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Effect of Osteogenic Protein-1 on the Development and Mineralization of Primary Cultures of Avian Growth Plate Chondrocytes: Modulation by Retinoic Acid

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Abstract Osteogenic protein-1 (OP-1), a member of the TGF-β family of proteins, induces endochondral bone formation. Here we studied the effect of OP-1 on the development of primary cultures of avian growth plate (GP) chondrocytes in either serum-free or serum-containing medium, in the absence or presence of retinoic acid (RA). OP-1 was added on day 7 of culture and continued for 7 days, or until the cultures were harvested, typically on day 21. Alone, OP-1 caused ~2-fold increase in proteoglycan synthesis into both the medium and the cell:matrix layer. Additionally, OP-1 caused a dosage-dependent increase in alkaline phosphatase (ALP) activity, and an increase in protein, when given from days 7–14 and examined on day 14. This stimulation was greater in cells grown in serum-free than in serum-containing media (3–5-fold vs. 2–3-fold increase in ALP; ~40% vs. ~20% increase in protein). Such stimulation of ALP activity and proteoglycan (PG) synthesis in cultured GP cells indicates that OP-1 elicits differentiation of chondrocytes. OP-1 minimally affected cell division (DNA content); however, a slight increase was seen when examined early in the culture. Alone, OP-1 increased mineral (Ca and Pi) content of the cultures by ~2-fold in both types of media. As early as day 14, clusters of mineral encircled many of the OP-1 treated cells. Thus, as in vivo, OP-1 strongly promoted mineral formation by the cultured GP chondrocytes. When present together, OP-1 and RA generally blocked the action of the other. Separately OP-1 and RA each stimulated protein synthesis, ALP activity, and Ca^{2+} deposition; together they were inhibitory to each. Also, RA blocked the stimulation of PG synthesis induced by OP-1; whereas OP-1 decreased cell division engendered by RA. Thus, this GP chondrocyte culture system is a good model for studying factors that influence differentiation and mineral deposition during bone growth in vivo. J. Cell. Biochem. 67:498–513, 1997. © 1997 Wiley-Liss, Inc.

Key words: chondrocytes; osteogenic protein-1; retinoic acid; mineralization; ALP; proteoglycans

Implantation of demineralized bone matrix powder intramuscularly or subcutaneously at non-bony sites induces cartilage and bone formation [Urist, 1965; Reddi and Huggins, 1972]. This induction recapitulates the pathway of normal embryonic bone development, including the migration of mesenchymal cells, condensation, proliferation, and differentiation to cartilage and bone-forming cells [Reddi, 1981]. Several bone morphogenetic proteins (BMPs) involved in ectopic bone formation have been purified from active fractions of the bone matrix [Wang et al., 1988; Luyten et al., 1992; Sampath et al., 1990]. Subsequently, cloning of cDNAs encoding BMPs 2–7 [Wozney et al., 1988; Celeste et al., 1990], BMP 10 [Cunningham et al., 1995], and the osteogenic proteins, OP-1 and OP-2 (also known as BMP-7 and -8) [Özkaynak et al., 1990, 1992], has led to the discovery that these proteins are members of the TGF-β superfamily. These proteins share a distinctive pattern of seven cysteine residues in their carboxy-terminal domains. Localization studies have shown that OP-1 (BMP-7) is present in the hypertrophic zone of developing long bones, suggesting its involvement in the physiological

Abbreviations: ALP, alkaline phosphatase; BMP, bone morphogenetic protein; GP, growth plate; LDH, lactate dehydrogenase; OP-1, osteogenic protein 1; PG, proteoglycan; RA, retinoic acid.

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regulation of endochondral bone formation [Vukicevic et al., 1994; Houston et al., 1994; Helder et al., 1995]. The mechanisms of action of OP-1 in osteogenesis are not yet clearly established.

Various in vitro studies have demonstrated that recombinant BMPs and OP-1 can: (1) *enhance* the osteoblastic characteristics of *osteoblast-like* cells (BMPs 2–4, OP-1) [Takuwa et al., 1991; T.L. Chen et al., 1991; Sampath et al., 1992; Asahina et al., 1996], and *induce* osteoblastic phenotype in *osteoprogenitor* cells (BMP-2) [Yamaguchi et al., 1991; Katagiri et al., 1990; Thies et al., 1992; Iwasaki et al., 1994]; (2) maintain chondrocytic phenotype in cultured articular chondrocytes (BMPs 2–4) [Luyten et al., 1992, 1994; Hiraki et al., 1991; Vukicevic et al., 1989], chick limb bud chondrocyte precursor cells (BMPs 2–4) [Carrington et al., 1991; P. Chen et al., 1991; Rosen et al., 1994], and embryonic sternal chondrocytes (OP-1) [Chen et al., 1995]; and (3) induce chondrocytic properties in non-cartilage cells (OP-1) [Asahina et al., 1996] and (BMP-2) [Katagiri et al., 1994]. Thus, OP-1 and its relatives have both chondrogenic and osteogenic differentiation capabilities.

We have developed a long-term primary culture system for studying growth plate (GP) chondrocyte maturation [Wu et al., 1989, 1995]. Only when supplemented with ascorbic acid and physiological levels of inorganic phosphate [Ishikawa and Wuthier, 1992] do the cells become capable of inducing matrix mineralization. In the present study, we examined the effect of recombinant OP-1 on GP chondrocytes grown in both serum-free and serum-containing media, administered in the absence or presence of retinoic acid (RA). The recombinant human OP-1 (rhOP-1) used was a disulfide-linked homodimer with an apparent MW of 36,000 [Sampath et al., 1992]. Cells were exposed to rhOP-1 either transiently or continuously, before or after confluence, to observe any chondro-inductive effects. In later studies, this regimen of OP-1 exposure was combined with RA. Levels of chondrocytic markers such as synthesis of proteoglycans and ALP were measured. Cell division (DNA content), and collagen and protein content, were also compared. Mineralization of the cultures was analyzed biochemically and histologically. The combined effects of RA and OP-1 were investigated to provide clues as to how these factors interact during chondrogenesis and bone formation.

MATERIALS AND METHODS

Chondrocyte Cultures

Chondrocytes were isolated from epiphyseal growth plate cartilage of the tibiae of 6–8-week-old hybrid broiler-strain chickens and plated in 35-mm dishes as previously described [Wu et al., 1989, 1995]. For serum containing DATP5 medium, cells were supplied with Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 8 amino acids found to be enriched in the extracellular fluid of the avian growth plate [Ishikawa et al., 1985], insulin-transferrin-selenite (ITS), and 1 mM Na_2HPO_4 [Ishikawa and Wuthier, 1992] from day 6 or 7 onward. For serum-free HL-1 medium, on day 6 or 7, cells were supplied with a medium composed of a 1:1 mixture of DMEM (containing 10% FBS) and serum-free HL-1 medium, and from day 10 onward, the cells were given only HL-1. The culture medium was changed every 3–4 days. Ascorbate was provided from day 3 onward at a concentration of 50 µg/ml. To prepare the stock solution, OP-1 was dissolved in 50% ethanol-0.1% trifluoroacetic acid and added to the treatment group after dilution to the desired concentration. Control culture dishes received the same volume of vehicle (2.0–2.5 µl per 2 ml of medium). OP-1 was supplied by Dr. T.K. Sampath (Creative BioMolecules, Inc., Hopkinton, MA). RA stock solution (1 mM) was made in ethanol, with further dilution in ethanol as required. RA was added to the cultures on day 7 for preconfluent treatment, and was added on day 14 for postconfluent treatment, in each case for the duration specified in the figures and tables.

Cell Harvest

On the specified day, chondrocytes were harvested after removal of the medium. The cell layer was rinsed twice with 1 ml of TMS (50 mM Tris, pH 7.5, 1.5 mM $MgCl_2$, and 10% sucrose) and scraped from the culture dishes after addition of 2 ml of TMS. Scraping was repeated twice with 1 ml of TMS to ensure removal of all cell and matrix material. This suspension was sedimented at 3,000 rpm for 30 min and 1 ml of TMT (10 mM Tris, pH 7.5, 0.5 mM $MgCl_2$, 0.05% Triton X-100) was added and stored frozen at −20°C. The harvested material was dispersed by sonication, and used for the analyses described below.

