Human Osteogenic Protein-1 Induces both Chondroblastic and Osteoblastic Differentiation of Osteoprogenitor Cells Derived from Newborn Rat Calvaria

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Abstract. Osteogenetic protein-1 (OP-1), a member of the TGF-β superfamily, induces endochondral bone formation at subcutaneous sites in vivo and stimulates osteoblastic phenotypic expression in vitro. Primary cultures of newborn rat calvarial cells contain a spectrum of osteogenic phenotypes ranging from undifferentiated mesenchymal osteoprogenitor cells to parathyroid hormone (PTH)-responsive osteoblasts. We examined whether treatment of this cell population with recombinant human osteogenic protein-1 could induce chondrogenesis in vitro. Markers of chondroblastic versus osteoblastic differentiation included alcian blue staining at pH 1, alkaline phosphatase-specific activity, osteocalcin radioimmunoassay, and expression of collagen mRNAs. 6 d of treatment (culture days 1–7) with 4–100 ng OP-1/ml caused dose-dependent increases in alcian blue staining intensity and alkaline phosphatase activity (4.7- and 3.4-fold, respectively, at 40 ng/ml), while osteocalcin production decreased twofold. Clusters of round, refractile, alcian blue-stained cells appeared by day 3, increased in number until day 7, and then became hypertrophic and gradually became less distinct. Histochemically, the day 7 clusters were associated with high alkaline phosphatase activity and became mineralized. mRNA transcripts for collagen types II and IX were increased by OP-1, peaking at day 4, while type X collagen mRNA was detectable only on day 7 in OP-1–treated cultures. Delay of OP-1 exposure until confluence (day 7) amplifies expression of the normal osteoblastic phenotype and accelerates its developmental maturation. In contrast, early OP-1 treatment commencing on day 1 strongly amplifies chondroblastic differentiation. In the same protocol, TGF-β1 alone at 0.01–40 ng/ml fails to induce any hypertrophic chondrocytes, and in combination with OP-1, TGF-β1 blocks OP-1–dependent chondroinduction. OP-1 is believed to act on a subpopulation of primitive osteoprogenitor cells to induce endochondral ossification, but does not appear to reverse committed osteoblasts to the chondrocyte phenotype.

Bone matrix contains various growth factors which control bone formation and resorption, and these factors clearly play important roles in the development and growth of cartilage and bone (Hauschka et al., 1986; Centrella et al., 1987; Hauschka, 1990). Implantation of demineralized bone matrix at intramuscular or subcutaneous sites induces cartilage and bone formation (Reddi and Huggins, 1972; Urist, 1965). Recently, several proteins involved in ectopic bone formation have been purified from bovine demineralized bone matrix, allowing the cloning of their full-length cDNAs and discovery of related cDNAs: bone morphogenetic proteins (BMPs)[1] (BMP-1 through BMP-7) (Wong et al., 1988; Wozney et al., 1988; Celeste et al., 1990); osteogenin (BMP-3) (Luyten et al., 1989); and osteogenic protein-1 (OP-1/BMP-7) (Özkaynak et al., 1990; Sampath et al., 1990) and OP-2 (Özkaynak et al., 1992). cDNA sequencing has revealed that these proteins are members of the superfamily of TGF-β–related proteins which share a distinctive pattern of seven cysteine residues in their COOH-terminal domains (Celeste et al., 1990; Ozkaynak et al., 1990; Wozney et al., 1988).

The members of the TGF-β superfamily play diverse and significant roles in growth and differentiation. For example, *Drosophila* decapentaplegic complex (dpp) is involved in embryonic dorsal/ventral polarity (Segal and Gelbart,

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1. *Abbreviations used in this paper*: BMP, bone morphogenetic protein; OP, osteogenic protein; TFA, trifluoracetic acid.

1985). Vg-1 is thought to act as an intercellular signal for mesoderm induction in *Xenopus* (Rebagliati et al., 1985). Vgr-1 (BMP-6) and BMP-2 (BMP-2a) expression has been analyzed in various tissues during mouse embryogenesis (Lyons et al., 1989). BMPs and OP-1 are thought to be involved in cartilage and bone development.

Formation of new bone is induced when OP-1 and other BMPs are implanted with carriers in nonbony sites in rats. This induction always involves the cascade of de novo endochondral bone formation: cartilage is induced and becomes calcified before being replaced by mineralized bone (Reddi, 1981; Sampath et al., 1992). Several in vitro studies have addressed the mechanism of action of bone-inducing proteins. These studies have shown that BMPs and OP-1 have two osteogenic effects. First, they enhance the osteoblastic characteristics of osteoblast-like cells, including MC3T3-E1 mouse calvaria-derived osteoblasts (BMP-2 and BMP-3) (Hiraki et al., 1991; Takuwa et al., 1991; Vukicevic et al., 1990), and rat calvaria-derived primary osteoblast-like cells (BMP-3 [Vukicevic et al., 1989]; BMP-4 [Chen, T., et al., 1991]; OP-1 [Sampath et al., 1992]). Second, these factors induce osteoblast phenotypic expression by osteoprogenitor cells such as rat calvaria-derived cells (BMP-2; Yamaguchi et al., 1991), bone marrow stromal cells (BMP-2; Thies et al., 1991), and C3H10T1/2 mouse embryo-derived mesenchymal cells (BMP-2; Katagiri et al., 1990). Other studies focussing on cartilage showed that native preparations of BMP-2/BMP-3 (Hiraki et al., 1991), BMP-3 alone (Vukicevic et al., 1989), and BMP-4 (Luyten et al., 1992) could enhance cartilage characteristics of cultured articular chondrocytes. Osteogenin (BMP-3) and BMP-4 have also been noted to induce the cartilage phenotype in chondrocyte precursor chick limb bud cells (Carrington et al., 1991; Chen, P., et al., 1991), and to promote reexpression of cartilage phenotype by dedifferentiated articular chondrocytes (Harrison et al., 1991). However, there has been no evidence until our study that OP-1 or other BMPs could induce chondrogenesis in noncartilage cells in vitro.

In contrast to the endochondral development of long bones, membranous flat bones of the skull (e.g., calvaria) develop by intramembranous ossification, bypassing the calcified cartilage stage characteristic of the endochondral process. In postfetal life, however, when injury or insult occurs, skull bones repair through a cellular process which includes both intramembranous and endochondral ossification (Marden et al., 1993). Might OP-1 and other BMPs play an intrinsic role in osseous healing? It is possible that chondrocytes and osteoblasts are developed by differentiating from the same common lineage of uncommitted mesenchymal osteoprogenitor cells, although the identity of the OP-1 target cell and the precise lineage leading to chondrocyte and osteoblast phenotypes is not clearly established.

While exploring the temporal dependence of OP-1 regulation of primary rat osteoblasts in vitro, we observed strong chondroinduction resulting from early OP-1 treatment, whereas if left untreated, these cultures would have naturally progressed to mature, mineralizing osteoblasts without evidence of cartilage. We hypothesized that OP-1 has chondroinductive effects on a subset of uncommitted osteoprogenitor cells derived from neonatal rat calvaria, and that the combined OP-1 effects of chondroinduction (this study) and the known osteogenic stimulation of committed osteoblasts (Sampath et al., 1992) could account for the in vivo observations that OP-1 induces the cascade of endochondral ossification.

Materials and Methods

Preparation of Recombinant Human OP-1

Recombinant human OP-1 (OP-1) was prepared as described previously (Sampath et al., 1992). Briefly, the full-length OP-1 cDNA was inserted into a mammalian expression vector and transfected into dhfr(−) CHO cells which were subjected to methotrexate-mediated gene amplification. The resulting selected cell line was cultured in roller bottles, and the conditioned media was collected. OP-1 was purified from conditioned media using three chromatography steps: S-Sepharose, phenyl-Sepharose, and reverse phase HPLC (Sampath et al., 1992). Mature OP-1 is a glycosylated 36-kD homodimer of 139-amino acid residue chains. Stock solutions of OP-1 were prepared in 50% ethanol, 0.1% trifluoroacetic acid (TFA) and standardized for protein concentration by amino acid analysis.

