerolphosphate was added to all wells. On day 15, cells were fixed and stained following the von Kossa method. The total nodule area in each culture well was measured by densitometry using the Visage 110 imager (BioImage). Values are normalized to the vehicle control (n = 8).

[a] $P < 0.01$ *vs.* OP-1 alone.

comparison, incubation of FRC cells with IGF-I for 24 h followed by OP-1 treatment for an additional 48 h resulted in an increase in AP activity to a level similar to that caused by OP-1 alone (data not shown).

Effects of exogenous IGF-I on gene expression of the OP-1 receptor

A possible mechanism of synergism between IGF-I and OP-1 would be that IGF-I stimulates OP-1 receptor gene expression. To test this hypothesis, the mRNA levels of the type I and type II OP-1 receptor candidates (26–32), ActR-I (ALK-2), BMPR-IA (ALK-3), BMPR-IB (ALK-6), and BMPR-II, were determined by Northern analysis. Figure 5 shows representative Northern blots. The quantified data from the average of four to six independent experiments are shown in Table 3. IGF-I alone did not change the mRNA levels for type I or type II OP-1 receptors. OP-1 alone increased ActR-I, BMPR-IA, and BMPR-II mRNA levels, but did not change the BMPR-IB level. Combined

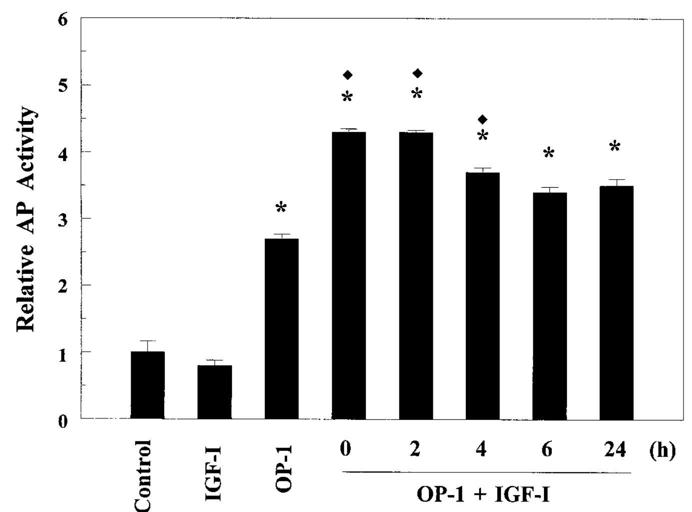

FIG. 4. Effect on AP activity of IGF-I added at different times to FRC cells treated with OP-1. Confluent cells in 48-well plates were incubated with serum-free αMEM containing solvent vehicle, IGF-I (25 ng/ml), or OP-1 (200 ng/ml). IGF-I (25 ng/ml) was added to the cultures 0, 2, 4, 6, or 24 h after OP-1 treatment. After 48 h of incubation, cells were lysed, and the level of AP activity was measured and normalized to the control, which is expressed as 1. Shown are the mean ± SE from six culture wells from each experiment and are representative of four independent FRC cell preparations. *, $P < 0.01$ compared with solvent control. ◆, $P < 0.01$ compared with OP-1 alone.

OP-1 and IGF-I did not change these levels any further. Whether there are changes in the level of cell surface receptor protein or in the translation rate of the OP-1 receptor mRNA is not known at present.

Effects of exogenous IGF-I on OP-1-induced expression of components of the IGF-I system

Previously, OP-1 has been shown to change the steady state mRNA levels of several components of the IGF-I system

Downloaded from https://academic.oup.com/endo/article/138/10/4181/2987658 by guest on 16 August 2022