Biochemical Analyses

All analyses were measured using a Labsystems IEMS microplate reader MF (Needham Heights, MA). Cellular and matrix protein content was analyzed using bovine serum albumin as a standard [Lowry et al., 1951]. Alkaline phosphatase (ALP) activity was determined using *p*-nitrophenylphosphate as a substrate by incubating aliquots (20 µl) of the TMT sonicate with 250 µl of substrate at 37°C for 5–10 min [Wu et al., 1995]. Proteoglycan content of the cell-matrix layer was analyzed in aliquots (20 µl) of the TMT sonicate, and proteoglycan secreted into the medium was analyzed in aliquots (10–30 µl) of medium, 5 mM benzamidine, 15 mM EDTA, and 250 µl of dimethylmethylene blue reagent and measuring the absorbency difference at 595 and 520 nm [Chandrasekhar et al., 1987]. For determination of calcium and phosphate mineral content in the cell layer, the TMT sonicate was centrifuged at 3,000 rpm for 40 min and the TMT supernatant was removed. The resulting cell-matrix pellet was incubated in 0.1 N HCl (1 ml per dish) at 4°C overnight to dissolve the mineral [Wu et al., 1995]. The HCl extract obtained after centrifugation was used for Ca^{2+} and Pi analyses. Ca^{2+} was measured using *o*-cresol phthalein complexone [Baginski et al., 1973], and Pi was analyzed using the ammonium molybdate method [Ames, 1966]. Cellular DNA content was determined using fluorescent dye, *bis*-benzimidazole (Hoechst 33258) with aliquots of the TMT cell sonicate; the DNA standard curve was constructed using the same level of TMT vehicle and measured using a Labsystems Fluoroskan II fluorescent microplate reader [Rago et al., 1990]. Lactate dehydrogenase (LDH) activity, measured to assess anaerobic metabolism, was assayed in 10-µl aliquots at room temperature in 250 µl of 0.2 M Tris · HCl buffer, pH 7.5, containing 0.2% Triton X-100, 0.15 mM NADH, and 1 mM sodium pyruvate. For statistical analyses of the data, Student's *t*-tests were performed; differences were considered increasingly significant as *P* values decreased ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

The amount of collagen produced in the presence or absence of OP-1 was monitored in both the medium and cell-matrix fractions [Wu et al., 1995; Miller and Rhodes, 1982]. For isolation of medium collagen, the media were pooled from 4 dishes, protease inhibitors were added (2.5 mM EDTA, 0.2 mM PMSF, and 1 mM benzamidine), and the mixture clarified by centrifugation. A 1.25 ml portion of the resulting supernatant was transferred to a microfuge tube and 0.54 ml of saturated $(NH_4)_2SO_4$ was added (30% saturation), incubating at 4°C for 16 h. The medium collagen was then sedimented at 16,000*g* for 20 min and the precipitate was solubilized in 0.25 M acetic acid. An aliquot was boiled in Laemmli sample buffer after neutralization. For isolation of the cell-matrix collagen and cellular proteins, the cell layer was extracted with TMT buffer using sonication, and aliquots of the TMT suspension from each of the 4 dishes were pooled and boiled in Laemmli sample buffer. Proteins were analyzed by 7.5–15% gradient or 8% acrylamide SDS-PAGE [Laemmli, 1970]. Additionally, collagen content of the cell-matrix layer (the whole TMT sonicate, see Cell Harvest) was determined by hydroxyproline analysis after 6 N HCl acid hydrolysis. Amino acid analyses were carried out using a Beckman (Palo Alto, CA) System 6300/System Gold amino acid analyzer.

RESULTS

Effect of OP-1 on Enzyme Activity, Protein Synthesis, Cell Division, and Proteoglycan Secretion

Figure 1 shows that alkaline phosphatase (ALP) activity was stimulated by OP-1 addition to the growth plate chondrocyte cultures. An increase in ALP activity was seen after 1 week of treatment (days 7–14, harvested on day 14; or days 14–21, harvested on day 21), or after 10 days of treatment (days 7–17, harvested on day 17) in cells grown in either serum-containing DATP5 medium or serum-free HL-1 medium. OP-1 at a dosage of 10 or 25 ng/dish (2.0 ml media) potently stimulated ALP, increasing activity as much as 3-fold. If treatment was prolonged (days 7–21), the increase in ALP activity was somewhat diminished. In the presence of OP-1, early measurement was necessary in order to observe the rise in ALP activity before it was obliterated by the increased mineralization [Genge et al., 1988].

Figure 2 shows that protein content of the cultures was also influenced by OP-1. A small (15–20%) increase in protein content was seen during treatment of the chondrocytes with 25 ng of OP-1 from days 7–14 in serum-containing DATP5 medium, when harvested at day 14. Similar enhancement of protein synthesis (20–45%) was seen in serum-free medium when