Cell Culture

Calvaria from 1-d-old Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were dissected, and five sequential 20-min digests (I-V) were performed on suture-free calvarial fragments at 37°C in Hefley's buffer (Hefley et al., 1981) containing 2 mg/ml collagenase B (Boehringer-Mannheim Corp., Indianapolis, IN) and 0.25% trypsin (GIBCO BRL, Gaithersburg, MD). Single cell suspensions obtained from digests III to V were pooled. In some experiments, calvaria were separated into four anatomical regions (frontal bone, parietal bone, occipital bone, and sutures) and digested separately as described above. Cells were plated on day 0 in MEM (Sigma Chemical Co., St. Louis, MO) supplemented with 10% FBS (Bioproducts for Science Inc., Indianapolis, IN) and antibiotics (100 U/ml penicillin G and 100 μg/ml streptomycin; GIBCO BRL) in 12-multiwell plates (Elkay, Shrewsbury, MA) at a density of 1.2×10^5 cells per 1-ml well. Treatment with OP-1 and 50 μg/ml L-ascorbic acid (Sigma Chemical Co.) was started 1 d after plating, and medium was replaced completely every 3 d beginning on day 1. To examine the effect of OP-1 on mineralization, 10 mM β-glycerophosphate (Sigma Chemical Co.) was added to the medium from day 1.

All OP-1 additions to cultured cells involved rapid dispersal of $\leq 10^{-3}$ vol of OP-1 stock solution (in 50% ethanol, 0.1% TFA) into the culture medium immediately before refeeding cells; solvent vehicle alone was added to control medium.

Human, recombinant TGF-β1 (R & D Systems, Minneapolis, MN) was prepared at 20 μg/ml in 4 mM HCl containing 1 mg/ml BSA. TGF-β1 or control vehicle was added to medium before refeeding.

For experiments examining the effect of OP-1 on chondrogenesis versus osteogenesis, primary cultures were compared with subcultivated cell cultures. Subcultivation was performed by trypsin digestion of day 7 confluent primary cultures grown in the absence of OP-1 and ascorbic acid. The washed cell suspension was diluted to 1.2×10^5 cells/ml and replated under the same conditions as the primary culture.

Cell Growth

Effects of OP-1 on cell growth were examined by determining the rate of [^{3}H]thymidine incorporation into total acid-insoluble DNA and by measurement of total DNA in cell layers.

DNA synthesis rates were determined after every 3 d of treatment with OP-1 by adding [^{3}H-methyl]thymidine (50 Ci/mmol; Amersham Corp., Arlington Heights, IL). Cells were labeled with 2 μCi/ml of [^{3}H]thymidine for 4 h before termination of the culture. The 5% TCA-precipitated radioactive DNA in the cell layer was solubilized with 0.5 N NaOH and quantitated by liquid scintillation counting.

DNA content was determined by fluorometric assay (Vytasek, 1982). Cells were precipitated with 5% TCA, dissolved in 1 N NaOH, hydrolyzed in 1 N perchloric acid at 70°C for 20 min, and incubated with diaminobenzoic acid dihydrochloride at 37°C for 1 h. DNA concentration was determined against a calf thymus DNA standard (Sigma Chemical Co.) by measuring the fluorescence emission at 500 nm during excitation at 408 nm using a Perkin Elmer LS-5 fluorescence spectrophotometer (Perkin-Elmer Corp., Norwalk, CT).

Measurement of Chondrogenesis

The degree of chondrogenesis was evaluated by staining with alcian blue. Cultures were washed with PBS, fixed 10 min with 4% paraformaldehyde, stained with 0.5% alcian blue (Fluka, Ronkonkoma, NY) in 0.1 N HCl, pH 1.0, overnight, and rinsed with distilled water. The quantity of sulfated glycosaminoglycan representative of cartilage matrix was estimated by measuring the amount of extractable dye (San Antonio and Tuan, 1985). Alcian blue-stained cultures were extracted with 6 M guanidine-HCl for 2 h at room temperature. Optical density of the extracted dye was measured at 650 nm in a 96-well plate reader (Molecular Devices, Menlo Park, CA).

Alkaline Phosphatase Activity

Cell layers were extracted with 20 mM TBS, pH 7.4, containing 1% Triton X-100 and stored at −20°C until the assay. Enzyme activity was assayed with 10 mM (final) *p*-nitrophenyl phosphate as a substrate in 50 mM sodium carbonate buffer, pH 10, containing 5 mM $MgCl_2$. After 10 min of incubation at 37°C, the reaction was stopped with 0.5 N NaOH and absorbance was measured at 405 nm. Protein in each sample was determined with the BCA protein assay kit (Pierce, Rockford, IL). Alkaline phosphatase specific activity is presented as μmol *p*-nitrophenol produced/min/μg protein.

Osteocalcin Radioimmunoassay

Rat osteocalcin levels in the media were measured by radioimmunoassay as described previously (Gundberg et al., 1984). Conditioned medium samples were collected and stored at −20°C. Radioimmunoassay was performed using goat anti-rat osteocalcin serum as first antibody and donkey anti-goat IgG as second antibody. The intraassay and interassay variance was ± 5% and ± 8%, respectively. Data are expressed as total ng rat osteocalcin in the 3-d-conditioned medium per μg cell layer DNA.

Histochemical Analysis

Cell layers were rinsed with PBS or 0.9% NaCl, and fixed with 4% paraformaldehyde. Fixed cell layers were stained with 0.5% alcian blue or 0.1% toluidine blue to examine chondrogenesis. For alkaline phosphatase, fixed cells were incubated with 0.125% β-naphthyl phosphate and 0.5% fast blue RR salt in 100 mM Tris buffer, pH 8.8, containing 5 mM $MgCl_2$. Mineral deposition was assessed by a modified von Kossa staining technique. After 30 min of incubation with 3% $AgNO_3$ in the dark, cell layers were exposed for 30 s to 254-nm ultraviolet light on a viewing box to rapidly develop the black silver phosphate nodules. To visualize cells undergoing DNA replication (S-phase), [^{3}H]thymidine-treated cultures were coated with Kodak NTB2 emulsion for autoradiography (Eastman Kodak Co., Rochester, NY) and counterstained with Giemsa after development.

Northern Analysis

For Northern analysis, cells were plated in 6-multiwell plates at a density of 3.6×10^5 cells per 2-ml/well and cultured for 4, 7, and 10 d in media containing 0, 4, and 40 ng OP-1/ml. Total RNA was extracted by the acid guanidine thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). Briefly, after washing with cold PBS, the cells were homogenized in Solution D, which contained 4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% *N*-lauroyl-sarcosine, 10 mM *trans*-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid (CDTA), and 100 mM β-mercaptoethanol, pH 7.15. One volume of water-saturated phenol, 0.2 volume of chloroform-isoamyl alcohol (49:1), and 0.1 volume of 2 M sodium acetate, pH 4.0, were added to the homogenate, and total RNA was extracted into the aqueous phase after centrifugation. Isopropanol-precipitated RNA was again solubilized in Solution D and reextracted by the same procedure to increase purity.

5-μg samples of total RNA were electrophoretically resolved in 1% formaldehyde agarose gels and transferred to nylon membrane filters (Nytran; Schleicher & Schuell, Keene, NH) by capillary blotting. Filter blots were prehybridized 4 h at 45°C with buffer containing 50% formamide, 5× SSPE, 5× Denhardt's solution, 1% SDS and 25 μg/ml of salmon sperm DNA. Hybridizations were carried out for 16 h at 45°C with buffer containing 50% formamide, 5× SSPE, 1% SDS, 10% dextran sulfate, 25 μg/ml of salmon sperm DNA, and the desired cDNA probe labeled with [α^{32}P]-dCTP by random priming (Kit; Boehringer Mannheim Corp.). Probes included the cDNA for rat α_1(I) collagen (Genovese et al., 1984), rat pro-α_1(II) collagen (Kohno et al., 1984), rat α_1(IX) collagen and rat α_1(X) collagen (Nishimura, I., unpublished observation), and mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Sabath et al., 1990). After washing the filters, autoradiograms were made at −80°C by exposure to Kodak XAR-5 film (Eastman Kodak Co.).

Results

Cell Growth

Effects of OP-1 on cell proliferation were evaluated in two ways: [^{3}H]thymidine incorporation into acid-insoluble DNA, and total DNA content of cell layers. OP-1 stimulates DNA synthesis in the early stages of culture where cells are sparse and generally proliferating. A 120% stimulatory effect of OP-1 is significant at day 4, but there are no apparent differences in [^{3}H]thymidine incorporation between OP-1–treated cultures and control cultures after confluence (day 10–13) (Fig. 1 *B*). At day 7, [^{3}H]thymidine incorporation is increased 55 and 50% by 20 and 40 ng/ml of OP-1, respectively. OP-1 at 100 ng/ml does not significantly elevate labeling, in part because cultures with this high dose have reached confluence by this 7-d assay point (Fig. 1 *A*). These results on cell proliferation are confirmed by measurements of DNA content. Although OP-1 increases DNA content by 30–60% in a dose-dependent manner at day 7 (Fig. 1, *C* and *D*), significant differences in DNA content are not observed after day 10 (Fig. 1 *D*).