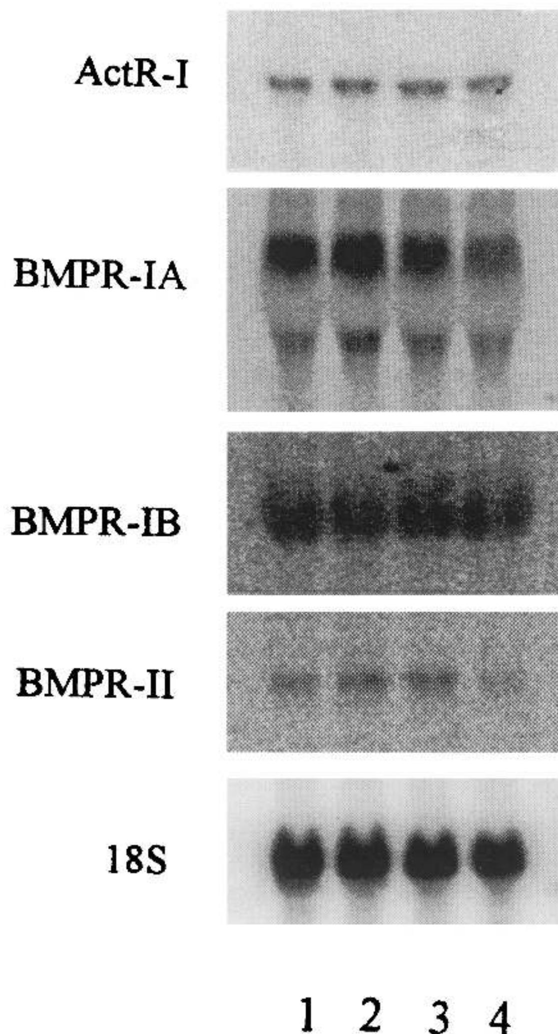

FIG. 5. Northern analysis of OP-1 type I and type II receptor gene expression in FRC cells. Total RNA was isolated from FRC cells treated with solvent vehicle (lane 1), OP-1 (200 ng/ml; lane 2), OP-1 (200 ng/ml) plus IGF-I (25 ng/ml; lane 3), or IGF-I (25 ng/ml; lane 4) for 48 h. The RNA was denatured, resolved on 1% agarose gel containing formaldehyde, and transferred onto a Nytran Plus membrane. The blot was hybridized with the cDNA probe for the individual receptor type or with an oligonucleotide for 18S rRNA. After washings, the blot was exposed to a PhosphorImager screen.

in primary cultures of osteoblastic cells (18). To determine whether the combination of exogenous IGF-I and OP-1 influences the expression of components of the IGF-I system beyond the effects exerted by OP-1 alone, mRNA levels for these components in cells treated with OP-1 and IGF-I were examined. The level of IGF-I receptor mRNA in FRC cells treated with OP-1 and IGF-I was not changed (data not shown). Representative blots for the IGFBP mRNAs are shown in Fig. 6, and the quantitative data are shown in Fig. 7. Consistent with previous results (18), the steady state level of IGFBP-2 mRNA did not change in OP-1-treated cells relative to that in control cells. Neither treatment with combined OP-1 and IGF-I nor that with IGF-I alone changed the IGFBP-2 mRNA level. The IGFBP-3 mRNA level in cells treated with OP-1 alone or IGF-I and OP-1 together was

TABLE 3. Effects of OP-1 and IGF-I on OP-1 receptor gene expression in FRC cells

Treatment	Relative level of receptor mRNA (%)			
	ActR-I	BMPR-IA	BMPR-IB	BMPR-II
Control	100	100	100	100
IGF-I	102 ± 6	104 ± 8	102 ± 9	91 ± 6
OP-1	108 ± 3[a]	120 ± 7[a]	103 ± 5	125 ± 7[b]
OP-1 + IGF-I	106 ± 11	114 ± 6[a]	108 ± 9	113 ± 8

Confluent FRC cells grown in T-150 flasks were treated with solvent vehicle, OP-1 (200 ng/ml), IGF-I (25 ng/ml), or OP-1 (200 ng/ml) plus IGF-I (25 ng/ml). Treatments were repeated with fresh mixtures after 24 h. Total RNA was isolated after 48 h. The steady state mRNA level of each receptor type was determined by Northern analysis. The numbers of different RNA preparations used for the measurement of each receptor type were four, six, four, and four, for ActR-I, BMPR-IA, BMPR-IB, and BMPR-II, respectively.

[a] $P < 0.05$.
[b] $P < 0.01$.

slightly decreased (relative to that in controls) compared with that in cells treated with IGF-I alone. The steady state mRNA levels of IGFBP-4, -5, and -6 in FRC cells treated with both IGF-I and OP-1 decreased to those in cells treated with OP-1 alone (Fig. 7). Additionally, the IGFBP-4 and -6 mRNA levels in IGF-I-treated cells did not change significantly relative to the control (Fig. 7). By comparison, the IGFBP-5 mRNA level in FRC cells treated with IGF-I alone was elevated significantly relative to that in the vehicle-treated cultures (Fig. 7), an observation in agreement with an earlier report (33). Thus, OP-1 displayed a dominant effect over IGF-I in regulating IGFBP-5 gene expression.

Effects of des(1–3)-IGF-I on OP-1 action in FRC cells

The observations that synergism between OP-1 and IGF-I required the concomitant presence of OP-1 and IGF-I, and that OP-1 exerted a dominant effect over IGF-I on IGFBP gene expression suggest the involvement of IGFBPs in OP-1 action. Hence, whether des(1–3)-IGF-I, an analog with a decreased affinity for IGFBPs (34), would also exhibit a synergistic effect with OP-1 in FRC cells was examined. Confluent FRC cells were treated with serum-free MEM-containing vehicle or OP-1 in the absence or presence of either IGF-I or des(1–3)-IGF-I. The levels of AP activity in these cultures were determined. IGF-I alone was without any measurable effect (data not shown). In agreement with previous findings, OP-1 alone stimulated AP activity 3- to 4 fold above the control value (Fig. 2). IGF-I and OP-1 stimulated AP activity synergistically (Figs. 2 and 8). Des(1–3)-IGF-I also enhanced synergistically the action of OP-1 (Fig. 8; $P < 0.01$), but itself did not stimulate AP activity in FRC cells (data not shown). The AP activity in FRC cultures treated with low concentrations (5–10 ng/ml) of des(1–3)-IGF-I was about 4-fold ($P < 0.05$) greater than that in cells treated with the full-length IGF-I at the same concentrations (Fig. 8). Therefore, des(1–3)-IGF-I showed a more potent synergism with OP-1 than did IGF-I.