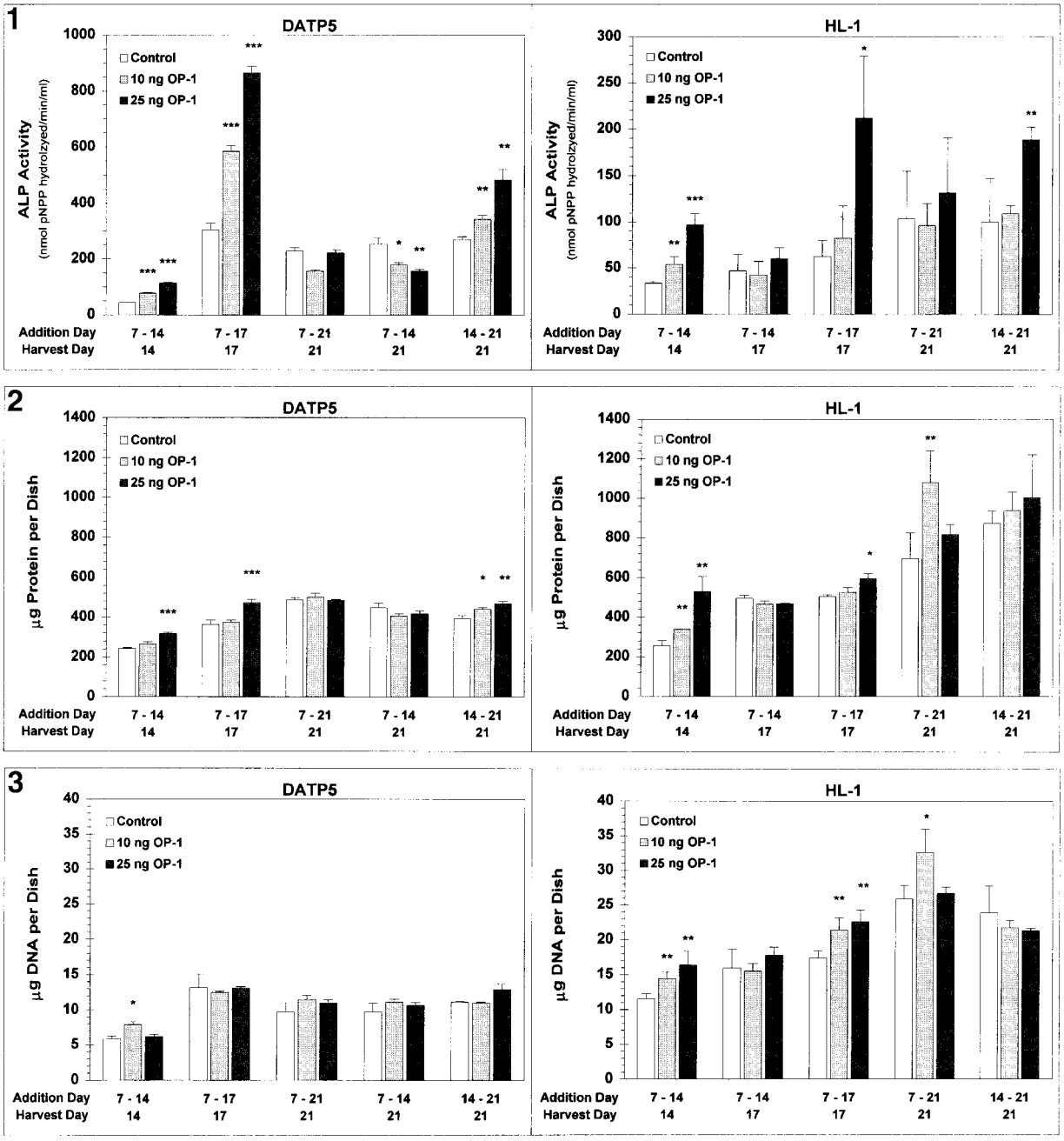


Fig. 1. Effect of OP-1 on ALP activity of primary cultures of growth plate chondrocytes. Primary cultures of avian growth plate chondrocytes were treated with OP-1 transiently or continuously in serum-containing DATP5 medium (**left**) or serum-free HL-1 medium (**right**). OP-1 was added to either pre-confluent cultures on day 7, or post-confluent cultures on day 14, and at each 3–4 day feeding interval thereafter as specified (addition day). Cells were harvested on days 14, 17, or 21, as indicated in the figure (harvest day). Values represent the mean ± SEM of 4 samples each. Open bars, control; dotted bars, OP-1 10 ng/dish; solid bars, OP-1 25 ng/dish. Asterisks (*) indicate values significantly different from the control: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Note that OP-1 greatly stimulated ALP activity, especially in early time points in cells grown in either DATP5 or HL-1 medium.

Fig. 2. Effect of OP-1 on synthesis of cellular protein by primary cultures of growth plate chondrocytes. OP-1 was added pre-confluently and post-confluently to cultured cells grown in serum-containing DATP5 medium (**left**) or serum-free HL-1 medium (**right**). Treatment with OP-1 at 0, 10, and 25 ng/dish was as described in Figure 1. Values are the mean ± SEM of 4 samples each. Asterisks indicate values significantly different from the control: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Fig. 3. Effect of OP-1 on DNA content of primary cultures of growth plate chondrocytes. OP-1 was added to the serum-free and serum-containing cultures as described in Figure 1. DNA was analyzed by Hoechst 33258 dye fluorescence [Rago et al., 1990]. Values represent the mean ± SEM of 4 samples each. Asterisks indicate values significantly different from the control: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

treated from days 7–14 or days 7–17, and harvested on the last day of treatment. The effect of OP-1 on protein synthesis was more prominent during the earlier time periods in both serum-free and serum-containing cultures.

The extent of cell division (amount of DNA per dish) was not changed significantly by OP-1 in serum-containing media (Fig. 3). However, in serum-free HL-1 grown cells, small increases in DNA content were observed when OP-1 was administered early (from day 7) in the culture. In post-confluent treatments (days 14–21), OP-1 did not promote cell proliferation in either serum-containing or serum-free systems.

One prominent feature exerted by OP-1 was a consistent increase in proteoglycan content of the cultures (Figs. 4 and 5). PG secretion into the medium was increased as early as day 10 after only 3 days of treatment (days 7–10). The presence of OP-1 for up to day 17 was required to sustain the increase in medium PG (Fig. 4). PG content in the cell/matrix layer was also markedly increased by OP-1 (Fig. 5). OP-1 at both 10 or 25 ng/dish caused a substantial increase in PG synthesis.

Effect of OP-1 on Calcium Phosphate Mineral Formation

We also investigated the effect of OP-1 on the terminal differentiation, which in growth plate

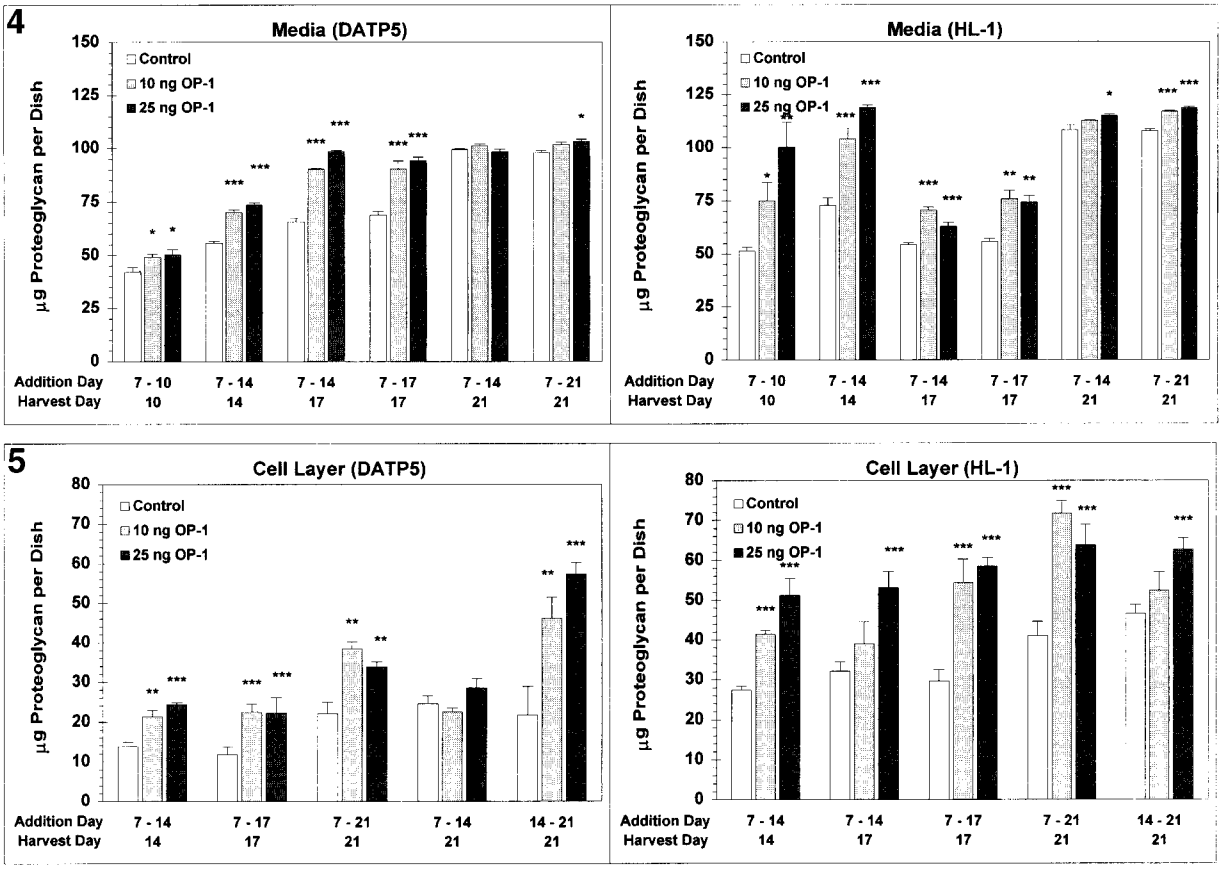
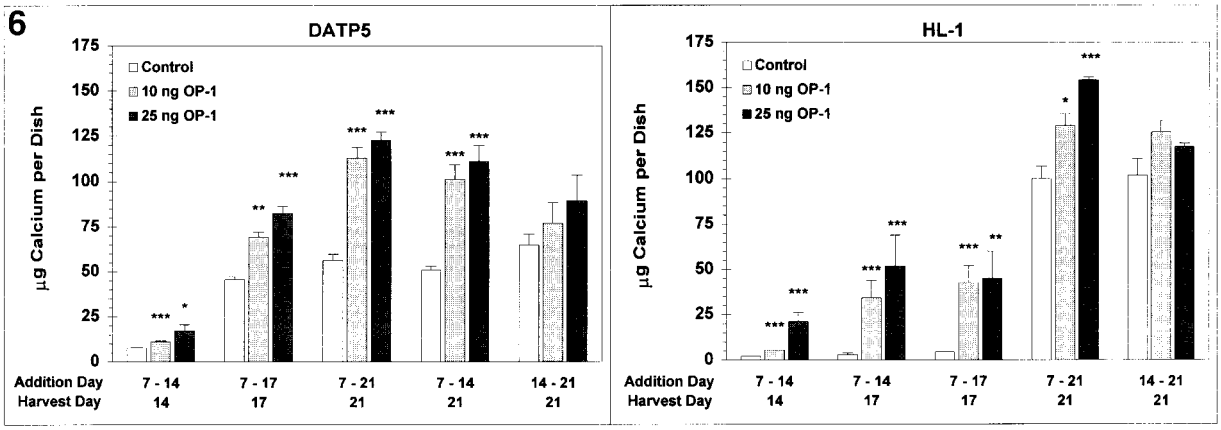


