Rapid Chondrocyte Maturation by Serum-Free Culture With BMP-2 and Ascorbic Acid

Phoebe S. Leboy,* Teresa A. Sullivan, May Nooreyazdan, and Rachel A. Venezian

Department of Biochemistry, School of Dental Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Abstract   In serum-containing medium, ascorbic acid induces maturation of prehypertrophic chick embryo sternal chondrocytes. Recently, cultured chondrocytes have also been reported to undergo maturation in the presence of bone morphogenetic proteins or in serum-free medium supplemented with thyroxine. In the present study, we have examined the combined effect of ascorbic acid, BMP-2, and serum-free conditions on the induction of alkaline phosphatase and type X collagen in chick sternal chondrocytes. Addition of either ascorbate or rhBMP-2 to nonconfluent cephalic sternal chondrocytes produced elevated alkaline phosphatase levels within 24–72 h, and simultaneous exposure to both ascorbate and BMP yielded enzyme levels at least threefold those of either inducer alone. The effects of ascorbate and BMP were markedly potentiated by culture in serum-free medium, and alkaline phosphatase levels of preconfluent serum-free cultures treated for 48 h with BMP + ascorbate were equivalent to those reached in serum-containing medium only after confluence. While ascorbate addition was required for maximal alkaline phosphatase activity, it did not induce a rapid increase in type X collagen mRNA. In contrast, BMP added to serum-free medium induced a three- to fourfold increase in type X collagen mRNA within 24 h even in the presence of cyclohexamide, indicating that new protein synthesis was not required. Addition of thyroid hormone to serum-free medium was required for maximal ascorbate effects but not for BMP stimulation. Neither ascorbate nor BMP induced alkaline phosphatase activity in caudal sternal chondrocytes, which do not undergo hypertrophy during embryonic development. These results indicate that ascorbate + BMP in serum-free culture induces rapid chondrocyte maturation of prehypertrophic chondrocytes. The mechanisms for ascorbate and BMP action appear to be distinct, while BMP and thyroid hormone may share a similar mechanism for induction. J. Cell. Biochem. 66:394–403, 1997.   © 1997 Wiley-Liss, Inc.

Key words: hypertrophic; growth plate; type X; alkaline phosphatase; sternum; chicken; avian

While some cartilages such as the nasal septum and articular cartilage persist through life, others undergo changes leading to cartilage calcification and replacement by endochondral bone. The maturational changes associated with calcifying cartilage include a switch from type II and type IX collagen to type X collagen synthesis, the induction of a high level of alkaline phosphatase, and the expression of several matrix proteins such as osteopontin and osteocalcin which are commonly associated with mineralizing bone matrix. A visible hallmark of these maturational changes is enlarged chondroatcytes, and the altered gene expression associated with this transition is usually subsumed under the term chondrocyte hypertrophy.

Several in vitro models have been developed to study chondrocyte maturation [Cancedda et al., 1995]. These include culturing prehypertrophic chondrocytes in gels or suspension culture to promote a more rounded cell shape [Gibson et al., 1984; Solursh et al., 1986; Iwamoto et al., 1989; Adams et al., 1991] or adding inducers such as ascorbic acid or retinoic acid [Habuchi et al., 1985; Tacchetti et al., 1987; Leboy et al., 1989; Pacifici et al., 1991; Iwamoto et al., 1993a; Lian et al., 1993]. Recently, several groups have reported that culture of avian chondrocytes in serum-free medium supplemented with thyroid hormone and insulin or insulin-like growth factor 1 (IGF-1) also induces chondrocyte maturation [Böhme et al., 1992, 1995; Quarto et al., 1992a].

Bone morphogenetic proteins (BMPs) are growth/differentiation factors of the transform-

ing growth factor beta (TGFβ) superfamily which were initially defined by their ability to induce formation of both cartilage and bone when implanted at ectopic sites [Urist, 1965; Reddi, 1981; Celeste et al., 1990]. While most in vitro studies have focused on the ability of BMPs to promote osteoblast formation, BMPs also promote chondrogenic differentiation in many types of mesenchymal cells [Vukicevic et al., 1989; Carrington et al., 1991; Asahina et al., 1993; Rosen et al., 1994]. The sequence of events following ectopic BMP implantation suggests that bone is formed via the endochondral pathway, with cartilage formation followed by chondrocyte hypertrophy, cartilage calcification, and finally osteogenesis [Reddi, 1992; Rosen and Thies, 1992]. It is therefore reasonable to expect that, in vitro, BMPs might promote not only chondrogenesis but also the further maturation of chondrocytes. Several recent studies have provided evidence that BMP treatment of chondrogenic cell lines does indeed promote chondrocyte maturation. Rosen et al. [1994] reported that a clonal mouse limb bud cell line in micromass culture developed a hypertrophic phenotype after 8 days of BMP-2 treatment. Similarly, a rat skeletal muscle cell line cultured on collagen-coated dishes with BMP-2 and subsequently treated with ascorbic acid was found to undergo chondrogenesis followed by chondrocyte maturation within 2–3 weeks [Aikawa et al., 1996]. We have now investigated the roles of serum-free culture, BMP, and ascorbic acid in the induction of chondrocyte maturation, using short-term preconfluent cultures of prehypertrophic sternal chondrocytes from 14 day chick embryos. Our results indicate that combining serum-free conditions with BMP-2 and ascorbic acid permits expression of the hypertrophic phenotype within 24–48 h in low density monolayer cultures.