Chondrogenic Differentiation

Alcian blue staining of sulfated glycosaminoglycan was used to evaluate chondrogenesis, since the production of this extracellular matrix component is a hallmark of chondrogenesis (Hunter and Caplan, 1983). The intensity of alcian blue staining shows dose-dependent increase caused by OP-1 (Fig. 2 *A*). Induction of alcian blue–stained colonies in OP-1–treated cultures peaks at day 7, and the intensity of the staining decreases gradually at later times (Fig. 2 *B*). Clusters of round, refractile, alcian blue–stained cells appear within two days after treatment with OP-1 (day 3), and increase in both size and number until day 7. These cells then become enlarged and hypertrophic, and gradually disappear. Although the intensity of alcian blue staining in control cultures increases gradually by day 13 (Fig. 2 *B*), this is caused by diffuse binding of dye to the entire cell layer; histological examination shows total absence of alcian blue–stained colonies, in contrast to their abundance in OP-1–treated cultures.

Analysis of the dose dependence of the OP-1 induction of chondrocytic cell clusters was carried out by plating cells on day 0 in the presence of different concentrations of OP-1 (Fig. 2 *C*). On day 5, wells were scored for total numbers of "clonal" clusters containing three or more cells. Fig. 2 *C* shows that concentrations of 10 ng/ml OP-1 or greater induce a highly significant increase in the number of these clusters, with a fourfold increase over background at 10 ng/ml and a 50-fold increase at 320 ng/ml.

Northern analysis of mRNA levels was performed with collagen type I, II, IX, and X cDNAs to evaluate the chondrocytic properties of OP-1–induced cells. Type II collagen mRNA is increased by OP-1 treatment in a dose-dependent manner, while type I collagen message decreases slightly (Fig. 3, *I* and *II*). Interestingly, short form type IX collagen message is expressed even in control cultures, while long form type IX collagen message is expressed only in OP-1

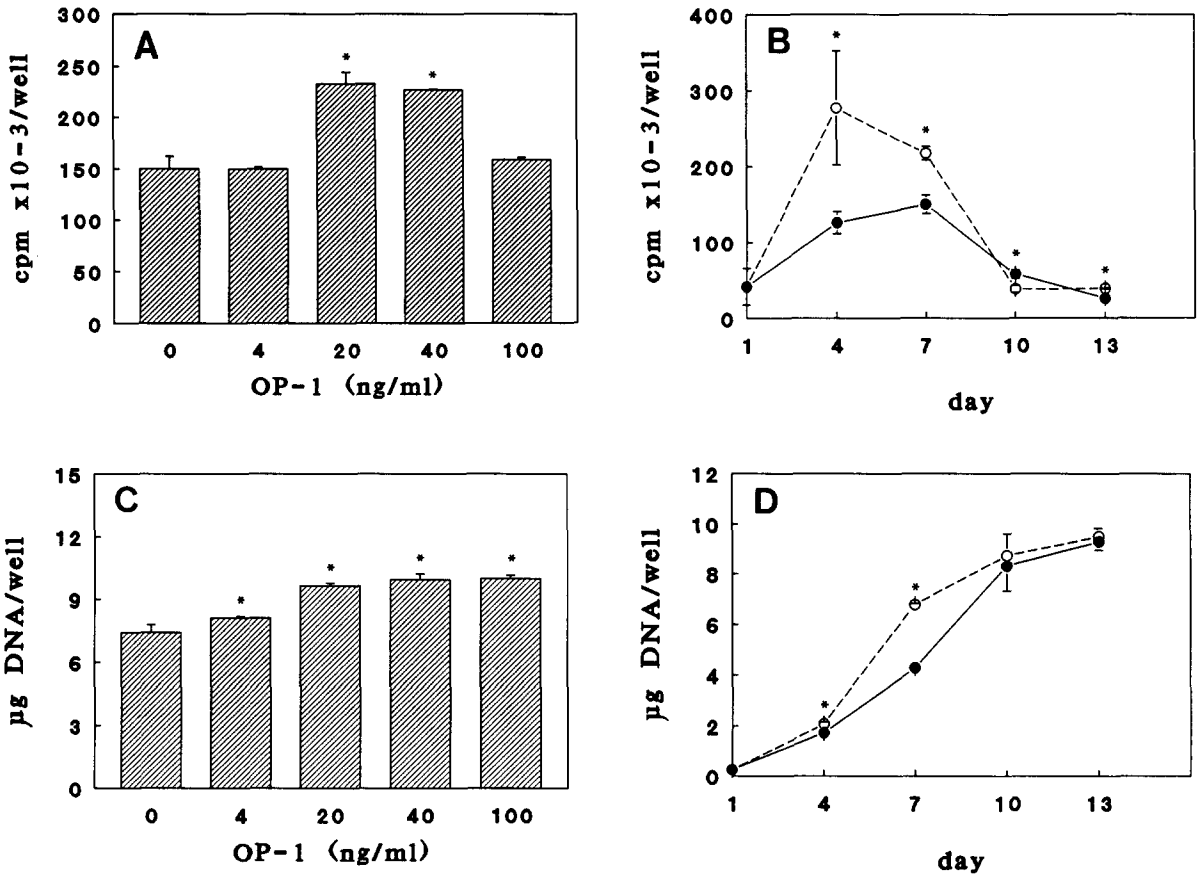

Figure 1. OP-1 effects on cell proliferation, showing the dose dependence (*A*) and time course (*B*) of [^{3}H]thymidine incorporation into acid-insoluble DNA, and the dose dependence (*C*) and time course (*D*) of changes in DNA content of cell layers. Cell layers were pulse labeled with [^{3}H]thymidine (2 μCi/ml, 4 h) before harvest (*A* and *B*). DNA was quantitated in cell layers (*C* and *D*) by the fluorometric diaminobenzoic acid method. For dose dependence, cells were cultured with graded concentrations of OP-1 for 6 d from day 1 and harvested at day 7. For kinetics, cells were cultured without OP-1 (•) or with 40 ng/ml OP-1 (○) from days 1–13, and harvested every 3 d beginning on day 1. Data represent the mean ± SD of three cultures for each treatment and are representative of three separate experiments. Asterisk, significant difference from control; $P \leq 0.05$.

treated cultures (Fig. 3, *IX*). Type II and IX collagen messages peak at day 4 and then gradually decrease. OP-1 induced the expression of type X collagen message only at day 7 in the culture treated with 40 ng/ml of OP-1 (Fig. 3, *X*).

Alkaline Phosphatase Activity

Alkaline phosphatase-specific activity is significantly increased by OP-1 in a dose-dependent manner; 3.4- and 4.5-fold at 40 and 100 ng/ml OP-1, respectively (Fig. 4 *A*). Activity in the OP-1 treated cultures peaks at day 7 and then decreases slightly (Fig. 4 *B*). In contrast, alkaline phosphatase in control cultures increases gradually throughout the culture period (Fig. 4 *B*).

Osteocalcin Production

Although the amount of osteocalcin production in early primary cultures (1–2 ng/μg DNA) is far less than in mature cultures, OP-1 decreases osteocalcin production in a dose-dependent manner at day 7 (Fig. 5 *A*). However, once the cultures have become confluent (days 10 and 13), the osteocalcin production becomes 10- to 20-fold greater than on day 7, and OP-1 significantly increases osteocalcin production (Fig. 5 *B*).

OP-1 Responsiveness of Different Calvarial Cell Populations

The histology of the developing rat calvaria features plates of membranous bone separated by fibrous sutures. At the outer limbs of the lambdoidal suture are remnants of the chondrocranium, from which Rifas et al. (1982) were able to derive chondrocytes by selective culture conditions. We examined the OP-1 response of cells from anatomically different regions of calvaria (frontal, parietal, and occipital bones, and sutures) (Table I). Cells from all regions showed OP-1–dependent increases of 1.5–3-fold in alcian blue staining, and 2.1–6-fold increases in alkaline phosphatase specific activity. It is noteworthy that untreated cultures from all regions have low alcian blue staining (Table I), indicating that few, if any, differentiated chondrocytes exist in any of these anatomical regions prior to culture in the presence of OP-1. However, the occipital and parietal populations appear to have a greater number of cells with OP-1–dependent chondrogenic potential.