Fig. 4. OP-1 stimulates proteoglycan secretion into the culture medium by primary cultures of avian growth plate chondrocytes. PG secretion into serum-containing DATP5 (**left**) or serum-free HL-1 (**right**) culture medium was analyzed after OP-1 treatment. OP-1 at 0, 10, and 25 ng/dish was added to the cultures either continuously from day 7 to day 21, with media being collected on days 10, 14, 17, and 21; or transiently between days 7 and 14, with media being collected on days 17 and 21. Aliquots (30 µl) were taken from the medium for PG analysis [Chandrasekhar et al., 1987] as described in Materials and Methods. Values represent the mean ± SEM of 4 samples each. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Fig. 5. OP-1 stimulates proteoglycan secretion into the cell/matrix layer by primary cultures of avian growth plate chondrocytes. PG secretion into the cell/matrix layer of serum-containing DATP5 (**left**) or serum-free HL-1 (**right**) cultures was analyzed after OP-1 treatment. OP-1 at 0, 10, and 25 ng/dish was added to the cultures as described in Figure 1. The cell/matrix layer was harvested on the specified day in TMT buffer (see Cell Harvest). PG content was analyzed using 20 µl of the TMT sonicate as described in Materials and Methods. Values represent the mean ± SEM of 4 samples each. $**P < 0.01$, $***P < 0.001$.

chondrocytes is calcification. Biochemical analyses of Ca^{2+} and Pi content of the cultures (Figs. 6 and 7), and phase-contrast micrographs revealing an abundance of refractile rings around calcifying cells and massive mineralized areas (Fig. 8), demonstrated this effect. OP-1 stimulated the accumulation of Ca^{2+} in serum-containing DATP5 cultures as early as day 14 (Fig. 6, left panel). Even relatively brief exposure to OP-1 (either 10 or 25 ng/dish) from day 7 to day 14 increased the Ca^{2+} content by 100% when examined on day 21. Deposition of Ca^{2+} was also increased when OP-1 was added to post-confluent cells (days 14–21); however, the effect was somewhat weaker. In serum-free cultures (Fig. 6, right panel) OP-1 caused up to 10-fold increase in Ca^{2+} deposition when cells were treated from days 7–14 or days 7–17. Untreated control cells started to mineralize only by day 21; however, cells treated with OP-1 at 25 ng/dish for only 7 days (days 7–14) had significant calcification. This increase in Ca^{2+} deposition in the matrix layer caused by OP-1 continued to be evident until day 21.

The deposition of Pi, the counterpart to Ca^{2+} in bone mineral, was also increased by OP-1 (Fig. 7). The effects on Pi deposition were closely parallel to those on Ca^{2+}, and while consistent, were slightly less dramatic in the very early phases (days 7–14). Phase contrast microphoto-



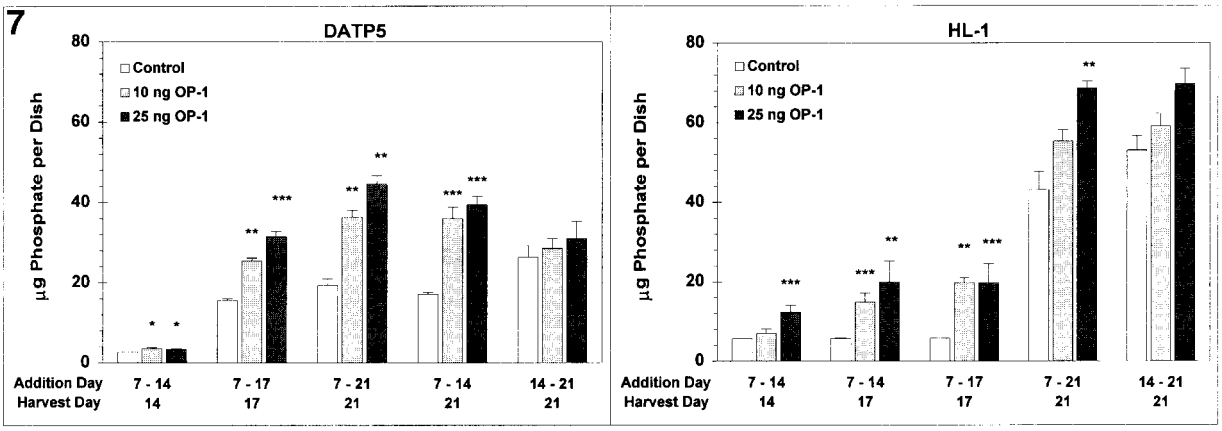


Fig. 6. OP-1 stimulates calcium deposition by primary cultures of avian growth plate chondrocytes. Cultures were treated with 0, 10, or 25 ng/dish of OP-1 in either serum-containing DATP5 (**left**) or serum-free HL-1 medium (**right**) as specified in Figure 1. The amount of mineral deposited in the cultures was analyzed after extraction of the cell/matrix layer with 0.1 N HCl. Aliquots of the acid extract were analyzed for calcium by the Baginski et al. method [1973] (see Materials and Methods). Note that mineral formation by the chondrocytes was greatly stimulated by OP-1 treatment, by as much as 10-fold in serum-free HL-1 medium. Values represent the mean ± SEM of 4 samples each. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Fig. 7. OP-1 stimulates phosphate deposition by primary cultures of avian growth plate chondrocytes. Cells grown in either serum-containing DATP5 (**left**) or serum-free HL-1 medium (**right**) were treated with 0, 10, or 25 ng/dish of OP-1 as in Figure 6. The phosphate counterpart of mineral deposited in the cultures was analyzed after extraction of the cell/matrix layer with 0.1 N HCl. Aliquots of the acid extract were analyzed for phosphate by the Ames method [1966] (see Materials and Methods). Note that OP-1 treatment also greatly stimulated mineral formation by the chondrocytes, as indicated by phosphate deposition. Values represent the mean ± SEM of 4 samples each. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