MATERIALS AND METHODS
Chondrocyte Culture

Cells were isolated from the cephalic portion of sternae from 14 day chick embryos (Truslow Farms, Chestertown, MD) by digestion for 3.5 h at 37°C in calcium- and magnesium-free Hank's balanced salt solution (CMF-HBSS) containing 0.6 mg/ml collagenase and 0.04% trypsin. The cells were resuspended in a complete medium containing high glucose Dulbecco's modified Eagle's medium (DMEM, Bedford, MA) with 10% NuSerum IV (Collaborative Biomedical

Products/Becton Dickinson, Bedford, MA), 2 mM L-glutamine, and 100 units/ml penicillin/streptomycin. The chondrocytes were plated at three sterna per 100 mm plate and were maintained on Corning (Corning, NY) tissue culture dishes at 37°C in 5% CO_2. After 5 days, the floating chondrocytes were separated from the attached fibroblasts and harvested by centrifugation. Cells were counted and plated at 3.6×10^4 cells per square centimeter on tissue culture dishes in complete medium supplemented with 4 units/ml hyaluronidase to promote attachment. To assess the effects of serum-free culture, the medium was removed after 24 h and the cell layer washed three times with CMF-HBSS before addition of DMEM (with glutamine, Pen/Strep, and hyaluronidase) supplemented with 10 pM triiodothyronine (T_3) (Sigma, St. Louis, MO), 60 ng/ml insulin, and 1 mM cysteine. Unless otherwise noted, 30 ng/ml rhBMP-2 (Genetics Institute, Cambridge, MA) or 56 μM ascorbic acid was added at 24 h. Freshly prepared ascorbic acid was added daily, and the culture medium was replaced after 48 h.

Alkaline Phosphatase Assays

Cultures were rinsed twice with CMF-HBSS and extracted with 0.15 M Tris, pH 9, 0.1 mM $ZnCl_2$, 0.1 mM $MgCl_2$, and 1% Triton X-100 for 30 min at 37°C. An aliquot of the solubilized cell layer extract was reacted with 7.5 mM p-nitrophenyl phosphate (Sigma 104 reagent) in 1.5 M Tris, pH 9, 1 mM $ZnCl_2$, and 1 mM $MgCl_2$ at room temperature, and absorption was measured at 410 nm over a 6 min time period. Enzyme levels are expressed as nanomoles of p-nitrophenol formed/minute/microgram of DNA with 1 A_{410} = 64 nmol of product.

DNA Analyses

The DNA content of the extracts used for alkaline phosphatase assays was determined as previously described [Teixeira et al., 1995]. Briefly, DNA from 75 μl aliquots was precipitated by addition of ethanol followed by 1–2 day storage at −20°C. The DNA was resuspended in alkaline EDTA, the sample was neutralized, and an equal volume of Hoechst dye 33258 was added. Fluorescence was measured in four-sided plastic cuvettes (Fisher Scientific, Fair Lawn, NJ) using a Photon Technology International (South Brunswick, NJ) spectrofluorometer, with an excitation wavelength of 365 nm and an emission wavelength of 460 nm.

mRNA Analysis

Cultures were rinsed with sterile saline and placed in 4 M guanidine isothiocyanate solution containing 0.5% sarkosyl, 5 mM EDTA, 20 mM sodium acetate (pH 5.2), and 0.1 M β-mercaptoethanol. Cells were homogenized by repeated extrusion through a 21 gauge needle; the homogenate was then extracted with an equal volume of acid phenol-chloroform (Ambion, Austin, TX). The aqueous phase was removed and precipitated twice with 2.5 volumes of ethanol. RNA samples (2–4 μg) were denatured by glyoxylation, electrophoresed on agarose gels for 2 h at 7 V/cm, and transferred onto nylon membrane (GeneScreen Plus; Dupont, Wilmington, DE). Levels of specific mRNAs were determined by hybridizing Northern blots to ^{32}P-labelled riboprobes. The chick type II and type X collagen probes have been described previously [Leboy et al., 1989]. The extent of hybridization to blots was quantitated with a Molecular Dynamics (Sunnyvale, CA) FluorImager.

Analysis of Cell Size

Cells were harvested by treatment with 0.35% trypsin and 1 mM EDTA in HBSS. They were then compared, based on their forward and side light scatter patterns, on a Becton Dickinson FacStar Plus flow cytometer using a 360 band pass filter.

RESULTS

Initial studies compared the effects of 56 μM ascorbic acid and 30 ng/ml rhBMP-2 on the induction of alkaline phosphatase activity in chondrocytes cultured in NuSerum vs. serum-free medium. Guided by previous studies [Böhme et al., 1992; Quarto et al., 1992a; Ballock and Reddi, 1994], we supplemented the serum-free medium with 60 ng/ml insulin, 10 pM T_3, and 1 mM cysteine. All cultures were initially plated in NuSerum, with transfer to serum-free conditions and addition of ascorbate and/or rhBMP-2 occurring 1 day later. The alkaline phosphatase activity of chondrocytes at day 3 is shown in Figure 1. Enzyme levels in control cultures (containing neither ascorbate nor BMP) were low in both media but were higher in serum-free control cultures than in NuSerum control cultures (2.9 nmol product/min/μg DNA vs. 0.6 nmol product/ml/μg DNA). Addition of either 30 ng/ml BMP-2 or 56 μM ascorbate for 48 h induced a two- to threefold

increase in enzyme levels both in NuSerum and serum-free cultures. The combination of BMP + ascorbate in serum-free cultures resulted in alkaline phosphatase activity three times higher than with either inducer alone, and the levels reached in serum-free culture with BMP + ascorbate were at least fourfold higher than those seen in NuSerum cultures with BMP + ascorbate. These results indicate that the combination of BMP and ascorbate was markedly more effective for inducing alkaline phosphatase activity than either ascorbate or BMP alone and that the effects were greater in serum-free cultures.