Effect of exogenous IGFBP-5 on OP-1-induced AP activity

The above data for des(1–3)-IGF-I imply that one mechanism of synergism between OP-1 and the native, full-length

Downloaded from https://academic.oup.com/endo/article/138/10/4181/2987658 by guest on 16 August 2022

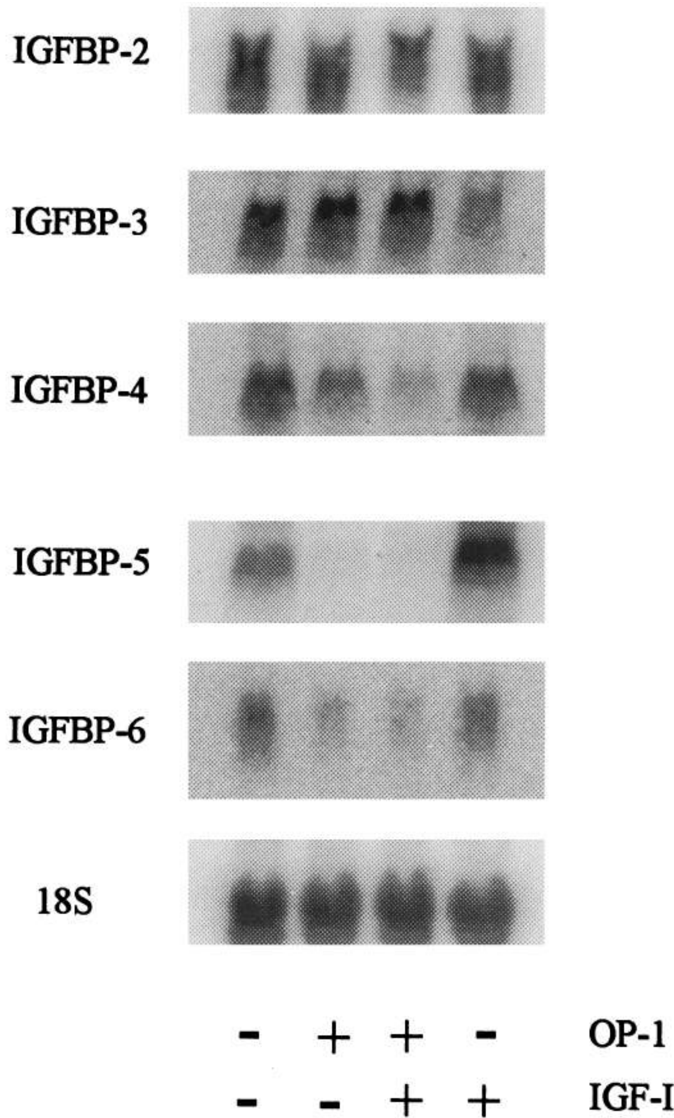

FIG. 6. Northern analysis of IGFBP-2 to -6 expression in FRC cells. Total RNA was isolated from FRC cells treated as described in Fig. 5. The blot was hybridized with the cDNA probes for the individual IGFBPs or with an oligonucleotide for 18S rRNA. The blot was exposed to a PhosphorImager screen.

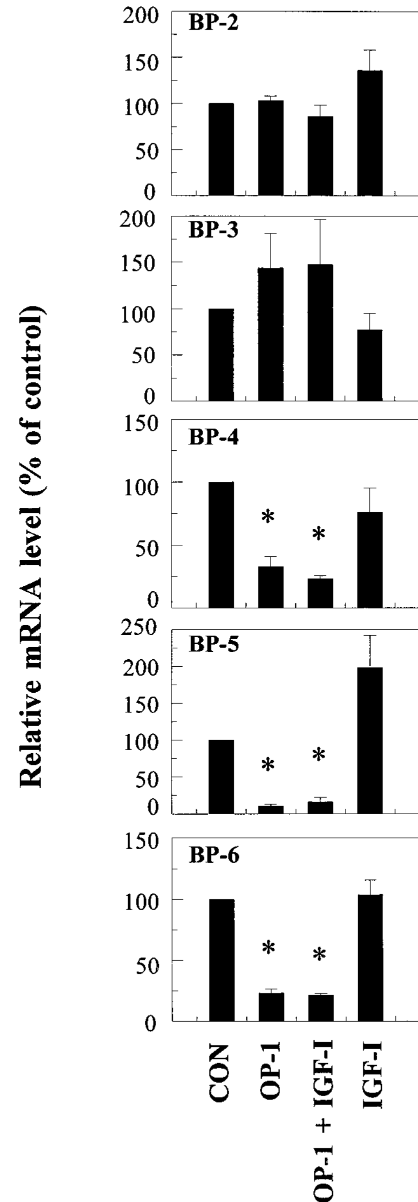

FIG. 7. Quantitative analysis of the steady state mRNA levels of IGFBPs. The intensity of the hybridized RNA species on Northern blots, as shown in Fig. 6, was quantified by phosphorimaging. The relative mRNA level was normalized to the control value (100%). Results are the mean ± SE from four independent experiments using four different FRC cell preparations and are corrected for 18S rRNA. *, $P < 0.01$ compared with the control.