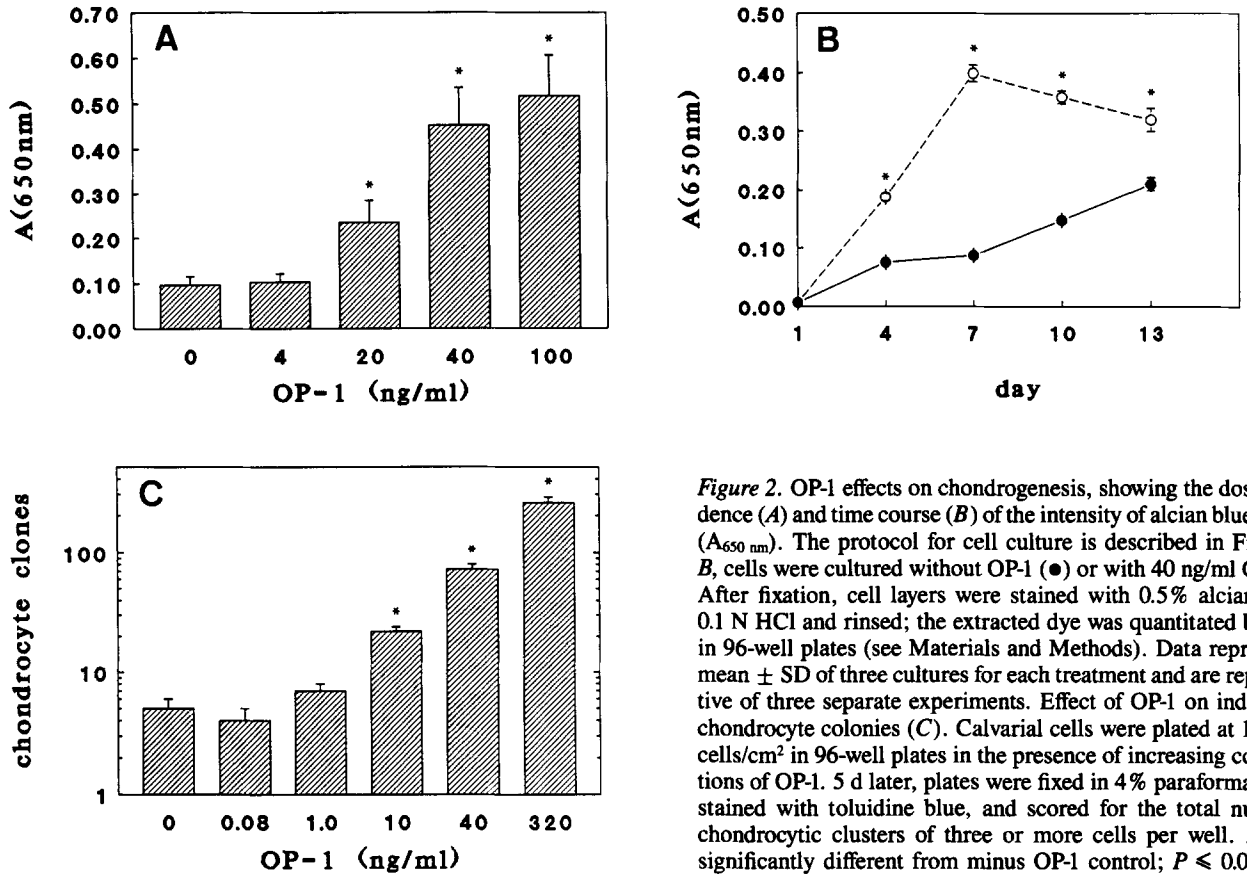

Figure 2. OP-1 effects on chondrogenesis, showing the dose dependence (*A*) and time course (*B*) of the intensity of alcian blue staining ($A_{650\ nm}$). The protocol for cell culture is described in Fig. 1; for *B*, cells were cultured without OP-1 (●) or with 40 ng/ml OP-1 (○). After fixation, cell layers were stained with 0.5% alcian blue in 0.1 N HCl and rinsed; the extracted dye was quantitated by $A_{650\ nm}$ in 96-well plates (see Materials and Methods). Data represent the mean ± SD of three cultures for each treatment and are representative of three separate experiments. Effect of OP-1 on induction of chondrocyte colonies (*C*). Calvarial cells were plated at 1.1×10^5 cells/cm² in 96-well plates in the presence of increasing concentrations of OP-1. 5 d later, plates were fixed in 4% paraformaldehyde, stained with toluidine blue, and scored for the total number of chondrocytic clusters of three or more cells per well. Asterisk, significantly different from minus OP-1 control; $P \leq 0.05$.

Sequential enzymatic digestion of calvarial bone segments in the standard protocol (frontal + parietal + occipital, trimmed free of sutures) releases cells with increased osteoblastic properties in later digests (Wong and Cohn, 1975). Similarly, we found that the chondrogenic potential increases significantly in later digests. OP-1 treatment (40 ng/ml, 6 d) causes increases in alcian blue staining of 1.66-fold in digest I cells, 2.54-fold in digest II, and 3.04-fold in pooled cells from digests III–V. The alkaline phosphatase in all three cell populations was elevated 5–6-fold by OP-1.

Effect of OP-1 on Osteoblastic Cell Populations of Different Age

Chondrogenic differentiation in this model system is observed only when primary cultures are treated early with OP-1 from day 0 (Fig. 2 *C*) or day 1 (Fig. 2, *A* and *B*; Table II). Quantitatively, alcian blue staining of the day 1–7 cultures increases 3.9-fold in response to OP-1, while the same duration of OP-1 treatment of confluent day 7–13 or subcultivated cultures causes little chondroinduction (Table II). Although the intensity of alcian blue dye uptake (A_{650nm}) by day 7–13 and subcultivated cultures with or without OP-1 exhibits a relatively high background, no alcian blue–stained cartilage-like clusters are observed histologically. Alkaline phosphatase specific activity, a marker for chondrocytes and osteoblasts, is also enhanced to a greater magnitude by OP-1 in primary cultures treated with OP-1 from day 1 (3.21-fold) than in more mature day 7–13 cultures or subcultivated cultures (Table II). However, the mature osteoblast marker os-

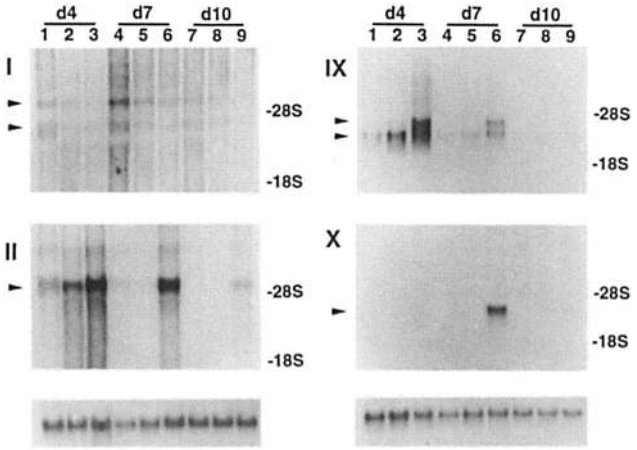

Figure 3. Northern analysis of gene expression, showing the effects of OP-1 on mRNA synthesis of collagen type I (*I*), type II (*II*), type IX (*IX*), and type X (*X*). Cells were cultured for 3 d (lanes *1*, *2*, and *3*), 6 d (lanes *4*, *5*, and *6*), and 9 d (lanes *7*, *8*, and *9*), with OP-1 treatment at 0 ng/ml (lanes *1*, *4*, and *7*), 4 ng/ml (lanes *2*, *5*, and *8*), and 40 ng/ml (lanes *3*, *6*, and *9*) beginning on day 1. Total RNA was extracted from each culture and Northern analysis was performed as described. Equivalent loading of total RNA (5 μg/lane) was verified by UV absorbance, ethidium bromide staining, and hybridization with a mouse GAPDH cDNA probe (*bottom panels*). For the analysis of types I and II collagen, and types IX and X collagen, the same filters were used. Arrows mark the collagen gene transcript size(s) for each collagen type detected by the specific cDNA probe. The positions of 28 S and 18 S ribosomal RNA are also indicated.

