Modulation of Chondrocyte Proliferation by Ascorbic Acid and BMP-2

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Chondrocytes show an unusual ability to thrive under serum-free conditions as long as insulin, thyroxine, and cysteine are present. Studies with sternal chondrocytes from chick embryos indicate that thymidine incorporation in chondrocytes cultured under serum-free conditions is 30–50% of that seen with fetal bovine serum (FBS). In contrast, skin fibroblast proliferation in serum-free culture is <5% of that seen with serum. Addition of 30–50 μM ascorbic acid to serum-free medium stimulates chondrocyte proliferation 4–5×, resulting in levels of thymidine incorporation higher than that seen with 10% serum. Three to five hours of ascorbate exposure is sufficient to stimulate proliferation, with maximal stimulation seen after 12–15 h. Bromo-deoxyuridine (BrdU) labelling indicated that approximately 25% of chondrocytes transit S phase during a 4-h period (16–20 h after ascorbate). Once maximal stimulation is reached, the proliferation rate remains fairly constant over at least 40 h. Ascorbate therefore increases the steady-state level of chondrocytes in the cycle. Because the stimulation of chondrocyte proliferation was greater than the net increase in cell numbers, we examined the level of apoptosis. Nuclear morphology, terminal uridine nucleotide end-labelling (TUNEL) assay, and 7-AAD/Hoechst dye FACS analyses all indicated that approximately 15% of the ascorbate-treated chondrocytes were undergoing apoptosis, while only 5% of the control chondrocytes were apoptotic. When prehypertrophic chondrocytes from the cephalic region of embryonic sternae were stimulated to undergo hypertrophy with rhBMP-2 + ascorbate, levels of apoptosis were similar to that seen with ascorbate alone. In contrast, treatment of caudal chondrocytes with BMP plus ascorbate does not induce hypertrophy, and the proportion of apoptotic cells was less than that seen with ascorbate alone. These results imply that in chondrocytes the transition to hypertrophy is associated with a decreased number of proliferating cells and a relatively high level of apoptosis. **J. Cell. Physiol. 174:331–341, 1998.** © 1998 Wiley-Liss, Inc.

In adults, cartilage exists primarily as hyaline cartilage, present in respiratory passages of the nose, larynx, trachea and bronchi, and at the ends of bone (articular cartilage). During development, additional sites of cartilage are found at regions destined to become endochondral bone such as growth plate and sternum. The process of endochondral bone formation involves further differentiation of chondrocytes in growth plate and sternum, a process known as chondrocyte hypertrophy. This transition to hypertrophic chondrocytes requires cell proliferation, followed by changes in gene expression leading to production of a novel collagen (type X), high levels of alkaline phosphatase, and mineralization of the extracellular matrix. In developing chicken embryos, the cephalic portion of the sternum becomes hypertrophic and ossifies while the caudal portion remains cartilage.

Most forms of cartilage are relatively avascular, with a high ratio of extracellular matrix per cell. Because of the limited vascularization, chondrocytes would be expected to adapt to conditions with low oxygen tension and poor nutrient supply. Consistent with this expectation, lowered oxygen tension promotes chondrogenesis (Pawelek, 1969) and cultured chondrocytes show an unusually high rate of survival in serum-free culture (Glaser and Conrad, 1984; Tschan et al., 1990; Rosselot et al., 1992; Ishizaki et al., 1994). An important requirement for chondrocyte survival under serum-free conditions is addition of an antioxidant such as cysteine or dithioerythritol (Tschan et al., 1990). This antioxidant

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requirement can also be satisfied by high density culture or conditioned medium from high density cultures, suggesting that chondrocytes can produce autocrine antioxidants which permit survival (Bruckner et al., 1989; Ishizaki et al., 1994). In 1990, Tschan et al. noted that, while serum was required for further chondrocyte differentiation and type X collagen production, high density cultures of chondrocytes from chick embryo sternae maintained viability and phenotype in cysteine-containing medium, in the absence of any added hormones or growth factors.