IGF-I is through a decrease in the levels of the inhibitory IGFBPs by OP-1. In particular, IGFBP-5 appears to be a target for down-regulation by OP-1. To test this hypothesis, the effect of exogenous human IGFBP-5 on the OP-1-induced AP activity in FRC cells was measured. Figure 9 shows that exogenous IGFBP-5 alone did not inhibit the basal level of AP activity in FRC cells. The OP-1-induced AP activity was inhibited by exogenous IGFBP-5 (50 ng/ml). The synergy of IGF-I on OP-1 action was reduced by exogenous IGFBP-5 (50 ng/ml) to about 75% of the level in the OP-1- plus IGF-I-treated culture. Thus, together with the data showing that des(1–3)-IGF-I was more potent than IGF-I (present study) and the previously observed drop in the IGFBP-5 mRNA level after OP-1 treatment (18), the current findings strongly suggest that at least one mechanism by which OP-1 stimulates FRC cell differentiation involves down-regulation of the IGFBP-5 level. Furthermore, as exogenous IGFBP-5 also reduced the extent of synergism between OP-1 and IGF-I, at least one aspect of the mechanism of synergism involves IGFBP-5.

Effects of IGF-I and TGFβ1 on AP activity in FRC cells

As OP-1 is a member of the TGFβ superfamily, possible synergism between IGF-I and TGFβ1 was also examined. Figure 10 shows that TGFβ1 alone, in the concentration range tested, decreased AP activity in FRC cells in a dose-dependent manner (Fig. 10, lanes 3–5). Exogenous IGF-I (10–50

Downloaded from https://academic.oup.com/endo/article/138/10/4181/2987658 by guest on 16 August 2022

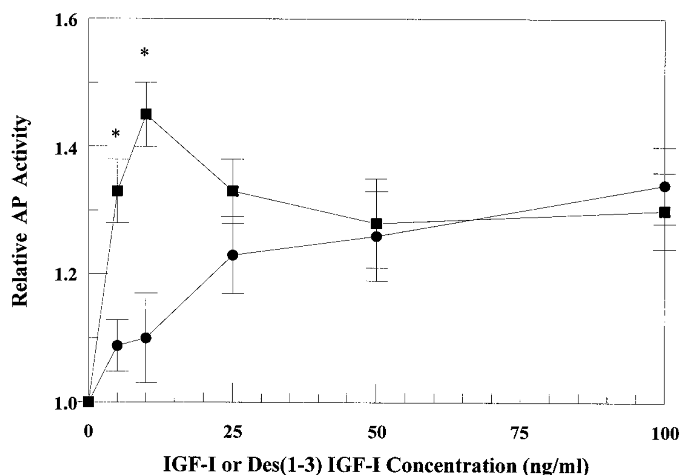

FIG. 8. Effects of des(1–3)-IGF-I on OP-1-stimulated AP activity in FRC cells. The level of AP activity in FRC cells treated with 200 ng/ml OP-1 and varying concentrations of IGF-I (●) or des(1–3)-IGF-I (■) was measured. Controls were treated with solvent vehicle only. Treatments were performed for 48 h, with a change of fresh medium at 24 h. Results are normalized to the AP activity in FRC cells treated with OP-1 alone, which is 5- to 7-fold higher than that in the solvent vehicle-treated culture. *, $P < 0.05$ compared with the control.

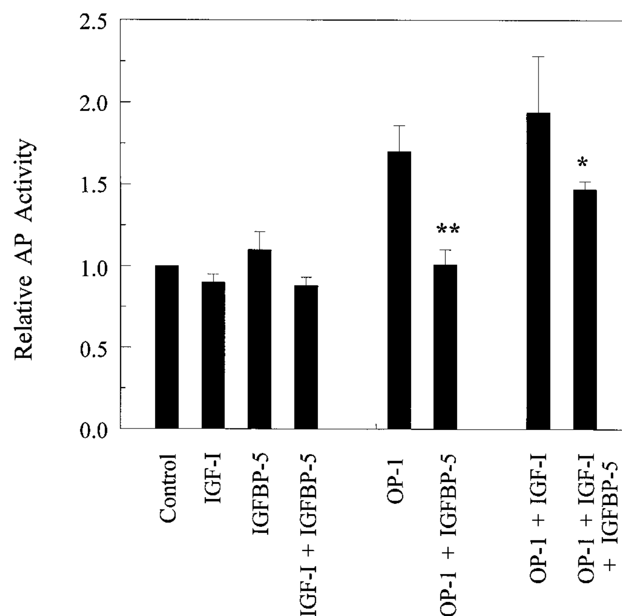

FIG. 9. Effects of exogenous IGFBP-5 on the OP-1-induced AP activity in FRC cells. Confluent FRC cells grown in 48-well plates were treated with vehicle (control), IGF-I alone (25 ng/ml), IGFBP-5 alone (50 ng/ml), IGF-I (25 ng/ml) plus IGFBP-5 (50 ng/ml), OP-1 (200 ng/ml) alone, OP-1 (200 ng/ml) plus IGFBP-5 (50 ng/ml), OP-1 (200 ng/ml) plus IGF-I (25 ng/ml), and OP-1 (200 ng/ml), IGF-I (25 ng/ml), and IGFBP-5 (50 ng/ml). All test substances were added simultaneously, and treatment was conducted for 48 h, with a change of fresh medium at 24 h. Results are normalized to the AP activity in solvent vehicle-treated controls. Values are the means of six replicates of each condition of two independent experiments using different FRC cell preparations. **, $P < 0.01$ compared with OP-1-treated culture; *, $P < 0.05$ compared with OP-1- and IGF-I-treated culture.

ng/ml) together with varying concentrations of TGFβ1 (0.05–2 ng/ml) did not significantly change the level of AP activity compared with the effect of TGFβ1 alone (Fig. 10, lanes 6–14).