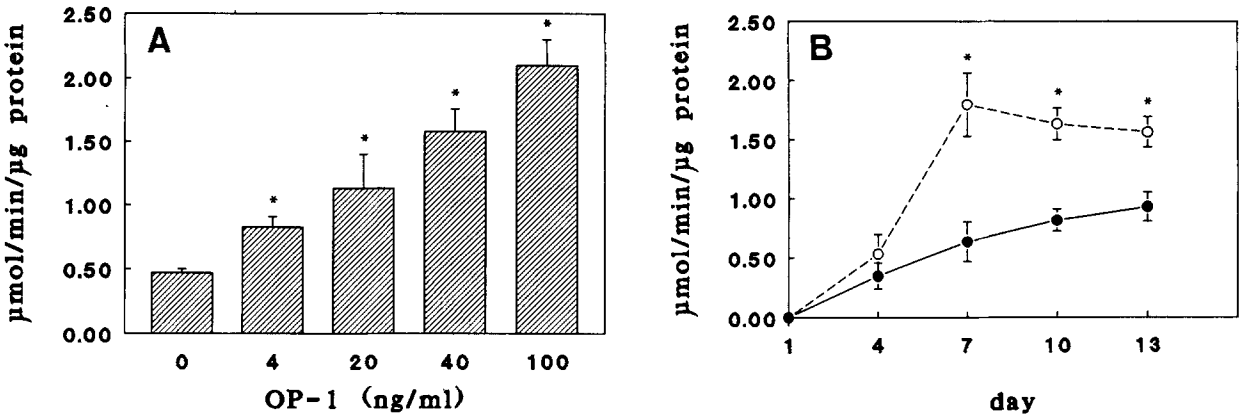

Figure 4. OP-1 effects on alkaline phosphatase specific activity, showing dose dependence (*A*) and time course (*B*) of the changes. The experimental protocol is described in Fig. 1; for *B*, cells were cultured without OP-1 (●) or with 40 ng/ml OP-1 (○). Alkaline phosphatase was measured spectrophotometrically in detergent-solubilized cell layers with *p*-nitrophenyl phosphate as a substrate. Enzyme specific activity is presented as μmol *p*-nitrophenol produced/min/μg protein. Data represent the mean ± SD of three cultures for each treatment and are representative of three separate experiments. Asterisk, significant difference from control; $P \leq 0.05$.

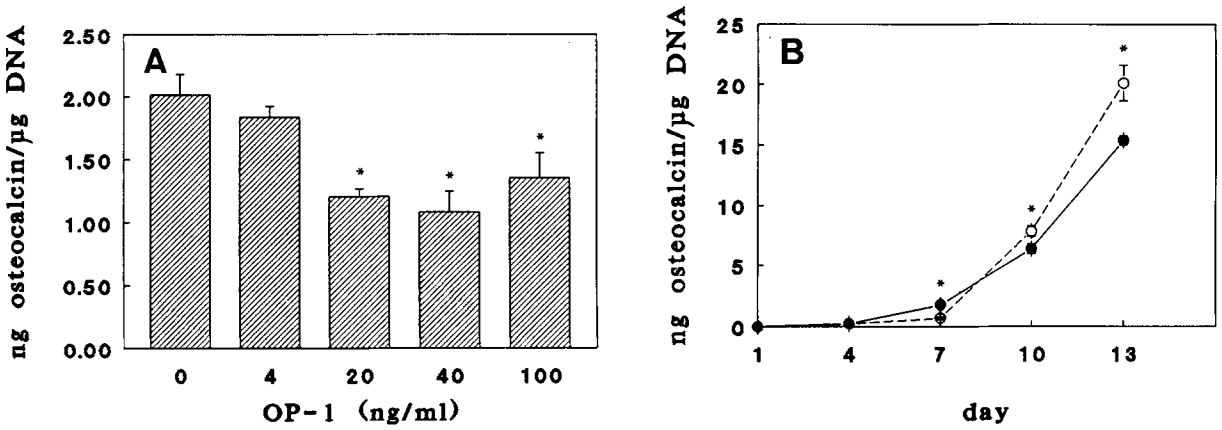

Figure 5. OP-1 effects on osteocalcin production, showing dose dependence (*A*) and time course (*B*) of osteocalcin levels in 3-d conditioned medium. The experimental protocol is described in Fig. 1; for *B*, cells were cultured without OP-1 (●) or with 40 ng/ml OP-1 (○). Osteocalcin was measured by specific radioimmunoassay. Data are expressed as total ng rat osteocalcin/μg cell layer DNA. Data represent the mean ±SD of three cultures for each treatment and are representative of three separate experiments. Asterisk, significant difference from control; $P \leq 0.05$.

Table I. OP-1 Effects on Different Calvarial Cell Populations

Calvarial region	OP-1	Alcian blue staining		Alkaline phosphatase	
		A(650) nm)	*T/C*	*μmol/min/μg protein*	*T/C*
Frontal	−	0.117 ± .010		0.279 ± .048	
	+	0.186 ± .007	1.59	0.709 ± .048	2.54
Parietal	−	0.190 ± .002		0.437 ± .025	
	+	0.503 ± .046	2.65	1.299 ± .168	2.97
Occipital	−	0.177 ± .018		0.121 ± .014	
	+	0.525 ± .033	2.97	0.731 ± .127	6.04
Suture	−	0.181 ± .006		0.579 ± .048	
	+	0.265 ± .014	1.46	1.216 ± .021	2.10

Calvarial regions were microdissected into pools containing anatomically defined regions (frontal bone, parietal bone, etc.). Cells from each region were enzymatically dissociated and plated at equal density on day 0 (see Materials and Methods); OP-1 treatment (40 ng/ml) was from days 1–7. Data represent mean ±SD from three replicate wells and are representative of several independent experiments. All ratios of OP-1 treated to control (*T/C*) showed significant OP-1 effects ($P \leq 0.05$).

teocalcin is decreased by OP-1 in primary cultures treated from day 1, but increased in subcultivated cell cultures (Table II). Thus, early treatment (days 1–7) with OP-1 elicits the greatest chondrogenic response (alcian blue, alkaline phosphatase) and a diminished osteogenic response (osteocalcin); late OP-1 treatment elicits minimal chondrogenic response and an enhanced osteoblastic response.

Effect of Ascorbic Acid and β-Glycerophosphate

OP-1 treatment in the absence of ascorbic acid increases chondrogenesis and alkaline phosphatase activity slightly, but these phenotypic properties are greatly increased when OP-1 is used in the presence of ascorbic acid (Table III). β-Glycerophosphate has no significant effect on the induction of chondrogenesis and alkaline phosphatase activity by OP-1 (Table III). Osteocalcin production is decreased by early OP-1 treatment (Table III; also Fig. 5 and Table IV). Although this OP-1–dependent decrease still occurs in the presence of ascorbate ± β-glycerophosphate, the addition of

Table II. OP-1 Effects on Osteoblastic Cell Populations of Different Age

Cell population	OP-1	Alcian blue staining		Alkaline phosphatase activity		Osteocalcin	
		A(650 nm)	*T/C*	*μmol/min/μg protein*	*T/C*	*ng/μg DNA*	*T/C*
Days 1-7	−	0.087 ± .005		0.490 ± .033		1.77 ± 0.07	
	+	0.339 ± .014	3.90*	1.573 ± .101	3.21*	0.68 ± 0.10	0.38*
Days 7-13	−	0.245 ± .015		0.637 ± .028		4.35 ± 0.34	
	+	0.289 ± .005	1.18*	0.785 ± .067	1.23*	4.97 ± 0.80	1.14
Subcult.	−	0.202 ± .009		0.560 ± .056		4.14 ± 0.26	
	+	0.212 ± .020	1.05	1.186 ± .031	2.12*	7.70 ± 1.54	1.86*

Cell cultures were established (see Materials and Methods) and treated with or without 40 ng/ml OP-1 for 6 d before analysis. *Days 1-7* indicates early treatment of subconfluent cells initiated on day 1 (cells were plated on day 0). *Days 7-13* indicates late treatment after cells have reached confluence. *Subcult.* indicates treatment of subcultivated cells which was initiated the day after trypsinization and replating of a day 7 culture. Data represent means ± SD from three replicate wells and are representative of several independent experiments.
* Ratios of OP-1 treated to control (*T/C*) which showed significant OP-1 effects ($P \leq 0.05$).