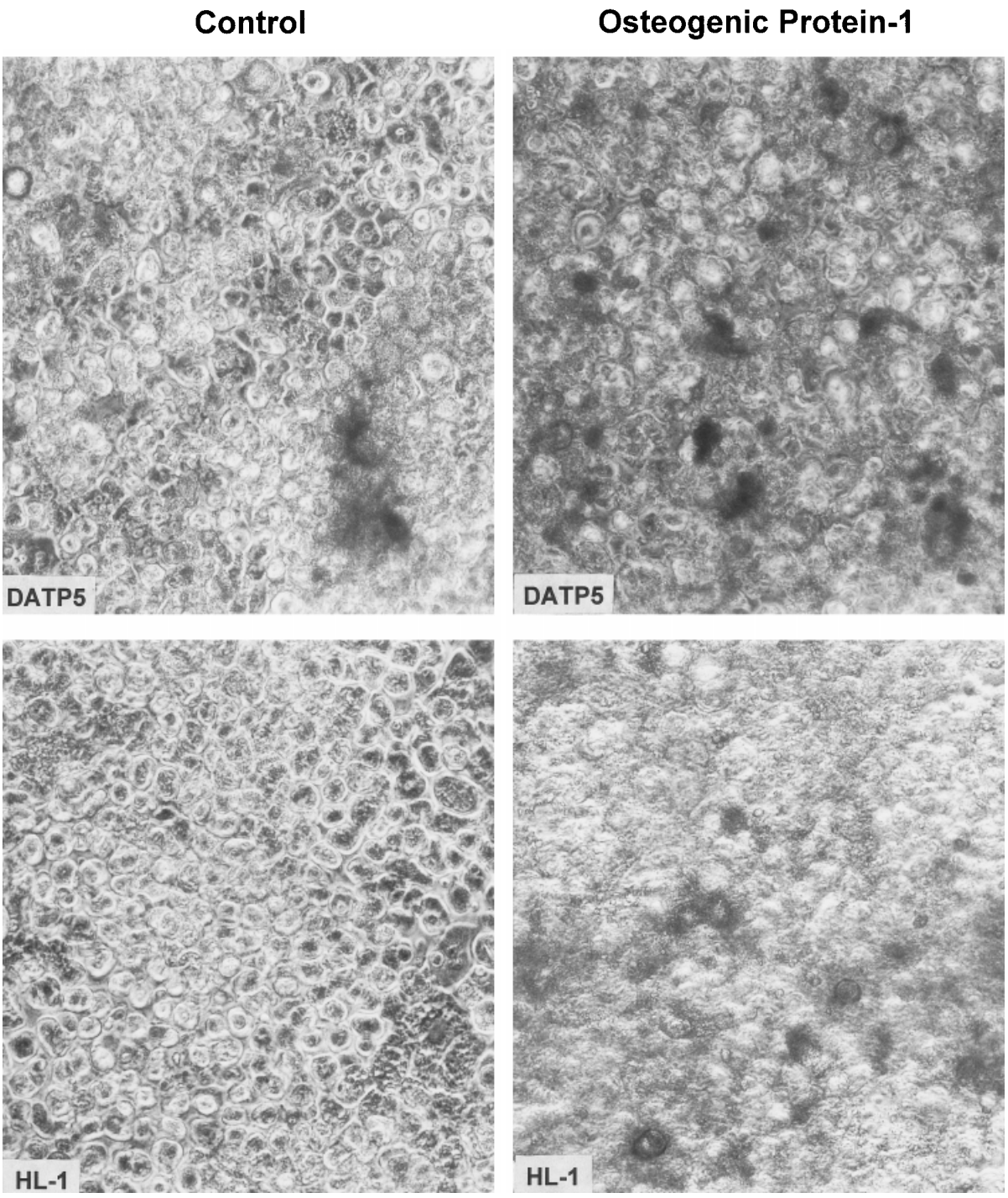


Fig. 8. Phase-contrast photomicrographs of primary cultures of growth plate chondrocytes treated with or without OP-1. The chondrocyte cultures were grown in either serum-containing DATP5 (**top**) or in serum-free HL-1 (**bottom**) in the absence of OP-1 (**left**) or in the presence of OP-1 (**right**). OP-1 was added from day 7 to day 20, with changes in medium and replenishment of OP-1 (25 ng/dish) every 3–4 days. Photographs were taken on day 20 with a Nikon DIAPHOT-TMD inverted phase-contrast microscope. Note the striking increase in mineral formation in the presence of OP-1, appearing as dark, refractile rings around numerous cells.

graphs of chondrocytes grown in the presence or absence of OP-1 are shown in Figure 8. While there was no obvious change in cell shape, OP-1 caused the formation of dark rings of mineral deposits around many of the cells grown in either DATP5 or HL-1 medium.

Modulation of the Effects of OP-1 by Retinoic Acid

The ability of retinoic acid (RA) to modulate the effects of OP-1 on chondrogenesis was also investigated. Cultured cells were treated with OP-1, alone or in combination with RA for various times. Tables I and II summarize the results of these studies using serum-containing DATP5 and serum-free HL-1 media, respectively. ALP activity was strongly induced by either RA or OP-1 when administered separately; however, when both were given in combination, the stimulatory effects were lost. In both serum-containing DATP5 (Table I) and serum-free HL-1 media (Table II), the combination of OP-1 and RA reduced ALP activity to a level even lower than that of the untreated controls.

The effects on levels of cellular protein synthesis and total DNA content (cell number or division) in the cultures was also studied. Either RA or OP-1 alone *increased* total cellular protein; however, the combination of these two agents tended to *reduce* protein synthesis. RA was previously shown to greatly stimulate cell proliferation as measured by DNA analysis [Wu et al., 1997]. Here again we show that RA alone stimulated cell division about 2-fold when added to the medium pre-confluently from day 7, but RA did not stimulate cell division if added post-confluently (i.e., from day 14). OP-1 alone had little effect on cell proliferation; when added in combination with RA, its effect was to partially block the stimulatory effect of RA.

Other very different effects are also illustrated in Tables I and II. RA greatly reduced PG content, whereas OP-1 markedly increased it. The combination of OP-1 and RA, however, was like RA alone, starkly reducing PG synthesis. Further, while both OP-1 and RA independently stimulated calcification of the GP chondrocytes, combination of the two blocked this stimulation, generally giving values similar to

TABLE I. Effect of OP-1, Retinoic Acid (RA), or a Combination of Both on Primary Cultures of Avian Growth Plate Chondrocytes Grown in Serum-Containing DATP5 Culture Medium†

Days of treatment/ day of harvest	DNA (μg/dish)	Protein (μg/dish)	Proteoglycan (μg/dish)	ALP (nmol/min/dish)	LDH (units/dish)	Calcium (μg/dish)
Days 7–14/day 14						
Control	8.5 ± 0.3	261 ± 7	21.8 ± 0.9	226 ± 30	—	19.4 ± 0.8
OP-1	10.2 ± 0.3*	339 ± 13**	45.6 ± 1.8***	487 ± 19***	—	27.7 ± 1.0***
RA	14.1 ± 1.6*	308 ± 40	4.9 ± 0.3***	630 ± 20***	—	17.9 ± 0.5
OP-1 + RA	10.1 ± 0.4*	232 ± 8*	6.9 ± 0.3***	181 ± 34	—	17.5 ± 0.4
Days 7–17/day 17						
Control	13.2 ± 0.9	362 ± 22	20.0 ± 2.3	304 ± 24	21 ± 1	45.5 ± 1.7
OP-1	13.1 ± 0.2	473 ± 18**	45.4 ± 2.3***	864 ± 24***	34 ± 1***	82.4 ± 3.8***
RA	28.0 ± 0.2***	530 ± 11***	7.7 ± 0.2**	2469 ± 61***	164 ± 16***	59.0 ± 1.9**
OP-1 + RA	15.9 ± 0.5	307 ± 7	7.4 ± 0.3**	231 ± 26	32 ± 1	50.2 ± 2.4
Days 7–21/day 21						
Control	12.1 ± 0.3	337 ± 10	24.0 ± 0.9	280 ± 12	45 ± 2	142 ± 3
OP-1	12.5 ± 0.5	436 ± 4***	46.6 ± 2.4***	674 ± 46***	31 ± 1***	239 ± 18**
RA	25.8 ± 0.9***	598 ± 22***	9.1 ± 0.4***	1047 ± 73***	240 ± 16***	207 ± 3***
OP-1 + RA	17.2 ± 0.3***	309 ± 12	10.7 ± 0.3***	234 ± 6*	130 ± 7***	178 ± 4***
Days 14–21/day 21						
Control	11.1 ± 0.1	336 ± 9	22.2 ± 1.7	270 ± 9	—	127 ± 7
OP-1	12.9 ± 0.8	443 ± 31**	47.9 ± 7.9*	438 ± 38**	—	141 ± 12
RA	12.5 ± 0.9	351 ± 14	8.9 ± 1.2**	216 ± 25*	—	132 ± 5
OP-1 + RA	10.3 ± 0.6	309 ± 9	9.3 ± 0.2**	145 ± 26**	—	133 ± 9

†Chondrocytes were treated with either OP-1 (25 ng/dish), RA (50 nM), or OP-1 + RA (25 ng/dish, 50 nM, respectively) as specified in Materials and Methods. Values are the mean ± SEM of 4 samples each. (−) LDH, lactate dehydrogenase activity not determined.

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, significance of differences between treated and control cultures.