BMP dose-response analyses (Fig. 2) indicated that 30–40 ng/ml of rhBMP-2 added to serum-free medium was sufficient to yield maximal stimulation of alkaline phosphatase both with and without ascorbate. Deleting insulin from the serum-free formulation had no consistent effect on alkaline phosphatase induction by either BMP or ascorbate; however, to permit comparison with previous reports, we included it in all subsequent experiments. We also tested the effects of adding dexamethasone (Dex) or deleting T_3. Addition of 1 pM Dex yielded results similar to those obtained without Dex, but raising the Dex concentration to 1 nM caused decreased alkaline phosphatase activity (Fig. 3A). In agreement with previous reports [Böhme
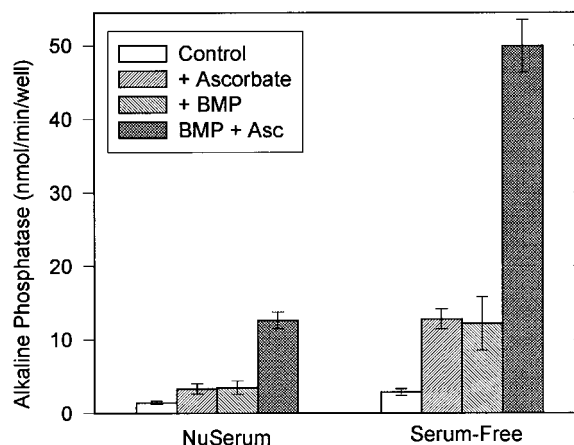
Fig. 1. Alkaline phosphatase levels in cephalic sternal chondrocyte cultures in NuSerum vs. serum-free medium. Chondrocytes were initially plated as monolayers in 12-well plates with DMEM containing 10% NuSerum and, where appropriate, transferred to DMEM supplemented with T_3, insulin, and cysteine (serum-free medium) after 1 day. Ascorbic acid (Asc) and/or BMP were added at the time of media change and alkaline phosphatase levels measured 48 h later. Values represent the average enzyme levels ± SEM for five independent experiments with duplicate samples.

et al., 1992; Quarto et al., 1992a; Ballock and Reddi, 1994; Alini et al., 1996], removing T_3 from serum-free medium containing ascorbic acid caused a marked decrease in alkaline phosphatase expression; levels with ascorbic acid and T_3 were more than twice those seen in the absence of T_3. However, deleting T_3 from medium which contained BMP had relatively little effect on alkaline phosphatase induction (Fig. 3B). When cultures were supplemented with both BMP and ascorbic acid, addition of T_3 caused a $1.2\times$ increase in alkaline phosphatase levels (data not shown).

The time course for induction of alkaline phosphatase in cephalic chondrocytes, expressed as nanomoles of product/minute/microgram of DNA, is presented in Figure 4A. Elevation of enzyme activity was seen within 24 h after addition of either 30 ng/ml BMP or 56 µM ascorbate. While BMP appeared to be slightly more effective than ascorbate, the results with BMP showed greater variation from experiment to experiment. At all time points, the combination of BMP + ascorbate resulted in alkaline phosphatase levels three- to fourfold higher than with ascorbate alone and two- to threefold those seen with BMP alone. Figure 4B presents an experiment in which chondrocytes were obtained from the caudal region of 14 day chick embryo sterna. These chondrocytes, derived from cartilage which is not prehypertrophic, show no elevation of alkaline phos-

phatase levels in response to BMP and/or ascorbate. Additional experiments indicated that exposure to BMP and ascorbate for up to 8 days did not produce elevated enzyme levels with caudal chondrocytes (data not shown).

Studies to examine the relative contributions of ascorbate and BMP were carried out by adding each of the inducers sequentially. Table I presents results of experiments in which inducer was changed after 24 h and alkaline phosphatase levels assayed after 48 h. Sequential addition of inducers was not as effective as the simultaneous presence of both BMP and ascorbate, but BMP followed by ascorbate (BMP/ascorbate) or ascorbate followed by BMP (ascorbate/BMP) yielded significantly more alkaline phosphatase than seen with cultures exposed
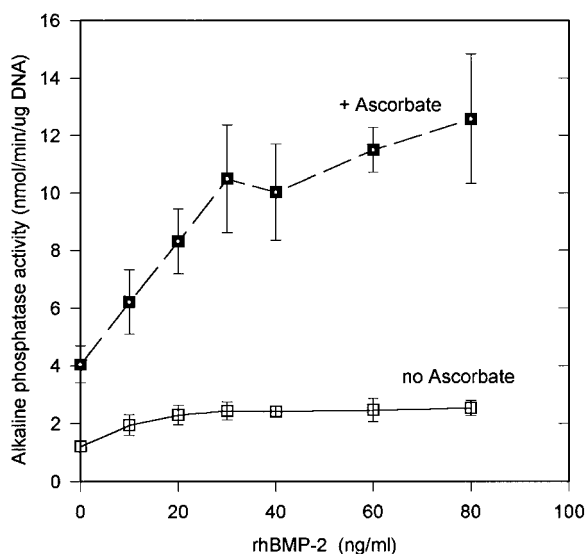