Discussion

The results presented in this report reveal that OP-1 alone is capable of inducing the proliferation of FRC cells, but the induction was significantly and synergistically stimulated by IGF-I. The current study also shows that exogenous IGF-I was without effect on the expression of AP activity and PTH responsiveness, which are characteristic biochemical markers of osteoblast differentiation. Combined OP-1 and IGF-I treatment of FRC cells produced a greater stimulation of AP activity and PTH responsiveness than did OP-1 alone. Furthermore, IGF-I enhanced the OP-1 action in stimulating nodule formation, a hallmark of bone formation in cell cultures. Taken together, the present studies provide biochemical and morphological evidence supporting the idea that IGF-I synergistically enhanced both the mitogenic action and the differentiation activity of OP-1 in primary cultures of osteoblastic cells. The present finding also endorses the premise that OP-1 acts in concert with another growth factor(s) to influence the bone formation process.

Although the synergistic effect could be observed when FRC cells were treated with OP-1 for up to 24 h before IGF-I addition, maximum synergism was observed when cells were treated with OP-1 and IGF-I simultaneously or when IGF-I was added within 2 h of OP-1. Published kinetic studies in OP-1-treated FRC cells demonstrate that the IGF-I mRNA levels do not increase significantly until 24 h posttreatment (18). It is possible that the observed synergism between OP-1 and exogenous IGF-I may be the function of an accelerated sequence of events induced after OP-1 stimulation of FRC cells. We previously showed that OP-1 caused a 2-fold increase in IGF-I mRNA level. A previous study in which measurements were made at both the mRNA and protein levels in osteoblastic cells under the same experimental conditions shows that the levels were correlated (35). Extrapolating from those results, we expect that the bioavailable IGF-I level in OP-1- plus IGF-I-treated cultures is higher than that in cultures treated with IGF-I alone. It is noteworthy that IGF-I alone elevated the IGFBP-5 mRNA level compared with vehicle control value, suggesting that OP-1 might exert a dominant effect on IGFBP-5 expression relative to that of IGF-I. These changes in IGFBPs, in turn, impact upon the response to endogenous and exogenous IGF-I (see below). In agreement with this supposition is the current observation that the cellular response to OP-1 was unchanged after IGF-I pretreatment. These observations suggest that sensitization of FRC cells to IGF-I action by OP-1 is a prerequisite for synergism between OP-1 and IGF-I. These protein factors influence cellular activities differentially and, consequently, affect the timing of changes in the cellular events that culminate in important effects on bone formation.

The data on the mRNA level of the IGFBPs in OP-1-treated FRC cells imply involvement of IGFBPs in the observed synergism between OP-1 and IGF-I. Two current results provide experimental evidence supporting this hypothesis: 1) des(1–3)-IGF-I, the IGF-I analog that shows a decreased binding affinity for the IGFBPs, and 2) effects of exogenous IGFBP-5 on OP-1 action. Des(1–3)-IGF-I, the truncated form

Downloaded from https://academic.oup.com/endo/article/138/10/4181/2987658 by guest on 16 August 2022