TABLE II. Effect of OP-1, Retinoic Acid (RA), or a Combination of Both, on Primary Cultures of Avian Growth Plate Chondrocytes Grown in Serum-Free HL-1 Culture Medium†

Days of treatment/ day of harvest	DNA (μg/dish)	Protein (μg/dish)	Proteoglycan (μg/dish)	ALP (nmol/min/dish)	LDH (units/dish)	Calcium (μg/dish)
Days 7–14/day 14						
Control	7.1 ± 0.2	356 ± 8	8.6 ± 0.8	52 ± 5	—	32 ± 6
OP-1	9.6 ± 0.5**	483 ± 9***	34.0 ± 1.5***	218 ± 11***	—	50 ± 12
RA	13.6 ± 0.9***	383 ± 22	4.4 ± 0.2**	283 ± 14***	—	36 ± 5
OP-1 + RA	13.0 ± 0.6***	323 ± 11*	5.7 ± 0.2*	24 ± 7*	—	35 ± 7
Days 7–17/day 17						
Control	9.6 ± 0.9	354 ± 19	19.8 ± 0.5	38 ± 2	23 ± 0.4	35 ± 5
OP-1	10.7 ± 0.2	439 ± 16*	28.4 ± 1.0***	98 ± 4***	21 ± 0.3*	94 ± 3***
RA	19.0 ± 0.8***	580 ± 4***	12.3 ± 0.5***	1007 ± 76***	52 ± 4.0***	77 ± 2***
OP-1 + RA	14.6 ± 0.8**	336 ± 29	11.4 ± 0.5***	26 ± 1***	26 ± 1.1	31 ± 2
Days 7–21/day 21						
Control	12.1 ± 0.4	436 ± 26	25.9 ± 1.6	43 ± 3	33 ± 1	55 ± 3
OP-1	13.0 ± 2.0	500 ± 33	31.6 ± 1.1*	54 ± 5	22 ± 1***	137 ± 2***
RA	24.3 ± 0.8***	768 ± 15***	24.8 ± 1.6	1511 ± 63***	69 ± 8**	137 ± 9***
OP-1 + RA	19.0 ± 1.1***	429 ± 8	20.7 ± 1.1*	43 ± 2*	35 ± 1	64 ± 8
Days 14–21/day 21						
Control	14.2 ± 0.7	669 ± 22	34.5 ± 1.3	49 ± 8	—	141 ± 2
OP-1	14.8 ± 2.3	738 ± 22	53.6 ± 1.2***	144 ± 12***	—	165 ± 7
RA	14.0 ± 1.2	371 ± 19***	8.0 ± 0.4***	37 ± 1	—	140 ± 13
OP-1 + RA	13.1 ± 1.5	400 ± 8***	9.6 ± 0.7***	10 ± 8**	—	110 ± 9*

†Chondrocytes were treated with either OP-1 (25 ng/dish), RA (50 nM), or OP-1 + RA (25 ng/dish, 50 nM, respectively) as specified in Materials and Methods. Values are the mean ± SEM of 4 samples each. (−) LDH, lactate dehydrogenase activity not determined.

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, significance of differences between treated and control cultures.

the untreated control cells. Also, in experiments studying the activity of lactate dehydrogenase (LDH), while RA alone markedly stimulated the activity of this enzyme, OP-1 alone had minimal effect. When combined together, OP-1 blocked the early, and blunted the later, stimulatory effects of RA.

Effect of OP-1 and RA on Collagen Synthesis

Using Western blot analysis of SDS-PAGE gels, we previously identified collagen present in the *culture medium* of untreated GP chondrocytes as type II and X [Wu et al., 1989, 1997], whereas retinoic acid induced expression of type I collagen [Wu et al., 1997]. Here, using SDS-PAGE analysis of the medium collagen precipitated with 30% ammonium sulfate, we show that OP-1 alone did not stimulate collagen secretion into serum-containing DATP5 medium, nor change the type of collagen expressed (Fig. 9). However, OP-1 did cause a slight increase in type II and X collagen secretion in serum-free HL-1 medium (Fig. 10). Note that type X collagen present in the medium sometimes appears as a doublet migrating at the 68- and 63-kDa positions of globular protein MW standards. These correspond to actual MW of 59- and 50-kDa, respectively. The lower (63 kDa) band is different from pepsin-digested type X collagen, migrating at ~50 kDa globular MW. Both the 68- and 63-kDa bands, but not pepsinized type X, reacted with type X 6F6 monoclonal antibody (gift from Dr. Gary Balian). Neither of the doublet bands is ovotransferrin [Gentili et al., 1993]; that protein is not precipitated by 30% ammonium sulfate saturation.

RA alone induced type I collagen synthesis, as shown by the presence of the α2(I) band. As noted above, this band was confirmed to be type I collagen by Western blot analysis using a rabbit antichicken type I antibody [Wu et al., 1997]. When collagen synthesis into the cell/matrix layer was analyzed by SDS-PAGE electrophoresis of TMT sonicates, it was found that OP-1 increased the deposition of both type II and X collagen in serum-free HL-1 cultures, but did so only at an early time point (day 14) (data not shown).

After OP-1 treatment, when samples of the cell layer TMT sonicate were subjected to amino acid analysis after acid hydrolysis, it was found that the molar percent of the individual amino

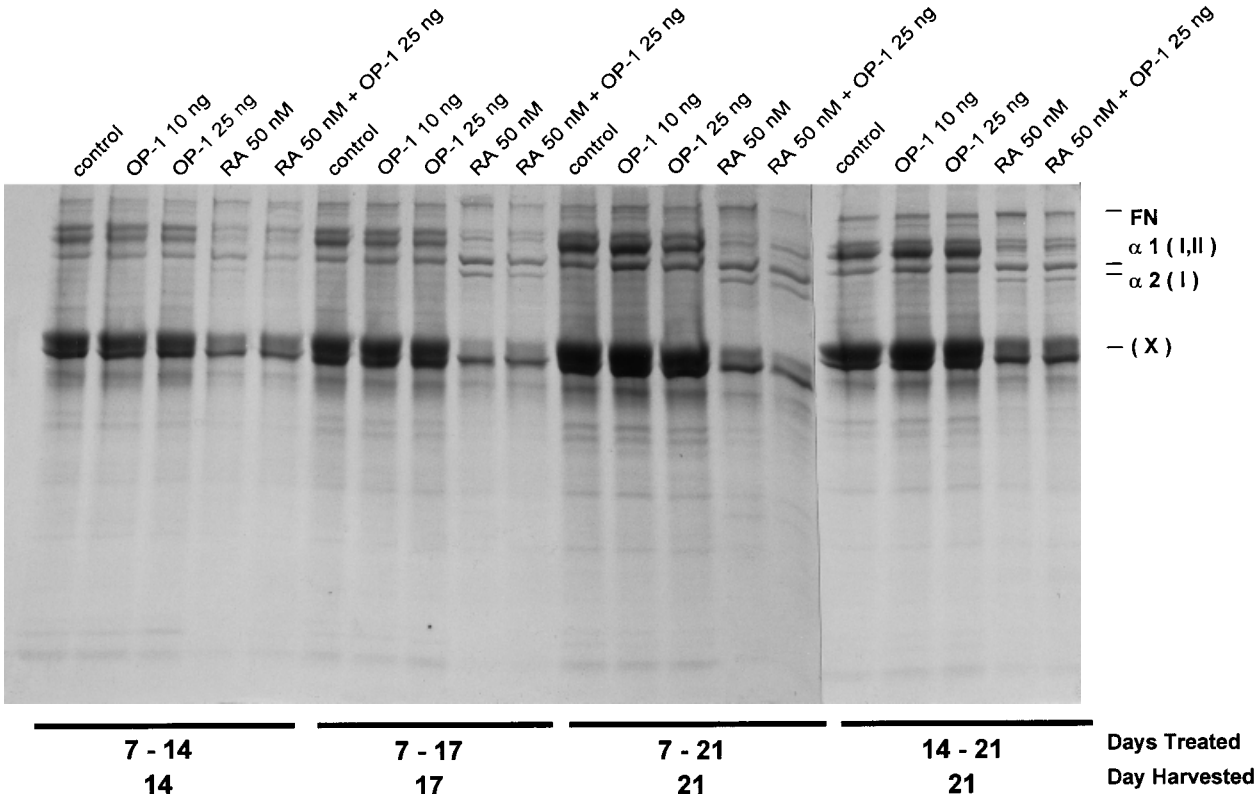


Fig. 9. SDS-PAGE analysis of the effect of OP-1 and RA on collagen secretion into serum-containing DATP5 culture medium. Chondrocytes were treated with either OP-1 (0, 10, or 25 ng/dish), RA (50 nM), or OP-1 + RA (25 ng/dish, 50 nM, respectively) as specified in Materials and Methods. Media from 4 dishes of each culture were harvested and pooled on days 14, 17, and 21. Ammonium sulfate was added to 30% saturation to precipitate the collagen. An aliquot equivalent to 150 µl of original medium was applied to each lane in Laemmli sample buffer. FN, fibronectin; X, type X collagen; α2(I), type I collagen α_2 band; α1(I,II), combined α_1 types I and II. (Only in the case of RA treatment was the type I collagen α_1 band present.)