Fig. 2. Dose response for BMP induction of alkaline phosphatase activity in serum-free cultures at 48 h. Results are the result of three independent experiments with duplicate wells for each condition.
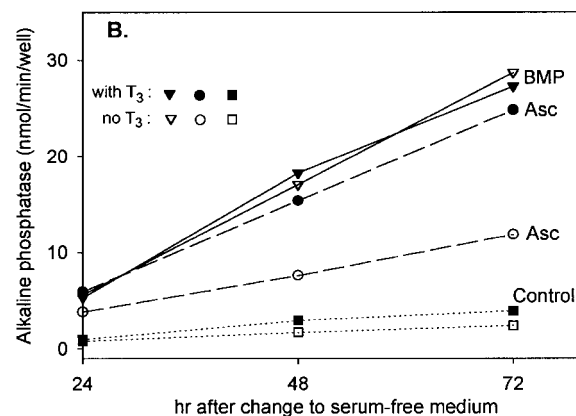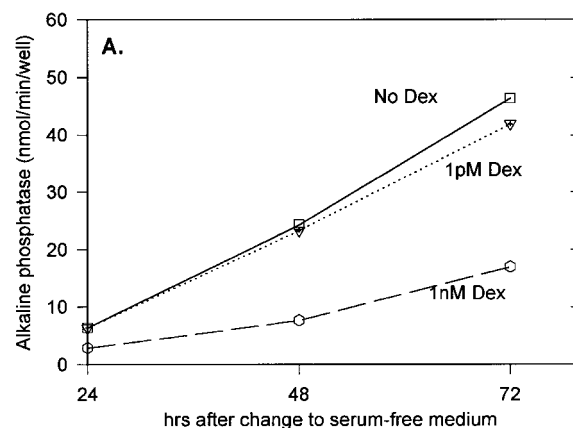
Fig. 3. Effect of dexamethasone and T_3 on alkaline phosphatase activity in chondrocyte cultures. **A:** Time course of alkaline phosphatase induction in cephalic sternal chondrocytes cultured in serum-free medium + 56 µM ascorbic acid, with varying levels of dexamethasone. **B:** Effect of adding 10^{-11} M triiodothyronine (T_3) to control cultures (dotted lines), ascorbic acid–treated cultures (dashed lines), and BMP-treated cultures (solid lines). Filled symbols = with T_3; open symbols = no T_3.
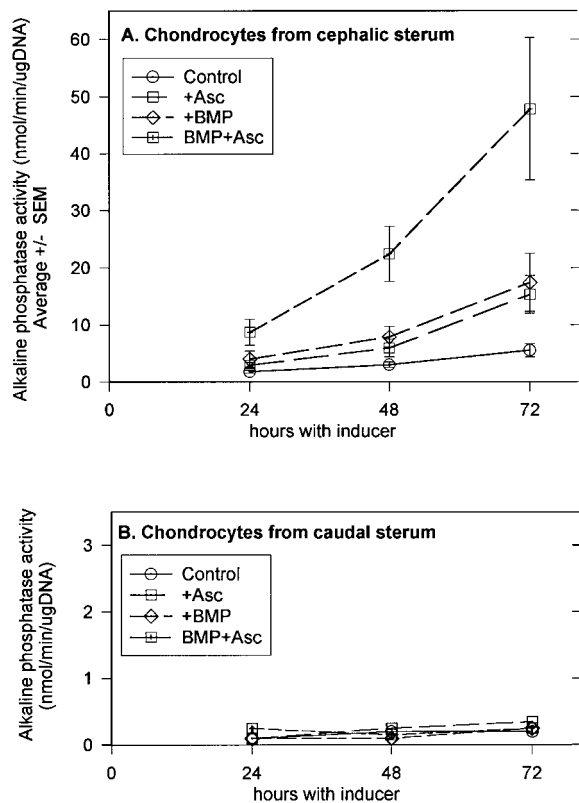
Fig. 4. Time course for alkaline phosphatase induction by 56 μM ascorbic acid and/or 30 ng/ml rhBMP-2. **A:** Measurement of alkaline phosphatase/microgram of DNA in cultures of cephalic sternal chondrocytes. Values represent the average enzyme levels ± SEM for at least five independent experiments with duplicate samples. **B:** Absence of alkaline phosphatase induction in caudal sternal chondrocytes. Note the 10× difference in scale for enzyme activity compared to A.

TABLE I. Alkaline Phosphatase Levels Following Sequential Exposure to Ascorbate or BMP-2 With Serum-Free Culture[†]

Additions		Relative Alkaline Phosphatase Activity (Asc/Asc = 1.0)
0–24 h	24–48 h	
None	None	0.46 ± 0.11*
Ascorbate	Ascorbate	1.0
BMP	BMP	1.37 ± 0.45
Ascorbate	BMP	2.18 ± 0.49*
BMP	Ascorbate	2.83 ± 0.62*
BMP + ascorbate	BMP + ascorbate	4.52 ± 0.97**

[†]Results are the average of three experiments with cephalic sternal chondrocytes.
*Difference between the mean and that for either BMP/BMP or ascorbate/ascorbate is significant at $P \leq 0.05$.
**Difference between the mean and that for either BMP/ascorbate or ascorbate/BMP is significant at $P < 0.01$.
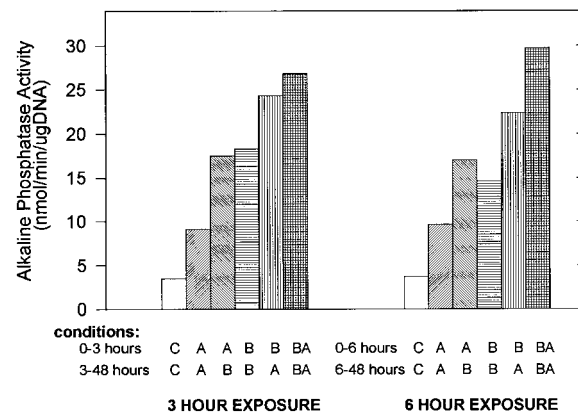

Fig. 5. Effect of switching inducers after 3 or 6 h on alkaline phosphatase levels. Cephalic sternal chondrocytes were cultured in serum-free medium with BMP (**B**) and/or ascorbic acid (**A**) for 3 h or 6 h. The medium was then replaced and culture continued for a total of 48 h.

to only one inducer. Furthermore, the results suggested that early exposure to BMP was more effective than later exposure. This possibility was examined by 3 or 6 h exposure to inducers, followed by media change and addition of fresh inducers. The results of such an experiment are presented in Figure 5. The pattern of alkaline phosphatase induction following 3 or 6 h exposure to inducer was similar to that seen when switching occurred at 24 h, and 3 h treatment with BMP followed by ascorbate treatment was almost as effective as simultaneous treatment with BMP + ascorbate. Brief exposure to ascorbate followed by BMP was not as effective, yielding alkaline phosphatase levels similar to those seen with BMP alone.