Table III. Dependence of OP-1 Effects on Ascorbate and β-Glycerophosphate (βGP)

Treatment group	OP-1	Alcian blue staining		Alkaline phosphatase activity		Osteocalcin	
		A(650 nm)	*T/C*	*μmol/min/μg protein*	*T/C*	*ng/ml*	*T/C*
Control	−	0.038 ± .003		0.011 ± .002		0.032 ± .043	
	+	0.048 ± .002	1.26	0.022 ± .003	2.00	0.007 ± .011	0.22
+Ascorbate	−	0.086 ± .002		0.053 ± .005		9.48 ± .25	
	+	0.276 ± .021	3.21*	0.204 ± .036	3.85*	6.57 ± .41	0.69*
+Asc +βGP	−	0.087 ± .002		0.053 ± .011		13.34 ± .65	
	+	0.316 ± .013	3.63*	0.201 ± .031	3.79*	9.66 ± 1.75	0.72*

Cell cultures were established on day 0 and treated from days 1-7 with ascorbate alone (50 μg/ml), or with ascorbate + β-glycerophosphate (10 mM), or without these additives (*Control*). These three groups were also treated with 40 ng/ml OP-1 (+) or vehicle (−) from days 1-7 before analysis. Data represent means ± SD from three replicate wells and are representative of several independent experiments.
* Ratios of OP-1 treated to control (*T/C*) which showed significant OP-1 effects ($P \leq 0.05$).

β-glycerophosphate elevates basal osteocalcin production compared to ascorbate alone (Table III).

Morphological and Histochemical Changes Induced by OP-1

The biochemical evidence for OP-1-dependent chondroinduction is consistent with histological evaluations. Cultures treated with OP-1 have abundant clusters of round cells which are stained with alcian blue intensively (Fig. 6 *B*). In contrast, there are no alcian blue-stained colonies in control cultures, while small colonies of tightly compacted, flattened cells are observed (Fig. 6 *A*). Staining of OP-1 treated cultures with toluidine blue shows red/purple metachromasia of these colonies (Fig. 6 *H*). Histochemically, strong alkaline phosphatase staining is observed for both the large, round chondrocyte-like cells and the small, compact cells surrounding these chondrocytes in OP-1-treated cultures (Fig. 6 *D*). In control cultures, the chondrocytic colonies are absent and alkaline phosphatase is restricted to tightly compacted cell colonies (Fig. 6 *C*). Mineralization of primary cultures occurs in the day 7-13 period only if 10 mM β-glycerophosphate is added to the medium. von Kossa staining shows strong mineralization in OP-1-treated cultures. Extensive mineralization occurs on the periphery of each large round cell in the chondrocyte-like cell clusters in OP-1-treated cultures (Fig. 6 *F*), while only a small amount of mineralization is observed in control cultures at day 7 (Fig. 6 *E*). Autoradiograms of [^{3}H]thymidine-labeled cultures at day 4 show that small cells surrounding the clusters of large round chondrocytic cells incorporate [^{3}H]thymidine prosperously, while the large cells within the colonies do not (Fig. 6 *G*).

TGF-β1 Antagonism of OP-1 Effects

Because OP-1 is a member of the TGF-β superfamily of growth and differentiation factors, comparative studies of OP-1 and TGF-β1 were initiated in this chondroinduction model system. These two factors share 34% identity in their 7-cysteine domain (Ozkaynak et al., 1990). Histological (Fig. 7) and biochemical (Table IV) changes associated with OP-1-dependent chondrogenesis are not seen with TGF-β1 over a dose range of 0.01-40 ng/ml. Furthermore, the chondrogenesis induced by 40 ng/ml of OP-1 alone (4.84-fold increase in alcian blue, 1.47-fold increase in alkaline phosphatase; Table IV) is strongly antagonized by TGF-β1 concentrations of 1 and 10 ng/ml (Fig. 7; Table IV). TGF-β1 also diminishes osteoblastic parameters osteocalcin and alkaline phosphatase (Table IV). Histologically, TGF-β1 changed the normal polygonal cell shape to a fibroblastic spindle shape (Fig. 7).

Discussion

Evidence That OP-1 Induces Chondrogenesis In Vitro

We present evidence that OP-1 induces chondrogenesis in a primary culture model system initiated from newborn rat calvaria. Morphologically, chondrocytic colonies appear within two days after treatment with OP-1, and these distinc-

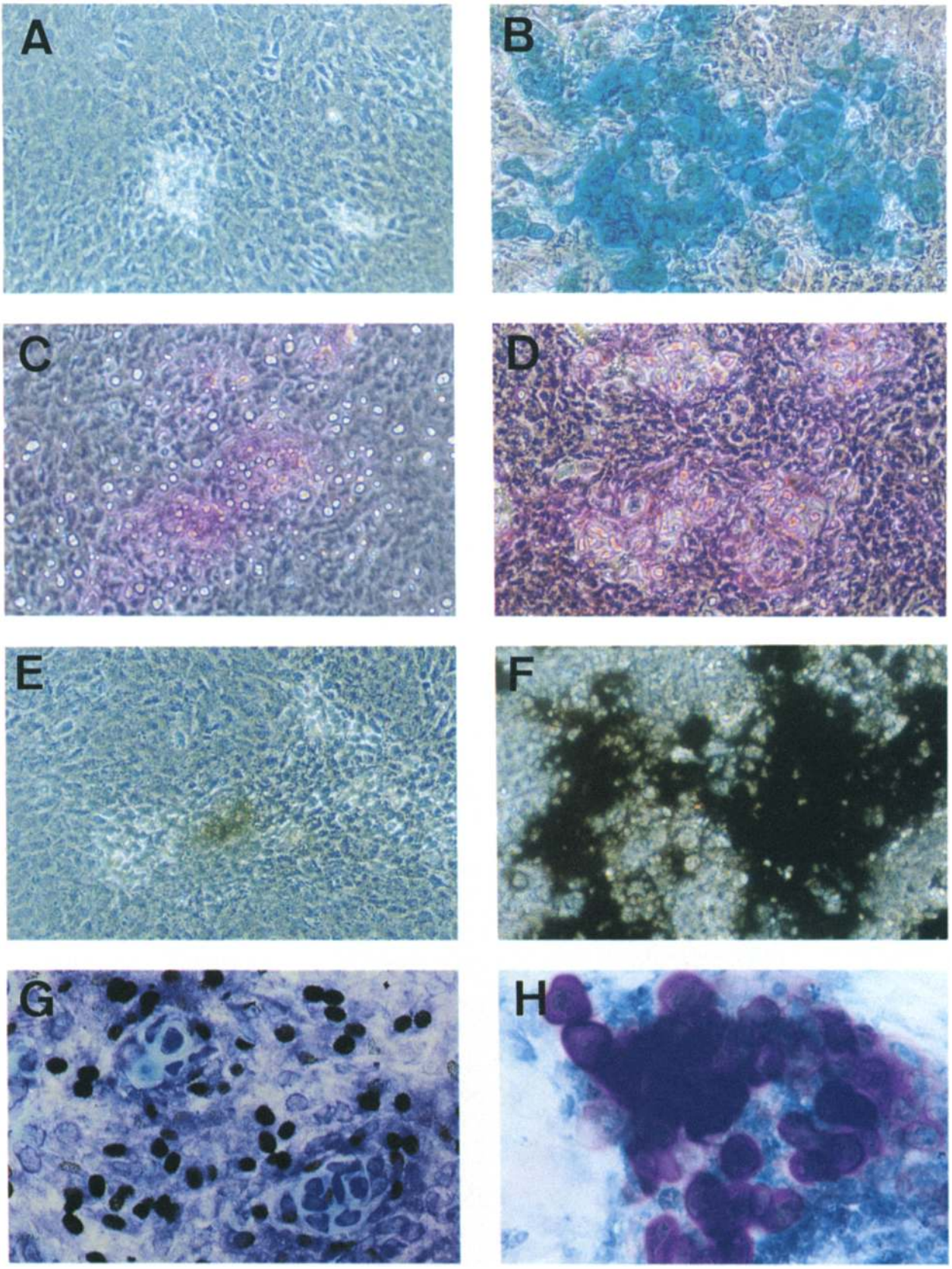

Figure 6. Histological changes induced by OP-1. Phase contrast micrographs of alcian blue staining (*A* and *B*), alkaline phosphatase staining (*C* and *D*), and von Kossa staining (*E* and *F*); autoradiogram of [^{3}H]thymidine incorporation into DNA (*G*); light micrograph of toluidine blue staining (*H*). Cells were cultured without OP-1 (*A*, *C*, and *E*) or with 40 ng/ml of OP-1 (*B*, *D*, *F*, and *H*) for 6 d from day 1, and the effects of OP-1 on chondrogenesis (*A*, *B*, and *H*) alkaline phosphatase activity (*C* and *D*) and mineralization (*E* and *F*) were examined at day 7. For autoradiography (*G*), cells were cultured with 40 ng/ml of OP-1 for 3 d from day 1, pulsed with [^{3}H]thymidine for 4 h, and autoradiographed.