These observations have led to development of defined media which permit analysis of hormone and growth factor requirements for chondrocyte growth and differentiation. The most common formulation contains Dulbecco's Minimal Essential Medium (DMEM) supplemented with 1 mM cysteine, thyroxine, and insulin or insulin-like growth factor I (IGF) (Böhme et al., 1992; Quarto et al., 1992). This serum-free medium has been used to assess the effects of hormones (Rosselot et al., 1992; Ballock and Reddi, 1994; Glade et al., 1994; Alini et al., 1996) as well as various growth and differentiation factors (Galéra et al., 1992; Wu et al., 1992; Tschan et al., 1993; Böhme et al., 1995; Chen et al., 1995) on cultured chondrocytes from both mammalian and avian sources. These studies have generally demonstrated that insulin, TGFβ, and bFGF promote proliferation of chondrocytes from articular cartilage or growth plate. In contrast, thyroxine, dexamethasone, and bone morphogenetic proteins (BMPs) induce proliferating chondrocytes derived from the growth plate or developing embryonic sternae to differentiate further, expressing the characteristic markers of hypertrophic chondrocytes.

Ascorbic acid is another modulator of chondrocyte hypertrophy with cultured cells derived from regions of cartilage destined for endochondral bone formation (Hall, 1981; Habuchi et al., 1985; Tacchetti et al., 1987; Leboy et al., 1989). However, ascorbic acid also shows effects on chondrocytes which appear unrelated to development of a hypertrophic phenotype; these include suppressed lactate formation and increased oxidative activity as well as increased chondrocyte attachment to tissue culture plastic (Shapiro et al., 1991; Leboy et al., 1992). A hallmark of these latter effects is that they are not limited to cells capable of hypertrophy but are seen both with chondrocytes from the cephalic region of embryonic sterum which forms endochondral bone, and with chondrocytes derived from the caudal region of sternum, which does not ossify. We now report that ascorbate has a rapid and profound mitogenic effect on both cephalic and caudal sternal chondrocytes in serum-free culture. The increased DNA synthesis seen with ascorbate treatment is associated with increased chondrocyte apoptosis. In cultures of prehypertrophic cephalic chondrocytes, the combination of increased replication and increased cell death results in little net change in cell numbers if the chondrocytes are induced to hypertrophy with BMP-2. However, with cultures of caudal chondrocytes which do not undergo hypertrophy, BMP partially suppresses apoptosis and permits significant increases in the cell population.

MATERIALS AND METHODS

Chondrocyte cultures

Cells were isolated from the cephalic and caudal portions of sternae from 14-day chick embryos by digestion for 3 hours at 37°C, 5% CO_2 in calcium/magnesium-free Hank's Balanced Saline Solution (CMF-HBSS) containing 0.6 mg/ml collagenase and 0.04% trypsin. Cells were resuspended in DMEM supplemented with 2 mM L-glutamine, 100 units/ml penicillin/streptomycin, and 10% NuSerum (complete medium) and plated in 100-mm Corning tissue culture dishes at a concentration of three sternae per dish. After 5 days in culture, the floating chondrocytes were removed and replated at 3.6×10^4 cells per cm^2 in complete medium containing four units of hyaluronidase/ml. On the following day (day 1 of secondary culture), the medium was changed for either fresh NuSerum-containing medium or serum-free culture medium. Serum-free medium was DMEM supplemented with 2 mM L-glutamine, 100 units/ml penicillin/streptomycin, 60 ng/ml insulin, 10^{-11} M tri-iodo-L-thyronine (Sigma, St. Louis, MO), 1 mM cysteine, and four units/ml hyaluronidase; 56 μM ascorbic acid and 30 ng/ml BMP-2 were added where appropriate. Fresh ascorbic acid (Sigma) was added daily. Recombinant human BMP-2 was a gift from Genetics Institute (Cambridge, MA).

Skin fibroblast culture

Cells were isolated from 12-day chick embryos and digested in 2.5% trypsin for approximately 3 minutes at 37°C, 5% CO_2. Cells were resuspended in complete medium, pelleted and the trypsin was removed. Cells were once again resuspended in complete medium. Feathers were allowed to settle and the supernatant, containing the cells, was filtered. The cells were counted using a hematocytometer and plated at 2.5×10^6 cells per 100-mm dish. After 5 days in culture, the cells were lifted with trypsin and replated at 3.6×10^4 cells/cm^2. On the following day (day 1 of secondary culture), the medium was changed as described for chondrocyte cultures.