Type II and type X collagen synthesis under serum-free conditions was assessed by Northern blot analysis of mRNAs. Figure 6 shows the hybridization of probes for type II and type X

collagens to RNA prepared from serum-free culture after 24 h. It is apparent that cells exposed to BMP or BMP + ascorbate are producing significant amounts of type X collagen mRNA within 24 h, while ascorbate alone for 24 h is not sufficient to induce type X mRNA. A modest rise in type X mRNA levels was seen after 48 h with ascorbate (data not shown), suggesting that the response to ascorbate is slower than the response to BMP. As expected, an increase in type X collagen expression was associated with a decrease in type II collagen by 24 h; thus, samples cultured with BMP showed a 20–40% decrease in type II levels, while ascorbate-treated samples had type II levels similar to

those in control cultures. A summary of collagen mRNA levels from blots using four independent sets of each RNA is presented in Table II. Addition of 0.5 μM cyclohexamide during the 24 h period did not block BMP induction of type X collagen expression, arguing that new protein synthesis is not required. When RNA from chondrocytes derived from the caudal region of sternae was hybridized to probe for type X collagen, no type X mRNA was detected even with BMP + ascorbate, with or without cyclohexamide.

Visual inspection of chondrocyte cultures suggested that neither BMP nor ascorbic acid was causing significant increases in cell size. However, since ascorbic acid promotes cell rounding which might obscure size changes, we assessed cell size by flow cytometry. Analyses of forward and side scatter indicated that the cells treated with BMP, ascorbic acid, or a combination of

Fig. 6. Representative Northern blot of RNA from cephalic sternal chondrocytes cultured for 24 h in serum-free medium containing no additions (C), 56 μM ascorbic acid (A), 30 ng/ml rhBMP-2 (B), or both (BA). Total cellular RNA (2 and 4 μg) from each sample was denatured with glyoxal and loaded on an agarose gel as described in Materials and Methods. After electrophoresis, samples were transfered to nylon membrane and hybridized to ^{32}P-labelled riboprobe for chick type X collagen (Col X). The blot was then rehybridized to probe for chick type II collagen (Col II).

both inducers for 48 h were not significantly larger than control cells (data not shown).

DISCUSSION

The results presented here demonstrate that rhBMP-2, in combination with serum-free medium and ascorbic acid, can rapidly induce the maturation of prehypertrophic chondrocytes derived from the cephalic sternae of 14 day chick embryo. The appearance of elevated alkaline phosphatase activity and type X collagen mRNA within 24 h suggests that this culture system permits chondrocyte maturation in vitro without the long-term culture customarily employed. However, although this short time period is sufficient to produce the changes in gene expression characteristic of hypertrophic chondrocytes, it does not permit significant cell enlargement.

Previous reports from several groups have indicated that transfer of chondrocytes to serum-free conditions can by itself promote maturation as long as thyroxine is present [for review see Cancedda et al., 1995]. Our results confirm these observations; while alkaline phosphatase activity and type X collagen mRNA levels in serum-free cultures were relatively low in the absence of inducers, they were higher than those found with NuSerum. Similarly, ascorbic acid or BMP supplementation under serum-free conditions was more effective than similar supplementation of media containing 10% NuSerum. We have previously reported data demonstrating that ascorbate stimulation of chondrocytes in NuSerum (which provides 2.5% fetal bovine serum (FBS) + several growth factors and hormones) is comparable to that seen with chondrocyte cultures containing
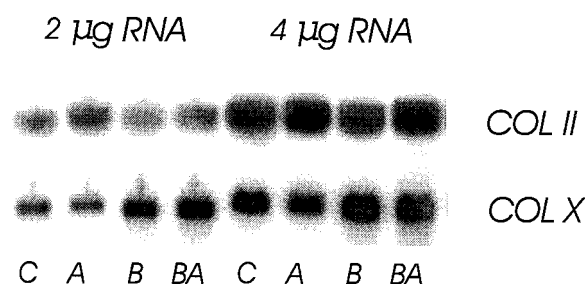
TABLE II. Relative Levels of Collagen mRNA After 24 h Culture in Serum-Free Medium[†]

	Level of collagen mRNA (no addition = 1.0)		
Addition to culture	Type II no cyclohexamide	Type X no cyclohexamide	Type X +0.5 μM cyclohexamide
No addition	1.0 (n = 4)	1.0 (n = 4)	1.0 (n = 3)
56 nM ascorbate	1.05 ± 0.06	0.75 ± 0.15	0.93 ± 0.15
30 ng/ml BMP-2	0.73 ± 0.06*	3.41 ± 1.60*	5.07 ± 0.92*
BMP-2 + ascorbate	0.89 ± 0.09**	3.72 ± 1.81*	4.90 ± 0.41*

[†]Two and four micrograms of each RNA were separated on glyoxal gels and hybridized to probes as described in Materials and Methods. Blots were scanned with a Molecular Dynamics FluorImager and the values expressed relative to the levels in control cultures (no addition). Results are average ± SD; n = number of RNA preparations assayed.
*Difference from ascorbate-treated values significant at $P < 0.01$.
**Difference from ascorbate-treated values significant at $P = 0.05$.