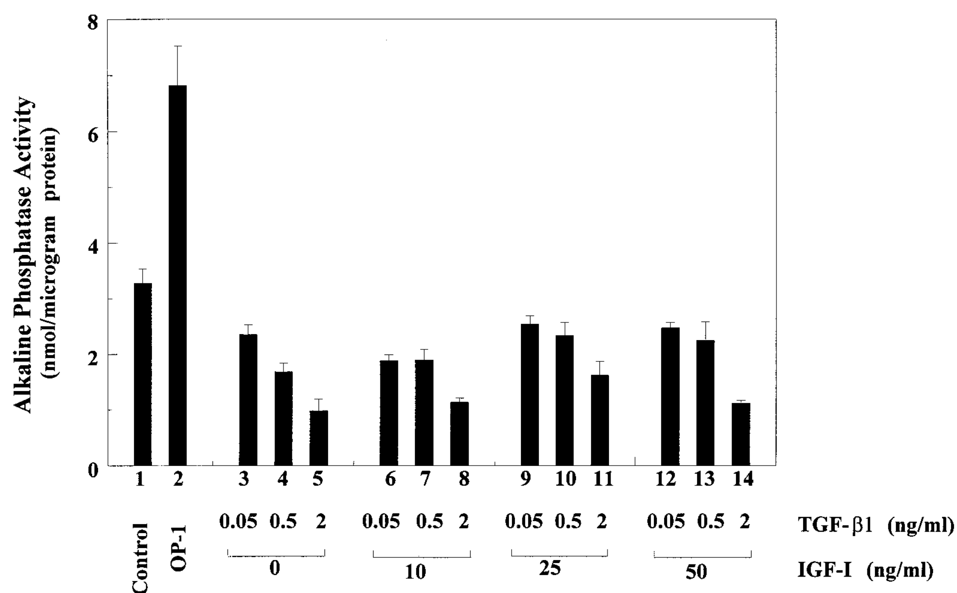

FIG. 10. Inhibition of basal AP activity by TGFβ1 in FRC cells. Confluent FRC cells in 48-well plates were incubated in serum-free αMEM containing vehicle (lane 1) or 200 ng/ml OP-1 (lane 2); in 0.05, 0.5, and 2 ng/ml TGFβ1 alone (lanes 3–5); or in a combination of varying concentrations of TGFβ1 and 10, 25, or 50 ng/ml IGF-I (lanes 6–14). The treatments (six wells per treatment) were refreshed after 24-h incubation. After a total of 48-h incubation, cells were lysed as described in Fig. 2. The activity of AP was expressed as nanomoles of *p*-nitrophenol per μg total protein. Values are the mean of three independent experiments of different preparations of FRC cells.

of IGF-I, like the full-length IGF-I molecule, exhibited a synergistic effect with OP-1 on AP activity in FRC cells. However, des(1–3)-IGF-I was more potent than the full-length IGF-I at low concentrations. The present observation agrees with the previous postulate that a decrease in the affinity of des(1–3)-IGF-I for IGFBPs resulted in an elevated level of unbound growth factor that interacted with the IGF-I receptor (34). The stimulation suggests that the mechanism by which OP-1 and the native, full-length IGF-I synergize to stimulate AP activity is through the decrease in the levels of inhibitory IGFBPs by OP-1. IGFBP-5, in particular, appears to be a target for down-regulation by OP-1. At higher concentrations, the levels of stimulation by both forms of IGF-I were similar. Presumably, at these high concentrations of des(1–3)-IGF-I and IGF-I, the IGF-I receptors were saturated, and the role of the IGFBPs in regulating the bioavailability of IGF-I was minimized. The data support the hypothesis that IGFBPs play a role in the action of OP-1 in FRC cells. The finding further implies that the synergism between OP-1 and IGF-I observed with the full-length IGF-I molecule was at least partially the result of an IGF-I receptor-mediated event. The observation that exogenous IGFBP-5 blocked the OP-1-induced AP activity and the combined OP-1 and IGF-I synergism supports the hypothesis that OP-1 sensitizes cells to IGF-I by reducing the level of IGF-I inhibitory binding proteins. As a result, OP-1 and IGF-I are able to synergistically stimulate FRC cell differentiation.

The observed synergism exhibited by OP-1 and IGF-I in FRC cells is the first reported between OP-1 and another growth factor. The actions of BMP-2 and BMP-3, which share 60% and 42% amino acid sequence homology with OP-1, have been reported to be potentiated by nonpeptide factors, such as vitamin D and retinoic acid in clonal cell lines (36–38). It is noteworthy that our results demonstrate that TGFβ1, which shares 35% amino acid sequence identity with OP-1, exhibited an antagonistic effect on AP activity in FRC cells, with half-maximum inhibition at about 0.5 ng/ml. Exogenous IGF-I did not change the TGFβ1 inhibitory effect. This observation agrees with earlier reports that TGFβ1 inhibits AP gene expression in FRC cells (39) and AP activity in MC3T3-E1 murine osteoblast-like cells (40) and mature MBA-15.6 human osteoblastic cell (38). However, TGFβ1 very slightly stimulates enzymatic activity in the human preosteoblastic cell MBA-15.4 (38). In osteoblastic cells, it is clear that the actions of OP-1 and TGFβ1 are diverse, thus emphasizing the physiological significance of OP-1 in osteoblastic cell development.

In summary, we have demonstrated a synergism between OP-1 and IGF-I in the stimulation of biochemical and morphological markers characteristic of bone cell differentiation in primary cultures of fetal rat calvaria cells. IGFBPs and IGFBP-5, in particular, are among the factors involved in the synergism between OP-1 and IGF-I.

Acknowledgments

The authors thank Stryker Biotech for the support. Plasmids used for the rat IGFBPs studies were kindly provided by Drs. S. Shimisaki and N. Ling, Whittier Institute (La Jolla, CA). We thank Dr. David R. Clemmons, University of North Carolina (Chapel Hill, NC), for providing purified human IGFBP-5; Dr. Joan Massague, Memorial Sloan-Kettering Cancer Center (New York, NY), for providing the human BMPR-II cDNA; and Dr. Lynda F. Bonewald, Department of Medicine, University of Texas Health Science Center (San Antonio, TX), for her valuable advice concerning the bone nodule formation assay. We also thank Megan Asher and Dr. Allison Kitten for preparation of initial supplies of frozen FRC cell stocks, and Karen P. Betchel for technical assistance.