Thymidine incorporation

Cells were treated with ^3H thymidine (1 μCi/ml) 3–6 hours prior to harvesting. Following this incubation, cultures were washed twice with CMF-HBSS, lifted in 0.25% trypsin, 1 mM EDTA in HBSS, and pipetted onto glass fiber filters (Whatman 2.5 cm GF/C) prewetted with 5% tri-chloro acetic acid (TCA). The wells were washed with CMF-HBSS and the wash was also loaded onto the filters. After allowing the filters to dry, they were washed once for 5 minutes in ice-cold 10% TCA with 1% sodium pyrophosphate, four times for 5 minutes with ice-cold 5% TCA, and once for 2 minutes with cold EtOH. Once the filters dried, they were transferred to vials, covered with scintillation fluid, and counted.

BrdU labelling

Analysis of DNA synthesis by bromo-deoxyuridine (BrdU) labelling was determined by the procedure of Dolbeare et al. (1990). Cells were treated with 1× BrdU labelling reagent (Boehringer Mannheim, Indianapolis, IN) prior to harvesting. Following incubation, cultures were washed twice with CMF-HBSS, lifted in 0.25% trypsin, 1 mM EDTA in HBSS, and pelleted. Cells were then fixed, permeablized in 0.5% Triton X-100 in 0.1 M HCl, heat denatured, and treated with primary mouse anti-BrdU and secondary goat anti-mouse FITC anti-