10% FBS [Leboy et al., 1989; Sullivan et al., 1994].

It has generally been assumed that induction of chondrocyte maturation requires not only the presence of soluble mediators acting to promote changes in gene expression but also extensive cell-cell and/or cell-matrix interactions [Cancedda et al., 1995]. This is based on observations suggesting that micromass culture, pellet culture, increased matrix secretion by ascorbic acid, and culture for 1–3 weeks are all factors which promote expression of high levels of alkaline phosphatase and type X collagen. Thus, when prehypertrophic chondrocytes are maintained in serum-containing cultures with inducers such as thyroxine or ascorbic acid, alkaline phosphatase levels reach 5–25 nmol product/min/µg DNA only in long-term, postconfluent, mineralizing cultures [Leboy et al., 1989; Gerstenfeld and Landis, 1991; Alini et al., 1996]. Results presented in Figure 4 show that this level of alkaline phosphatase activity is reached within 24–48 h by prehypertrophic chondrocytes plated at low density ($3–4 \times 10^4$ cells/cm^2) and cultured in serum-free media supplemented with ascorbic acid and BMP-2. Similarly, 24 h incubation in serum-free culture with BMP is sufficient to induce type X collagen mRNA, whereas this typically requires 1–3 weeks either in FBS-containing media supplemented with ascorbate [Solursh et al., 1986; Tacchetti et al., 1987; Leboy et al., 1989; Lian et al., 1993] or in serum-free media containing thyroxine [Böhme et al., 1992; Quarto et al., 1992a; Ballock and Reddi, 1994; Alini et al., 1996]. These results suggest that neither high cell density nor extensive matrix production is essential for the changes in gene expression associated with chondrocyte maturation. It is plausible that high cell density and/or abundant extracellular matrix cause prehypertrophic chondrocytes to secrete BMP which is bound to pericellular extracellular matrix [Reddi, 1992a; Carey and Liu, 1995], while addition of exogenous BMP circumvents this autocrine system.

While the mechanisms by which BMPs, ascorbic acid, and serum-free conditions promote transition to a hypertrophic phenotype are not yet defined, the results presented here argue that each contributes unique stimuli. Serum-free culture with ascorbic acid alone yielded alkaline phosphatase levels approximately 30% those of BMP + ascorbic acid, while serum-free cultures treated with BMP alone showed en-zyme activity averaging 40% of those with both inducers. Although assays for enzyme activity suggested that BMP was only marginally more effective than ascorbate, analyses of mRNA levels indicated that 24 h exposure to BMP but not ascorbate was sufficient time for stimulation of type X collagen transcription (Fig. 6). Similarly, a brief 3 h exposure to BMP prior to ascorbic acid treatment was sufficient to produce alkaline phosphatase levels almost as high as continuous exposure to both inducers (Fig. 5). These results, along with the observation that cyclohexamide does not block BMP induction of type X collagen mRNA, argue that BMP effects are mediated by a signal transduction pathway leading to transcriptional changes which do not require new protein synthesis [Hoodless et al., 1996; Liu et al., 1996].

Although several groups have reported that induction of chondrocyte maturation under serum-free conditions required the presence of thyroxine or triiodothyronine [Böhme et al., 1992; Quarto et al., 1992a; Ballock and Reddi, 1994; Alini et al., 1996], Ballock and Reddi [1994] noted that BMP-4 or BMP-7 can induce maturation of rat chondrocytes cultured under serum-free conditions even without thyroxine. Similarly, data presented in Figure 3B show that while ascorbic acid stimulation of alkaline phosphatase is suppressed when T_3 is absent, BMP-2 induction of alkaline phosphatase activity in cultures of avian chondrocytes does not require T_3. These data suggest that BMP and thyroxine may share a common pathway for inducing chondrocyte maturation. One possibility is that thyroxine promotes a gradual increase in production of one or more BMPs.

While BMP modulation of chondrocyte gene expression is relatively rapid, ascorbic acid effects are more gradual and probably involve a more indirect mechanism for altering gene expression. Since ascorbate promotes collagen hydroxylation and secretion, it has been postulated that ascorbate effects are mediated by changes in the extracellular matrix [Franceschi, 1992; Cancedda et al., 1995]. This mechanism appears to account for ascorbic effects on osteoblast differention in vitro [Franceschi, 1992]; however, previous studies from our lab have demonstrated that blocking collagen secretion with proline hydroxylation inhibitors does not prevent ascorbate-induced chondrocyte

maturation [Sullivan et al., 1994]. Thus, the mechanism for ascorbate-induced changes in gene expression leading to chondrocyte maturation does not appear to involve changes in cell-matrix interaction. An alternative possibility is that ascorbic acid functions as an antioxidant to protect the chondrocytes against toxic hydroxyl radicals and peroxide. However, the present studies showed that the presence of 1 mM cysteine in the culture medium did not replace ascorbic acid as an inducer of hypertrophic markers. Furthermore, compounds such as glutathionine or N-acetyl cysteine, which should mimic an antioxidant effect of ascorbic acid, do not induce significant alkaline phosphatase [Leboy, unpublished observations]. Our laboratory is presently engaged in studies to define the mechanism by which ascorbic acid promotes chondrocyte maturation.