References

1. **Ozkaynak E, Rueger DC, Drier EA, Corbett C, Ridge RJ, Sampath TK, Oppermann H** 1990 OP-1 cDNA encodes an osteogenic protein in the TGF-β family. EMBO J 9:2085–2093
2. **Sampath TK, Coughlin JE, Whetstone RM, Banach D, Corbett C, Ridge RJ, Ozkaynak E, Oppermann H, Rueger DC** 1990 Bovine osteogenic protein is composed of dimers of OP-1 and BMP-2A, two members of the transforming growth factor-β superfamily. J Biol Chem 265:13198–13205
3. **Wozney JM, Rosen V, Celeste AJ, Mitsock LM, Whitters MJ, Kriz RW, Hewick RM, Wang EA** 1988 Novel regulators of bone formation: molecular clones and activities. Science 242:1528–1534
4. **Cook SD, Baffes GC, Wolfe MW, Sampath TK, Reuger DC** 1994 Recombinant

Downloaded from https://academic.oup.com/endo/article/138/10/4181/2987658 by guest on 16 August 2022

human bone morphogenetic protein-7 induces healing in a canine long-bone segmental defect model. Clin Orthop Rel Res 301:302–312
5. **Cook SD, Baffes GC, Wolfe MW, Sampath TK, Reuger DC, Whitecloud TS** 1994 The effect of recombinant human osteogenic protein-1 on healing of large segmental bone defects. J Bone Joint Surg Am 76:827–838
6. **Cook SD, Baffes GC, Sampath TK, Rueger DC** 1992 Healing of large segmental defects using recombinant human osteogenic protein (rhOP-1). Trans Orthop Res Soc 17:581–587
7. **Knutsen R, Wergedal JE, Sampath TK, Baylink DJ, Mohan S** 1993 Osteogenic protein-1 stimulates proliferation and differentiation of human bone cells *in vitro*. Biochem Biophys Res Commun 194:1352–1358
8. **Sampath TK, Maliakal JC, Hauschka PV, Jones WK, Sasak H, Tucker RF, White KH, Coughlin JE, Tucker MM, Pang RHL, Corbett C, Ozkaynak E, Oppermann H, Rueger DC** 1992 Recombinant human osteogenic protein-1 (hOP-1) induces new bone formation *in vivo* with a specific activity comparable with natural bovine osteogenic protein and stimulates osteoblast proliferation and differentiation *in vitro*. J Biol Chem 267:20352–20362
9. **Asahina I, Sampath TK, Nishimura I, Hauschka PV** 1993 Human osteogenic protein-1 induces both chondroblastic and osteoblastic differentiation of osteoprogenitor cells derived from newborn rat calvaria. J Cell Biol 123:921–933
10. **Sodek J, Chen J, Nagata T, Kasugai S, Todescan Jr R, Li IW, Kim RH** 1995 Regulation of osteopontin expression in osteoblasts. Ann NY Acad Sci 760:223–241
11. **Kitten AM, Lee JC, Olson MS** 1995 Osteogenic protein-1 enhances phenotypic expression in ROS 17/2.8 cells. Am J Physiol 269:E918–E926
12. **Maliakal JC, Asahina I, Hauschka PV, Sampath TK** 1994 OP-1 (BMP-7) inhibits cell proliferation and stimulates the expression of markers characteristic of osteoblast phenotype in rat osteosarcoma (17/2.8) cells. Growth Factors 11:227–234
13. **Andrews PW, Damjanov I, Berends J, Kumpf S, Zappavigna V, Mavilio F, Sampath TK** 1994 Inhibition of proliferation and induction of differentiation of pluripotent human embryonal carcinoma cells by OP-1 (BMP-7). Lab Invest 71:243–251
14. **Hentunen TA, Lakkakorpi PT, Tuukkanen J, Lehenkari PP, Sampath TK, Vaananen HK** 1995 Effects of recombinant human osteogenic protein-1 on the differentiation of osteoclast-like cells and bone resorption. Biochem Biophys Res Commun 209:433–443
15. **Knutsen R, Honda Y, Strong DD, Sampath TK, Baylink DJ, Mohan S** 1995 Regulation of insulin-like growth factor system components by osteogenic protein-1 in human bone cells. Endocrinology 136:857–865
16. **Kanzaki S, Baxter RC, Knutsen R, Baylink DJ, Mohan S** 1995 Evidence that human bone cells in culture secrete insulin-like growth factor (IGF)-II and IGF binding protein-3 but not acid-labile subunit both under basal and regulated conditons. J Bone Miner Res 10:854–858
17. **Srinivasan N, Knutsen R, Baylink DJ, Sampath TK, Mohan S** 1995 Evidence that TGF-β, and BMP-7 increase insulin-like growth factor binding protein (IGFBP)-3 production *via* different protein kinase-C-dependent signal transduction pathways in human bone cells. J Bone Miner Res [Suppl 1] 10:S310
18. **Yeh LCC, Adamo ML, Kitten AM, Olson MS, Lee JC** 1996 Osteogenic protein-1-mediated IGF gene expression in primary cultures of rat osteoblastic cells. Endocrinology 137:1921–1931
19. **Aronow MA, Gerstenfeld LC, Owen TA, Tassinari MS, Stein GS, Lian JB** 1990 Factors that promote progessive development of the osteoblast phenotype in cultured fetal rat calvarial cells. J Cell Physiol 143:213–221
20. **McCarthy TL, Centrella M, Canalis E** 1988 Further biochemical and molecular characterization of primary rat parietal bone cell cultures. J Bone Miner Res 3:401–408