acids (e.g., hydroxyproline and hydroxylysine) was not altered consistently in either the serum-containing or serum-free cultures (data not shown). However, OP-1 did increase the total amino acids per dish (total protein synthesis), ~15% in serum-containing DATP5 and ~45% in serum-free HL-1 cultures. From many SDS-PAGE analyses, it became evident that the effects of OP-1 on collagen secretion were relatively minor, and if both OP-1 and RA were present together, RA still induced type I collagen synthesis.

DISCUSSION

The present study demonstrates that recombinant OP-1 can regulate the development and maturation of growth plate chondrocytes. OP-1 treatment had a relatively minor effect on general parameters such as total DNA and collagen synthesis, although some increase in collagen was seen at early time points (days 7–14). Stimulation of matrix/cellular protein was greater in serum-free than serum-containing cultures, perhaps due to synthesis of PG-related proteins. In contrast, several specific markers of GP chondrocyte phenotype were significantly stimulated by OP-1. For example, secretion of PG into the culture medium and the cell/matrix layer was significantly increased by OP-1. Synthesis of cartilage-specific PG is considered to be a marker of chondrogenesis. Therefore, the intensified production of PG indicates that OP-1 is capable of inducing chondrogenic differentiation. ALP activity was also markedly increased by OP-1 during early stages of the cultures, and at the post-confluent stage between days 14 and 21. The OP-1 effect either declined or reverted back to the control level when treatment continued for longer periods of time, or upon early withdrawal of OP-1. This apparent loss of effect is probably a reflection of the enhanced mineral deposition observed in

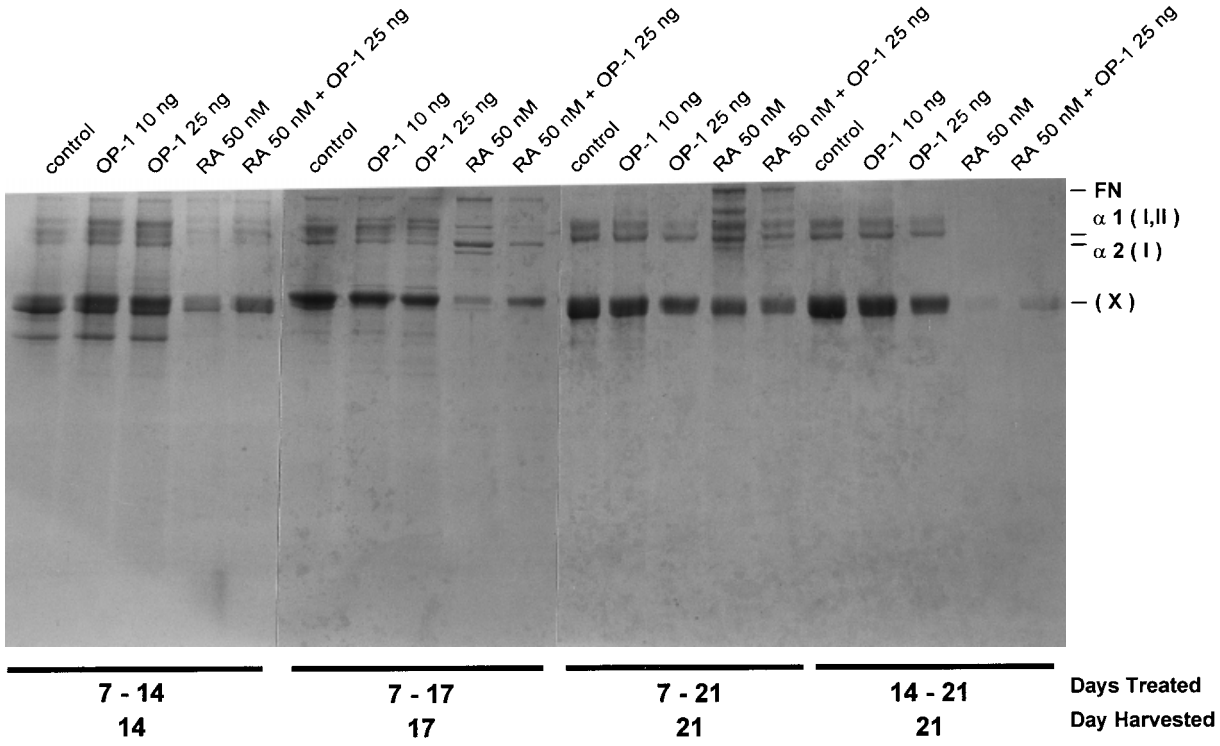


Fig. 10. SDS-PAGE analysis of the effect of OP-1 and RA on collagen secretion into serum-free HL-1 culture medium. Chondrocytes were treated with either OP-1 (0, 10, or 25 ng/dish), RA (50 nM), or OP-1 + RA (25 ng/dish, 50 nM, respectively) as specified in Materials Methods. Media from 4 dishes of each culture were harvested and pooled on days 14, 17, and 21. Ammonium sulfate was added to 30% saturation to precipitate the collagen. An aliquot equivalent to 150 µl of original medium was applied to each lane in Laemmli sample buffer. FN, fibronectin; X, type X collagen; α2(I), type I collagen α_2 band; α1(I,II), combined α_1 types I and II. (Only in the case of RA treatment was the type I collagen α_1 band present.)

the OP-1 treated cultures. Earlier studies have shown that mineralization itself markedly decreases ALP activity [Genge et al., 1988].

Here for the first time in vitro we show that mineralization, as assessed by Ca^{2+} and Pi levels in the cell/matrix layer, is markedly increased by OP-1, whether the GP cultures are treated only between days 7–14, or continuously from days 7–17 or 7–21. The increases in mineral deposition induced by OP-1 ranged from 2- to over 10-fold. This demonstrates that OP-1 is a powerful mineral-inductive factor for GP chondrocytes.

Although numerous findings have suggested that the BMPs initiate signals necessary to form the embryonic skeleton, such as mesenchymal condensation and chondrogenesis [Rosen et al., 1994], there is still uncertainty as to how this is brought about. Questions remain as to why all BMPs do not have the same bone-inducing effect, or why there are so many different kinds of BMP. For instance, BMPs 2, 3, 4, 5, and 7 have been shown to induce bone and cartilage formation in vivo [Kingsley, 1994]. Some BMPs are of embryonic origin, such as BMP-2 [Lyons et al., 1990], BMP-4 [Francis et al., 1994], BMP-5 [Kingsley et al., 1992], and BMP-7 [Vukicevic et al., 1994]. And some are present in adult tissues: in kidney (BMP-3, 4 and 7), lung (BMP-3, 4, 5, and 6), small intestine (BMP-3 and 7), heart (BMP-2, 4, 6, and 7), and teeth (BMP-3, 4, and 7). Interestingly, BMP-6 is expressed specifically in the prehypertrophic [Vortkamp et al., 1996] and hypertrophic [Lyons et al., 1989] cartilage zones. BMP-7 expression is confined to hypertrophic cartilage adjacent to invading metaphyseal blood vessels [Vukicevic et al., 1994; Helder et al., 1995; Houston et al., 1994].