Long-term culture (1–3 weeks) under serum-free conditions, without BMP supplementation, has been reported to promote maturation of avian chondrocytes cultured in monolayer [Böhme et al., 1992; Quarto et al., 1992a] or in suspension culture [Böhme et al., 1995] and mammalian chondrocytes in pellet culture [Ballock and Reddi, 1994; Alini et al., 1996]. One plausible hypothesis for the effect of serum-free culture on chondrocyte maturation is that, in the absence of serum-containing mitogens, chondrocytes stop proliferating and this cessation triggers terminal differentiation [Quarto et al., 1992b; Iwamoto et al., 1993b]. Arguing against this hypothesis are observations in the literature that chondrocytes display an unusual ability to survive and proliferate under serum-free conditions [Glaser and Conrad, 1984; Böhme et al., 1992]. We have observed that, during the 24–48 h period of serum-free culture, cell numbers increase 1.2–1.4× and that ascorbic acid, which stimulates maturation, also markedly stimulates thymidine incorporation in serum-free culture [Venezian and Leboy, manuscript in preparation]. These results would imply that the changes in gene expression associated with maturation do not require cessation of proliferation. An alternative hypothesis is that serum contains factors that inhibit chondrocyte maturation [Alini et al., 1996]. Recent analyses of transgenic mice lacking PTHrP or the PTHrP receptor suggest that PTHrP functions to suppress chondrocyte hypertrophy; these studies demonstrate that, in the absence of a functional PTHrP signalling mechanism, prehypertrophic chondrocytes show accelerated maturation [Lanske et al., 1996; Vortkamp et al., 1996]. Another candidate inhibitor is TGFβ, which has been reported to arrest the terminal differentiation of both avian and mammalian chondrocytes in culture [Kato et al., 1988; Böhme et al., 1995]. Our results would argue that, while absence of inhibitors in serum-free culture may provide a permissive environment for maturation, addition of BMP and ascorbic acid greatly accelerate the process.

Unlike the cephalic region of chicken sternae, which will undergo endochondral ossification before hatching, the caudal region remains cartilaginous throughout embryonic development [Solursh et al., 1986]. Chen et al. [1995] have reported that chick caudal sternal chondrocytes in serum-free culture with OP-1 (BMP-7) show elevation of type X collagen mRNA. However, this was not accompanied by the expected downregulation of type II collagen mRNA or elevated alkaline phosphatase, suggesting that the caudal chondrocytes were not proceeding through the entire maturation pathway. Data presented here indicate that, while BMP-2 promotes alkaline phosphatase and type X collagen gene expression in prehypertrophic chondrocytes from the cephalic portion of chick embryo sternae, it does not induce either alkaline phosphatase or type X collagen mRNA in caudal sternal chondrocytes. Our results are consistent with a hypothesis that BMPs are capable of inducing both chondrogenesis and chondrocyte maturation in undifferentiated multipotential cells and promoting maturation in prehypertrophic chondrocytes but that caudal sternal chondrocytes are at a stage in differentiation which does not respond to rhBMP-2.

ACKNOWLEDGMENTS

REFERENCES

Adams SL, Pallante KM, Niu Z, Leboy PS, Golden EB, Pacifici M (1991): Rapid induction of type X collagen gene expression in cultured chick vertebral chondrocytes. Exp Cell Res 193:190–197.

Aikawa T, Shirasuna K, Iwamoto M, Watatani K, Nakamura T, Okura M, Yoshioka H, Matsuya T (1996): Establishment of bone morphogenetic protein 2 responsive chondrogenic cell line. J Bone Miner Res 11:544–553.

Alini M, Kofsky Y, Wu W, Pidoux I, Poole AR (1996): In serum-free culture thyroid hormones can induce full expression of chondrocyte hypertrophy leading to matrix calcification. J Bone Miner Res 11:105–113.

Asahina I, Sampath TK, Nishimura I, Hauschka PV (1993): Human osteogenic protein-1 induces both chondroblastic and osteoblastic differentiation of osteoprogenitor cells derived from newborn rat calvaria. J Cell Biol 123:921–933.

Ballock RT, Reddi AH (1994): Thyroxine is the serum factor that regulates morphogenesis of columnar cartilage from isolated chondrocytes in chemically defined medium. J Cell Biol 126:1311–1318.

Böhme K, Conscience-Egli M, Tschan T, Winterhalter KH, Bruckner P (1992): Induction of proliferation or hypertrophy of chondrocytes in serum-free culture: The role of insulin-like growth factor-I, insulin, or thyroxine. J Cell Biol 116:1035–1042.

Böhme K, Winterhalter KH, Bruckner P (1995): Terminal differentiation of chondrocytes in culture is a spontaneous process and is arrested by transforming growth factor-β2 and basic fibroblast growth factor in synergy. Exp Cell Res 216:191–198.

Cancedda R, Cancedda FD, Castagnola P (1995): Chondrocyte differentiation. International Review of Cytology 159:265–358.

Carey DE, Liu X (1995): Expression of bone morphogenetic protein-6 messenger RNA in bovine growth plate chondrocytes of different size. J Bone Miner Res 10:401–405.

Carrington JL, Chen P, Yanagishita M, Reddi AH (1991): Osteogenin (bone morphogenetic protein-3) stimulates cartilage formation by chick limb bud cells in vitro. Dev Biol 146:406–415.

Celeste AJ, Iannazzi JA, Taylor RC, Hewick RM, Rosen V, Wang EA, Wozney JM (1990): Identification of transforming growth factor β family members present in bone-inductive protein purified from bovine bone. Proc Natl Acad Sci U S A 87:9843–9847.

Chen P, Vukicevic S, Sampath TK, Luyten FP (1995): Osteogenic protein-1 promotes growth and maturation of chick sternal chondrocytes in serum-free cultures. J Cell Sci 108:105–114.

Franceschi RT (1992): The role of ascorbic acid in mesenchymal differentiation. Nutr Rev 50:65–70.

Gerstenfeld LC, Landis WJ (1991): Gene expression and extracellular matrix ultrastructure of a mineralizing chondrocyte cell culture system. J Cell Biol 112:501–513.