21. **Bradford MM** 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
22. **Bellows CG, Aubin JE, Heersche JNM, Antosz ME** 1986 Mineralized bone nodules formed *in vitro* from enzymatically released rat calvaria cell populations. Calcif Tissue Int 38:143–154
23. **Drury RAB, Wallington EA** 1980 Modified von Kossa Method in Carleton's Histological Technique, ed 5. Oxford University Press, Oxford, pp 217–218
24. **McCarthy TL, Centrella M, Canalis E** 1989 Regulatory effects of IGF-I and IGF-II on bone collagen synthesis in rat calvarial cultures. Endocrinology 124:301–309
25. **Li IWS, Cheifetz S, McCulloch CAG, Sampath KT, Sodek J** 1996 Effects of osteogenic protein-1 (OP-1, BMP-7) on bone matrix protein expression by fetal rat calvarial cells are differentiation state specific. J Cell Physiol 169:115–125
26. **ten Dijke P, Yamashita H, Sampath TK, Reddi AH, Estevez M, Riddle DL, Ichijo H, Heldin C-H, Miyazono K** 1994 Identification of type I receptors for osteogenic protein-1 and bone morphogenetic protein-4. J Biol Chem 269:16985–16988
27. **Takeda K, Oida S, Ichijo H, Iimura T, Maruoka Y, Amagasa T, Sasaki S** 1994 Molecular cloning of rat bone morphogenetic protein (BMP) type IA receptor and its expression during ectopic bone formation induced by BMP. Biochem Biophys Res Commun 204:203–209
28. **Yamashita H, ten Dijke P, Huylebroeck D, Sampath TK, Andries M, Smith JC, Heldin C-H, Miyazono K** 1995 Osteogenic protein-1 binds to activin type II receptors and induces certain activin-like effects. J Cell Biol 130:217–226
29. **Rosenzweig BL, Imamura T, Okadome T, Cox GN, Yamashita H, ten Dijke P, Heldin C-H, Miyazono K** 1995 Cloning and characterization of a human type II receptor for bone morphogenetic proteins. Proc Natl Acad Sci USA 92:7632–7636
30. **Liu F, Ventura F, Doody J, Massague J** 1995 Human type II receptor for bone morphogenetic proteins (BMPs): extension of the two-kinase receptor model to the BMPs. Mol Cell Biol 15:3479–3486
31. **Estevez M, Attisano L, Wrana JL, Albert PS, Massague J, Riddle DL** 1993 The *daf-4* gene encodes a bone morphogenetic protein receptor controlling *C. elegans* dauer larva development. Nature 365:644–649
32. **ten Dijke P, Yamashita H, Sampath TK, Reddi AH, Estevez M, Riddle DL, Ichijo H, Heldin C-H, Miyazono K** 1994 Characterization of type I receptors for transforming growth factor-β and activin. Science 264:101–104
33. **Dong Y, Canalis E** 1995 Insulin-like growth factor (IGF) and retinoic acid induce the synthesis of IGF-binding protein 5 in rat osteoblastic cells. Endocrinology 136:2000–2006
34. **Francis GL, Ross M, Ballard FJ, Milner SJ, Senn C, McNeil KA, Wallace JC, King R, Wells JRE** 1992 Novel recombinant fusion protein analogues of insulin-like growth factor (IGF)-I indicate the relative importance of IGF-binding protein and receptor binding for enhanced biological potency. J Mol Endocrinol 8:213–223
35. **Canalis E, Gabbitas B** 1994 Bone morphogenetic protein 2 increases insulin-like growth factor I and II transcripts and polypeptide levels in bone cell cultures. J Bone Miner Res 9:1999–2005
36. **Kawamura M, Urist MR** 1988 Growth factors, mitogens, cytokines, and bone morphogenetic protein in induced chondrogenesis in tissue culture. Dev Biol 130:435–442
37. **Benayahu D, Fried A, Shamay A, Cunningham N, Blumberg S, Wientroub S** 1994 Differential effects of retinoic acid and growth factors on osteoblastic markers and CD10/NEP activity in stromal-derived osteoblasts. J Cell Biochem 56:62–73
38. **Benayahu D, Fried A, Wientroub S** 1995 PTH and 1,25$(OH)_2D_3$ priming to growth factors differentially regulates the osteoblastic markers in MBA-15 clonal subpopulations. Biochem Biophys Res Commun 210:197–204
39. **Harris SE, Bonewald LF, Harris MA, Sabatini M, Dallas S, Feng JQ, Ghosh-Choudhury N, Wozney J, Mundy GR** 1994 Effects of transforming growth factor beta on bone nodule formation and expression of bone morphogenetic protein 2, osteocalcin, osteopontin, alkaline phosphatase, and type I collagen mRNA in long term cultures of fetal rat calvarial osteoblasts. J Bone Miner Res 9:855–863
40. **Noda M, Rodan GA** 1986 Type β-transforming growth factor inhibits proliferation and expression of alkaline phosphatase in murine osteoblast-like cells. Biochem Biophys Res Commun 140:56–65

Downloaded from https://academic.oup.com/endo/article/138/10/4181/2987658 by guest on 16 August 2022