The almost universal presence of BMPs in so many tissues suggests that they must play diverse roles, in addition to those in skeletal development. Furthermore, BMPs are not only involved in embryonic skeletal development, but also in adult bone in the maintenance of bone mass, and in the repair and balance between bone and cartilage formation at articular surfaces [Kingsley, 1994].

Since BMP-7 is a member of the TGF-β superfamily, it is of interest to compare regulatory actions of different members of this family. TGF-β has been previously shown to induce chondrogenesis and osteogenesis in vivo [Noda and Camilliere, 1989; Joyce et al., 1990]; however, a major feature is that TGF-β must be implanted at a bony site to produce this effect. If implanted ectopically in nonskeletal sites, only BMPs are capable of inducing cartilage and bone formation in vivo.

The following in vitro findings distinguish some of the divergent effects of the BMPs and TGF-βs. BMP-2 can induce myoblast cell lines to differentiate into an osteoblast-like phenotype, whereas TGF-β cannot, even though both BMP-2 and TGF-β can inhibit myogenic differentiation [Yamaguchi et al., 1991; Katagiri et al., 1994]. The effects of BMP-4 and TGF-β_1 on fetal rat osteoblast-like cells also have been compared [T.L. Chen et al., 1991]. Both factors increased DNA synthesis, protein accumulation, and synthesis of type I and II collagen; however, while TGF-β_1 inhibited ALP activity, BMP-4 enhanced its activity 8-fold. Further, in studies on TGF-β_1 and OP-1 in rat calvarial cultures [Sampath et al., 1992], while both OP-1 and TGF-β_1 promote cell proliferation and collagen synthesis, only OP-1 is effective in inducing expression of markers of the osteoblast phenotype (increased ALP activity, osteocalcin, and mineralization).

In serum-free conditions, addition of OP-1 alone to 15-day chick embryo sternal cartilage cultures [Chen et al., 1995] supports cell proliferation, enhances ALP activity, and increases mRNA levels and synthesis of type II and X collagens; TGF-β_1 alone fails to do this. We also have studied the effects of TGF-β_1 (and bFGF) on chicken GP chondrocytes in serum-free cultures [Wu et al., 1992]. One week of exposure of preconfluent cells to TGF-β_1 alone stimulates cell division (^{3}H-thymidine uptake), synthesis of fibronectin and type II and X collagens, and increases ALP activity. When added to postconfluent cells, TGF-β_1 was mildly (10–20%) inhibitory to [^{45}Ca] deposition, but when added to preconfluent cells, caused a 2-fold increase. TGF-β_1 is unique in its ability to stimulate fibronectin synthesis [Wu et al., 1992]; our current studies show that OP-1 lacks this property.

The question of whether chondrogenesis is consistently stimulated by BMPs is uncertain. Both stimulation [Thies et al., 1992; Carrington et al., 1991; Chen et al., 1995] and no effect [Asahina et al., 1993] have been reported, based on quantitation of uronic acid synthesis, type II collagen gene expression, or Alcian blue PG staining. Here, we show in GP cells isolated from weight-bearing avian tibiae cartilage that OP-1 stimulates chondrogenesis and mineralization. These discrepancies probably result from differences in culture systems, stages of cell differentiation, or BMP subclasses.

At this point, the regulatory effect of retinoic acid (RA) on limb bud development needs to be mentioned. Application of RA to the anterior side of a limb bud produces mirror image duplication during limb development [Tickle et al., 1982; Summerbell, 1983]. In vitro, RA has been shown to cause stage-dependent modulation of growth and differentiation of embryonic sternal and GP chondrocytes. In general, RA promotes maturation of cultured embryonic sternal chondrocytes, enhancing expression of type X collagen characteristic of hypertrophic cells, progressing on to osteoblast-like cells producing type I collagen and undergoing mineralization [Cancedda et al., 1992; Iwamoto et al., 1993; Horton et al., 1987; Takishita et al., 1990; Ballock et al., 1994; Yasui et al., 1986; Dietz et al., 1993]. The differing roles that RA plays during early embryogenesis and late GP differentiation are not well understood.

During early embryogenesis there is evidence of a close relationship between RA and BMP-2. Application of RA to the anterior margin of the developing chick limb bud in vivo induces BMP-2 expression [Francis et al., 1994]. Further, the α- and γ-forms of the RA receptor have been implicated in the regulation of BMP-2 by RA in embryonal F9 carcinoma cells [Rogers, 1996]. On the other hand, we have shown recently with GP chondrocytes cultured from 6-week-old chickens that RA drastically reduces PG synthesis, causing a switch to type I collagen synthesis and enhancing MMP-2 activity, along with massive mineralization of the culture [Wu et al., 1997]. This inhibitory effect of RA on PG synthesis is opposite to the stimulation by OP-1 shown in this paper.

Thus, it is evident that OP-1 regulates GP chondrocytes by a mechanism different from that of RA. We show here that when OP-1 and RA interact, they tend to negate their independent actions (Tables I and II). The drastic reduction of ALP activity in the presence of both these agents is in striking contrast to the strong

stimulation in activity found with either OP-1 or RA alone. Similarly, although protein synthesis is stimulated by either OP-1 or RA alone, when present together they essentially neutralize each other's effect. In addition, while RA is able to annihilate the stimulatory effects of OP-1 on PG synthesis, it still induces the expression of type I collagen. On the other hand, we found that OP-1 partially blocks the stimulation of cell division (DNA content) produced by RA alone.

Previous studies have shown that co-treatment of immature osteoblast-like RCT-1 cells with BMP-2 and RA suppresses the induction of ALP activity seen with RA alone (Ogata et al., 1994). This antagonism between OP-1 and RA on ALP activity is also observed in the present study. It seems probable that these factors are part of a complex regulatory network that functions in the GP in vivo. For example, BMP-7 (OP-1) is expressed primarily in hypertrophic cells, immediately before the region of vascular invasion [Vukicevic et al., 1994; Helder et al., 1995; Houston et al., 1994]. Because the GP is avascular, and the PG-rich matrix is impermeable to protein-borne lipophilic factors, there would be little access to RA in this region. To illustrate this key point, normal GP cartilage is profoundly deficient in essential fatty acids [Adkisson et al., 1991]; delivery of these hydrophobic molecules (as well as RA) to the tissue is mediated by serum albumin. Thus, interplay between RA and OP-1 in the GP may be minimal in vivo, or at least confined to the terminal region where access of the cells to RA becomes reestablished by vascular penetration. At that site, RA, which blocks synthesis of PG and stimulates synthesis of type I collagen and mineral deposition [Wu et al., 1997], would block chondrogenesis and facilitate true bone formation.

It is also important to note in these cultured avian tibial GP chondrocytes that OP-1 by itself concomitantly stimulated both proteoglycan synthesis (Figs. 4, 5) and mineral deposition (Figs. 6, 7). This finding contradicts a widely held concept that proteoglycans must be removed in order for mineralization to occur. While it is clear that there is a large net loss of proteoglycan when GP cartilage is replaced by cancellous bone, during the actual calcification of cartilage, there does not appear to be a major loss of proteoglycan. This interpretation is consistent with the histological appearance of GP cartilage; the matrix at sites of mineral deposition stains as intensely with Alcian blue as regions that have not yet calcified [see Poole, 1991, for review]. Thus, while OP-1 appears to stimulate calcification of cartilage per se, it seems likely that retinoic acid is a key factor required for its replacement by true bone.

There are undoubtedly many other growth factors that influence GP chondrocyte proliferation, hypertrophy, and bone formation [Tabin, 1991; Hunziker, 1994; Eriebacher et al., 1995; Centrella et al., 1994]. Among these are Sonic hedgehog protein that acts as a signaling molecule for limb bud development [Riddle et al., 1993; Johnson and Tabin, 1995] and Indian hedgehog protein that regulates the rate of hypertrophic differentiation [Lanske et al., 1996; Vortkamp et al., 1996]. This complex interplay between the various systemic and local factors that regulate endochondral bone formation will require much additional study.

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