Gibson GJ, Beaumont BW, Flint MH (1984): Synthesis of a low molecular weight collagen by chondrocytes from the presumptive calcification region of the embryonic chick sterna: The influence of culture with collagen gels. J Cell Biol 99:208–216.

Glaser JH, Conrad HE (1984): Properties of chick embryo chondrocytes grown in serum-free medium. J Biol Chem 259:6766–6772.

Habuchi H, Conrad HE, Glaser JH (1985): Coordinate regulation of collagen and alkaline phosphatase levels in chick embryo chondrocytes. J Biol Chem 260:13029–13034.

Hoodless PA, Haerry T, Abdollah S, Stapleton M, O'Connor MB, Attisano L, Wrana JL (1996): MADR1, a MAD-related protein that functions in BMP2 signaling pathways. Cell 85:489–500.

Iwamoto M, Sato K, Nakashima K, Shimazu A, Kato Y (1989): Hypertrophy and calcification of rabbit permanent chondrocytes in pelleted cultures: Synthesis of alkaline phosphatase and 1,25-dihydroxycholecalciferol receptor. Dev Biol 136:500–507.

Iwamoto M, Shapiro IM, Yagami K, Boskey AL, Leboy PS, Adams SL, Pacifici M (1993a): Retinoic acid induces rapid mineralization and expression of mineralization-related genes in chondrocytes. Exp Cell Res 207:413–420.

Iwamoto M, Yagami K, Valle PL, Olsen BR, Petropoulos CJ, Ewert DL, Pacifici M (1993b): Expression and role of c-myc in chondrocytes undergoing endochondral ossification. J Biol Chem 268:9645–9652.

Kato Y, Iwamoto M, Koike T, Suzuki F, Takano Y (1988): Terminal differentiation and calcification in rabbit chondrocyte cultures grown in centrifuge tubes: Regulation by transforming growth factor B and serum factors. Proc Natl Acad Sci U S A 85:9552–9556.

Lanske B, Karaplis AC, Lee K, Luz A, Vortkamp A, Pirro A, Karperien M, Defize LHK, Ho C, Mulligan RC, Abou-Samra AB, Jüppner H, Segre GV, Kronenberg HM (1996): PTH/PTHrP receptor in early development and Indian hedgehog-regulated bone growth. Science 273:663–666.

Leboy PS, Vaias L, Uschmann B, Golub E, Adams SL, Pacifici M (1989): Ascorbic acid induces alkaline phosphatase, type X collagen and calcium deposition in cultured chick chondrocytes. J Biol Chem 264:17281–17286.

Lian JB, McKee MD, Todd AM, Gerstenfeld LC (1993): Induction of bone-related proteins, osteocalcin and osteopontin, and their matrix ultrastructural localization with development of chondrocyte hypertrophy in vitro. J Cell Biochem 52:206–219.

Liu F, Hata A, Baker JC, Doody J, Cárcamo JG, Harland RM, Massagué J (1996): A human Mad protein acting as a BMP-regulated transcriptional activator. Nature 381:620–623.

Pacifici M, Golden EB, Iwamoto M, Adams SL (1991): Retinoic acid treatment induces type X collagen gene expression in cultured chick chondrocytes. Exp Cell Res 195:38–46.

Quarto R, Campanile G, Cancedda R, Dozin B (1992a): Thyroid hormone, insulin, and glucocorticoids are sufficient to support chondrocyte differentiation to hypertrophy: A serum-free analysis. J Cell Biol 119:989–995.

Quarto R, Dozin B, Tacchetti C, Robino G, Zenke M, Campanile G, Cancedda R (1992b): Constitutive myc expression impairs hypertrophy and calcification in cartilage. Dev Biol 149:168–176.

Reddi AH (1981): Cell biology and biochemistry of endochondral bone development. Collagen Rel Res 1:209–266.

Reddi AH (1992): Regulation of cartilage and bone differentiation by bone morphogenetic proteins. Curr Opin Cell Biol 4:850–855.

Rosen V, Thies RS (1992): The BMP proteins in bone formation and repair. Trends Genet 8:97–102.

Rosen V, Nove J, Song JJ, Thies RS, Cox K, Wozney JM (1994): Responsiveness of clonal limb bud cell lines to bone morphogenetic protein 2 reveals a sequential relationship between cartilage and bone cell phenotypes. J Bone Miner Res 9:1759–1768.

Solursh M, Jensen KL, Reiter RS, Schmid TM, Linsenmayer TF (1986): Environmental regulation of type X collagen production by cultures of limb mesenchyme, mesectoderm and sternal chondrocytes. Dev Biol 117:90–101.

Sullivan TA, Uschmann B, Hough R, Leboy PS (1994): Ascorbate modulation of chondrocyte gene expression is independent of its role in collagen secretion. J Biol Chem 269:22500–22506.

Tacchetti C, Quarto R, Nitsch L, Hartmann DJ, Cancedda R (1987): In vitro morphogenesis of chick embryo hypertrophic cartilage. J Cell Biol 105:999–1006.

Teixeira CC, Hatori M, Leboy PS, Pacifici M, Shapiro IM (1995): A rapid and ultrasensitive method for measurement of DNA, calcium and protein content, and alkaline phosphatase activity of chondrocyte cultures. Calcif Tissue Int 56:252–256.

Urist MR (1965): Bone: Formation by autoinduction. Science 150:893–899.

Vortkamp A, Lee K, Lanske B, Segre GV, Kronenberg HM, Tabin CJ (1996): Regulation of rate of cartilage differentiation by Indian hedgehog and PTH-related protein. Science 273:613–622.

Vukicevic S, Luyten FP, Reddi AH (1989): Stimulation of the expression of osteogenic and chondrogenic phenotypes in vitro by osteogenin. Proc Natl Acad Sci U S A 86:8793–8797.