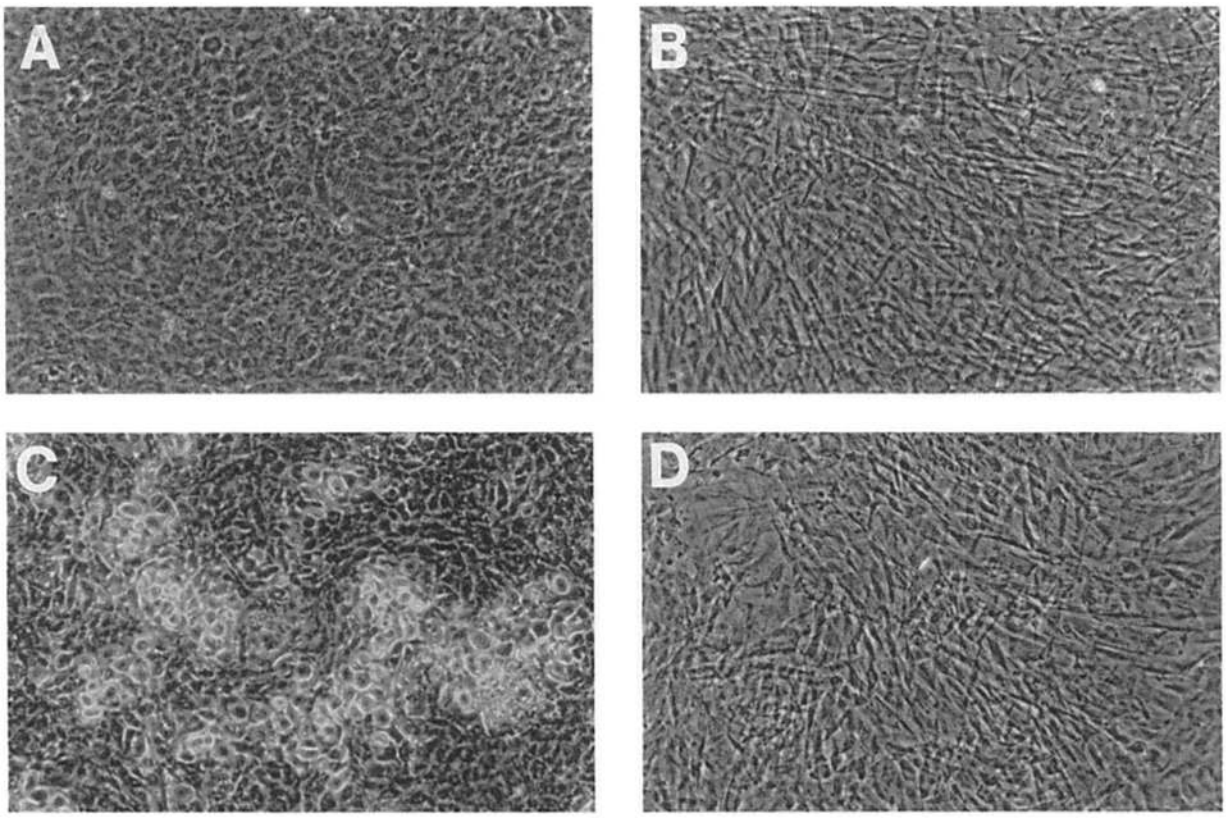

Figure 7. TGF-β1 antagonism of OP-1 chondroinduction. Phase-contrast micrographs are shown for the control culture (*A*), and cultures treated with either 1 ng/ml TGF-β1 (*B*), 40 ng/ml OP-1 (*C*), or 1 ng/ml TGF-β1 and 40 ng/ml OP-1 (*D*) for 6 d from day 1.

tive cell clusters increase in both number and size before becoming hypertrophied. Chondrocytic cell clusters stain heavily with alcian blue, stain metachromatically with toluidine blue, express high alkaline phosphatase activity, and act as loci for the initiation of mineralization which first occurs in and around the hypertrophic chondrocytic cells. These sequential changes have strong parallels to the process of endochondral bone formation in vivo, as discussed below.

Table IV. TGF-β1 Antagonism of OP-1 Induced Phenotypic Changes

Treatment		Ratio of treated/control		
TGF-β1	OP-1	Alcian blue staining	Alkaline phosphatase activity	Osteocalcin
ng/ml				
–	–	1.00	1.000	1.000
0.1	–	0.98	0.367*	0.094*
1	–	0.99	0.083*	0.056*
1	40	1.41*	0.250*	0.055*
10	–	1.09	0.058*	0.062*
10	40	1.43*	0.215*	0.059*
–	40	4.84*	1.465*	0.242*

Cell cultures were established (see Materials and Methods) and treated with or without various doses of TGF-β1 and/or OP-1 for 6 d (culture day 1–7) before analysis. Data were calculated from means of three replicate wells; standard deviations are ±5% for alcian blue staining, and ±12% for alkaline phosphatase activity and osteocalcin.

* Ratios of treated to control values which showed significant differences from the untreated control ($P \leq 0.05$).

Expression of cartilage-associated type II and IX collagen genes is enhanced in the early stages after exposure to OP-1, and then gradually decreases. It is noteworthy that long-form type IX collagen is expressed only in cultures treated with OP-1, while type II and short-form type IX collagens are expressed even in control cultures. Although the association of type II and IX collagens with chondrogenesis is well established (von der Mark, 1980; Heinegard and Paulsson, 1987), type II collagen is also expressed in a variety of other tissues during embryogenesis, thus it is not a definitive marker for the chondrocyte phenotype (Chah et al., 1991; Kosher and Solursh, 1989). Type IX collagen can be synthesized in two distinct forms due to differential mRNA transcription of the α1(IX) gene, using alternative promotor/transcriptional start sites (Nishimura et al., 1989). In cartilage, the upstream promoter/transcriptional start site is predominantly activated, resulting in processing of the long form containing the 5′ exons encoding the NC4 globular amino-terminal domain. It has been demonstrated that the downstream promoter/transcriptional start site (short form α1[IX] mRNA) is predominantly used in the nonchrondrogenic tissues, including chicken primary cornea (Svoboda et al., 1988; Nishimura et al., 1989), nonchondrogenic regions of developing chicken forelimb (Swiderski and Solursh, 1992*a*), and notochord (Swiderski and Solursh, 1992*b*). The OP-1–dependent appearance of long-form type IX collagen strongly supports our inference that OP-1 induces chondrogenesis.

Type X collagen is well known to be a specific molecule of hypertrophic cartilage (Linsenmayer et al., 1988), and it

appears with the onset of hypertrophic cartilage mineralization (Linsenmayer et al., 1991). While expression of both type II and IX collagen genes gradually diminishes through the culture period, type X collagen mRNA appears only transiently after 6 d of OP-1 treatment (Fig. 3). Thus it appears that OP-1 is capable not only of inducing chondrogenesis, but also of promoting the further differentiation of chondrocytes to a hypertrophic state analogous to that which occurs in the growth plates of long bones.

OP-1–dependent Chondrogenic Potential of Calvarial Cells

Development of the calvaria follows the classical process of intramembranous ossification. Osteoblasts are derived from mesenchymal fibroblast-like precursors, or osteoprogenitor cells. Osteogenesis proceeds without histological evidence of cartilage involvement. However, there are elements of the embryonic chondrocranium associated with the distal regions of the lambdoidal suture and small portions of the adjacent parietal and occipital bones. Rifas et al. (1982) showed that floating cells derived from ascorbate-free cultures of the calvarial chondrocranium could redifferentiate into chondrocytes and form cartilage nodules in the presence of ascorbate. To rule out the possibility that early OP-1 treatment was acting only on cells derived from the chondrocranium, calvarial segments were dissected and studied separately (Table I). It is clear that alcian blue–stained clusters of hypertrophic chondrocytes can form from cells derived from all calvarial regions, although they are somewhat more abundant in the occipital and parietal bones. Another conclusion from sequential calvarial digestion studies is that the greatest abundance of cells with OP-1–dependent chondrogenic potential occurs in digests III–V, cells indigenous to the mineralizing osseous matrix which are also known to have the greatest osteogenic potential. In contrast, cells from calvarial digest populations I and II, which are depleted of osteoprogenitor cells, show a poor chondrogenic response to OP-1. This supports the hypothesis that pluripotent osteoprogenitor cells are the target cells for OP-1–dependent chondroinduction.

In the early stages of the cultures, OP-1 stimulates DNA synthesis. Because chondrogenic differentiation is also stimulated in this early period, there is an apparent contradiction to the general rule of an inverse relationship between cell proliferation and differentiation. However, the autoradiogram of [^{3}H]thymidine incorporation into DNA at day 4 shows that the clusters of large round cells which seem to be incipient chondrocytes induced by OP-1 do not incorporate [^{3}H]thymidine, while small cells surrounding the colonies do so intensively (Fig. 6 *G*). It is possible that OP-1 stimulates DNA synthesis in undifferentiated progenitor cells, but is a weaker mitogen for differentiated cells. Judging from the typical hypertrophic chondrocyte cluster size of 2–20 cells, OP-1 allows ~1–4 rounds of cell division in cells responding to the chondroinductive influence.

Ascorbic acid is essential for the induction of chondrogenesis by OP-1. In rat primary osteoblast-like cell cultures, ascorbate is known to be required for expression of the osteoblastic phenotype (Aronow et al., 1990), and ascorbate induces alkaline phosphatase and mineralization in chick chondrocyte cultures (Leboy et al., 1989). Though the precise role of this vitamin in chondrogenesis and osteogenesis is still unclear, ascorbic acid is required for the hydroxylation of proline and lysine residues and promotes collagen secretion (Kivirikko and Myllyla, 1987). In a recent study of MC3T3-E1 osteoblasts, Franceschi and Iyer (1992) showed that expression of mature osteoblastic markers is reversibly blocked when collagen synthesis and secretion is inhibited by ascorbate deprivation or addition of proline analogs. It is likely that the existence of a properly assembled, mature collagenous extracellular matrix plays a critical role in the induction of chondrogenesis and osteogenesis.

OP-1 Regulates the Commitment of Osteoprogenitor Cells

Primary cultures prepared through sequential collagenase digestions of fetal or newborn rat calvaria have long been known to possess osteoblastic characteristics (Wong and Cohn, 1975; Bellows et al., 1986). A temporal appearance of osteoblastic phenotypic markers in these in vitro systems is analogous to that seen in vivo in developing fetal rat calvaria tissue (Aronow et al., 1990). A similar correlation of temporal expression exists for embryonic chicken osteoblasts (Gerstenfeld et al., 1987). Owen et al. (1990) proposed a model wherein primary rat calvarial cell cultures were considered to traverse three principal periods of osteoblast development, consisting of proliferation, matrix development/maturation, and mineralization. They presented evidence that proliferation was downregulated before the onset of events associated with osteoblast differentiation.

The rat calvarial cell culture system which we have used expresses osteoblastic characteristics after confluence, and this osteogenesis is known to be amplified by OP-1 treatment after confluence. We reported previously that OP-1 stimulates osteoblastic properties such as alkaline phosphatase activity, intracellular cAMP production in response to parathyroid hormone (PTH), osteocalcin synthesis, and mineralization (Sampath et al., 1992).

Chondroinductive effects of OP-1, the focus of this study, are observed in rat calvarial cell primary cultures when exposure occurs early, during the proliferation phase before confluence. It is noteworthy that early OP-1 treatment decreases osteocalcin production when the level of osteocalcin synthesis is relatively low (Fig. 5 and Tables II and IV). This suggests that OP-1 delays the osteoblastic differentiation of uncommitted osteoprogenitor cells, either by stimulating the proliferation of this subpopulation, or by diverting cells to a chondrogenic pathway. As the culture matures in the absence of added OP-1, these progenitors either die or become increasingly committed to the osteoblastic lineage, since later exposure to OP-1 promotes further maturation and expression of osteoblastic phenotypes by acting on the predominant target population of committed preosteoblasts and osteoblasts in mature cultures (Sampath et al., 1992). Reversal of these committed cells by OP-1 from osteogenic to chondrogenic fate apparently does not occur, and there is no obvious formation of chondrocytic clusters during late OP-1 treatment. Table II also supports this inference by demonstrating OP-1 enhancement of osteoblastic, but not chondrocytic, markers in subcultivated cells.

We have observed similar OP-1 effects on ROS 17/2.8 cells and MC3T3-E1 cells (unpublished data): OP-1 enhances both alkaline phosphatase activity and osteocalcin produc-

tion in these osteoblastic cell lines. These effects of OP-1 on mature osteoblasts are consistent with the studies using related TGF-β superfamily proteins BMP-2, BMP-4, and BMP-3 (osteogenin) (Chen et al., 1991; Takuwa et al., 1991; Vukicevic et al., 1989). In the calvarial cell primary culture model, the chondrogenic versus osteogenic effects of OP-1 are, therefore, primarily determined by the timing of the onset of OP-1 treatment.

The exact lineage of cells which differentiate into chondrocytes under the influence of OP-1 is not yet known. The primary rat calvarial cell population used in this study (digest III–V) is dominated by osteoprogenitor cells, preosteoblasts, and osteoblasts (Wong and Cohn, 1975; Bellows et al., 1986; Bellows and Aubin, 1989; Aronow et al., 1990). Clonal cell lines which have characteristics of immature versus mature stages of osteoblastic differentiation and developmental commitment have been derived from rat calvaria (Yamaguchi and Kahn, 1991). Another clonally selected rat calvarial cell line was found to express the chondrocytic phenotype (Bernier et al., 1990). Other evidence exists that a clonal cell line derived from fetal rat calvaria can differentiate into several cell types. RCJ3.1, clonally derived from the 21-d fetal rat calvaria, differentiates into four distinct phenotypes including myoblasts, mineralized osteoblasts, adipocytes, and chondrocytes, the latter after dexamethasone treatment (Grigoriadis et al., 1988). Kellerman et al. (1990) demonstrated that an SV-40 immortalized cell line derived from mouse teratocarcinoma differentiates into osteoblasts, chondrocytes, and adipocytes under various culture conditions. It has also been shown that chick periosteal cells (Nakahara et al., 1992), bone marrow cells (Berry et al., 1992), and calvarial cells (Manduca et al., 1992) contain osteoprogenitor cells that are capable of differentiating into osteoblasts or chondrocytes.

The hypothesis that early OP-1 treatment targets uncommitted osteoprogenitor cells is supported by our findings, but we cannot rule out the possibility that OP-1 stimulates cartilage phenotypic expression among cells already committed to the chondrocytic lineage. The frequency of hypertrophic chondrocyte colonies induced by OP-1 is ~0.7% of plated cells in our experiments. Bellows and Aubin (1989) determined an osteoprogenitor cell frequency of 0.3% in the rat calvarial cell culture system by limited dilution counting of mineralized nodules.

TGF-β1 appears to strongly influence osteoprogenitor cell commitment in this primary culture model. The phenotypic changes (Table IV) and the fibroblastic appearance of the TGF-β1–treated calvarial cells, even in the presence of 40 ng/ml OP-1 (Fig. 7, *B* and *D*), suggests that TGF-β1 causes diversion of the uncommitted osteoprogenitor cells from both chondrogenic and osteogenic fates. This contrasts with reported TGF-β1 effects in vivo where subperiosteal injections of 200 ng/day in the rat femur cause elevated chondrogenesis (Joyce et al., 1990).

OP-1 Treatment In Vitro as a Model for Endochondral Ossification

In primary calvarial cultures, OP-1 induces chondrogenesis and fosters further differentiation of chondrocytes to a hypertrophic state. These centers of hypertrophic chondrocytes which develop in response to OP-1 in vitro appear to "nucleate" and organize local osteogenesis and mineralized nodule development (Fig. 6), just as calcified cartilage in the endochondral growth plate acts as a substratum for osteoblastic bone formation in vivo.

Differentiated chondrocytes are known to produce growth factors such as basic FGF which may locally stimulate osteoblast proliferation (Hauschka et al., 1986). A heparan- and chondroitin-SO_4-rich proteoglycan, betaglycan, is the class III TGF-β receptor (Cheifetz et al., 1987) and in a soluble, mobile form, betaglycan has been implicated in TGF-β binding to the extracellular matrix (Andres et al., 1989). Might proteoglycans such as betaglycan, decorin (DS-PGII), and biglycan (DS-PGI) play a role in tethering OP-1 to the matrix surrounding hypertrophic chondrocytes, thereby allowing a localized osteogenic response?

We have presented evidence in support of the hypothesis that OP-1 induces the differentiation of chondrocytes from uncommitted osteoprogenitor cells. It is possible that OP-1 is naturally involved in the induction of chondrocytes from these progenitors, thus playing an essential role in normal developmental and tissue repair processes of the skeletal system in vivo.

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