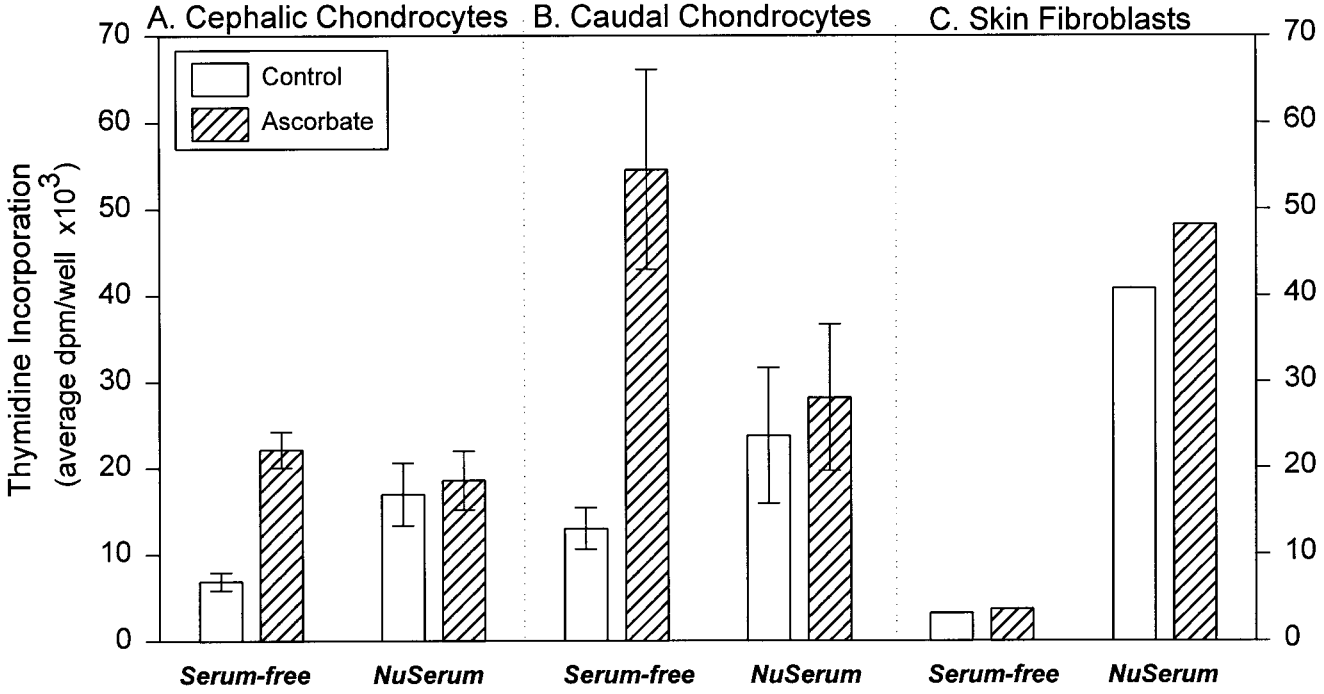

Fig. 1. Thymidine incorporation with serum-free and NuSerum media. Cells plated as monolayers in 24-well plates were placed in NuSerum or serum-free medium ± 56 μM ascorbic acid (Asc) for 24 h, with 3H-thymidine added for the last 6 h. **A:** Incorporation (dpm/well) into cephalic sternal chondrocytes; values are average ± S.E.M. of at least six independent experiments with triplicate samples. **B:** Incorporation into caudal sternal chondrocytes; values are average ± S.E.M. of at least six independent experiments with triplicate samples. **C:** Incorporation into chick embryo skin fibroblasts.

bodies. Finally, cells were analyzed by flow cytometry on a Becton Dickinson FacStar Plus.

Cell counts

Cells were lifted in 0.25% trypsin, 1 mM EDTA in HBSS. Aliquots of the lifted cells were then brought up to a final volume of 10 ml in Isoton II buffer (Fisher, Orangeburg, NY). Cell counts were made on 0.5-ml aliquots of the resuspended cells using a Coulter Counter model Z_F. The Coulter Counter settings were: amplification 4, threshold 5/10, and aperture current 4.

Flow cytometric analyses for apoptosis

Cultures were rinsed twice with CMF-HBSS and lifted in CMF-HBSS with 0.6 mg/ml collagenase and 0.041% trypsin. Cells were pelleted by centrifugation and the supernatant decanted.

7-AAD/Hoechst dye (Schmid et al., 1994). Cell pellets were resuspended in 0.3–1.0 ml of HBSS. One μg/ml Hoechst dye #33258 was added and the cells incubated in the dark for 7 min at 37°C. Cells were transferred to ice, 1 μg/ml of 7-amino-actinomycin D (7-AAD) was added, and the cells analyzed by flow cytometry. Nuclei of apoptotic cells have been shown to exhibit increased uptake of Hoechst stain while the ability to exclude 7-AAD provides a measure of membrane integrity. Hoechst fluorescence was excited by the primary laser operating at 310 nm (50 mW) and detected with a 424/44 bandpass filter. 7-AAD fluorescence was excited with a second laser at 488 nm (250 mW) and detected with a 680/30 bandpass filter. Both detections used logarithmic amplification.

TUNEL assay. dUTP end-labelling of DNA used the procedure of Zhang et al. (1995). Briefly, cell pellets were washed, fixed in 1% paraformaldehyde, and permeabilized with 0.1% Triton X-100. Biotinylated dUTP was used with a commercial nick translation kit (Life Technologies, Bethesda, MD) to label the cellular DNA. The biotin was then reacted with streptavidin conjugated to phycoerythrin. Cells were washed, resuspended in phosphate-buffered saline (PBS), and analyzed by flow cytometry on a Becton Dickinson FacStar Plus.

RESULTS

Ascorbate effects on 3H-thymidine incorporation

The effect of ascorbate treatment on proliferation of chondrocytes from 14-day chick embryo sternae was examined by measuring DNA synthesis. Chondrocytes derived from either the cephalic, prehypertrophic region, or the caudal region of the sternum were plated in DMEM medium plus 10% NuSerum and allowed to attach for 1 day in the presence of hyaluronidase. Half of the cultures were transferred to serum-free medium, while the other half were maintained in NuSerum. Cells were then incubated for 24 h, with or without ascorbate, and ^{3}H-thymidine added for the last 6 h. As demonstrated in Figure 1, ascorbate had no significant effect on cells in NuSerum. However, ascorbate had

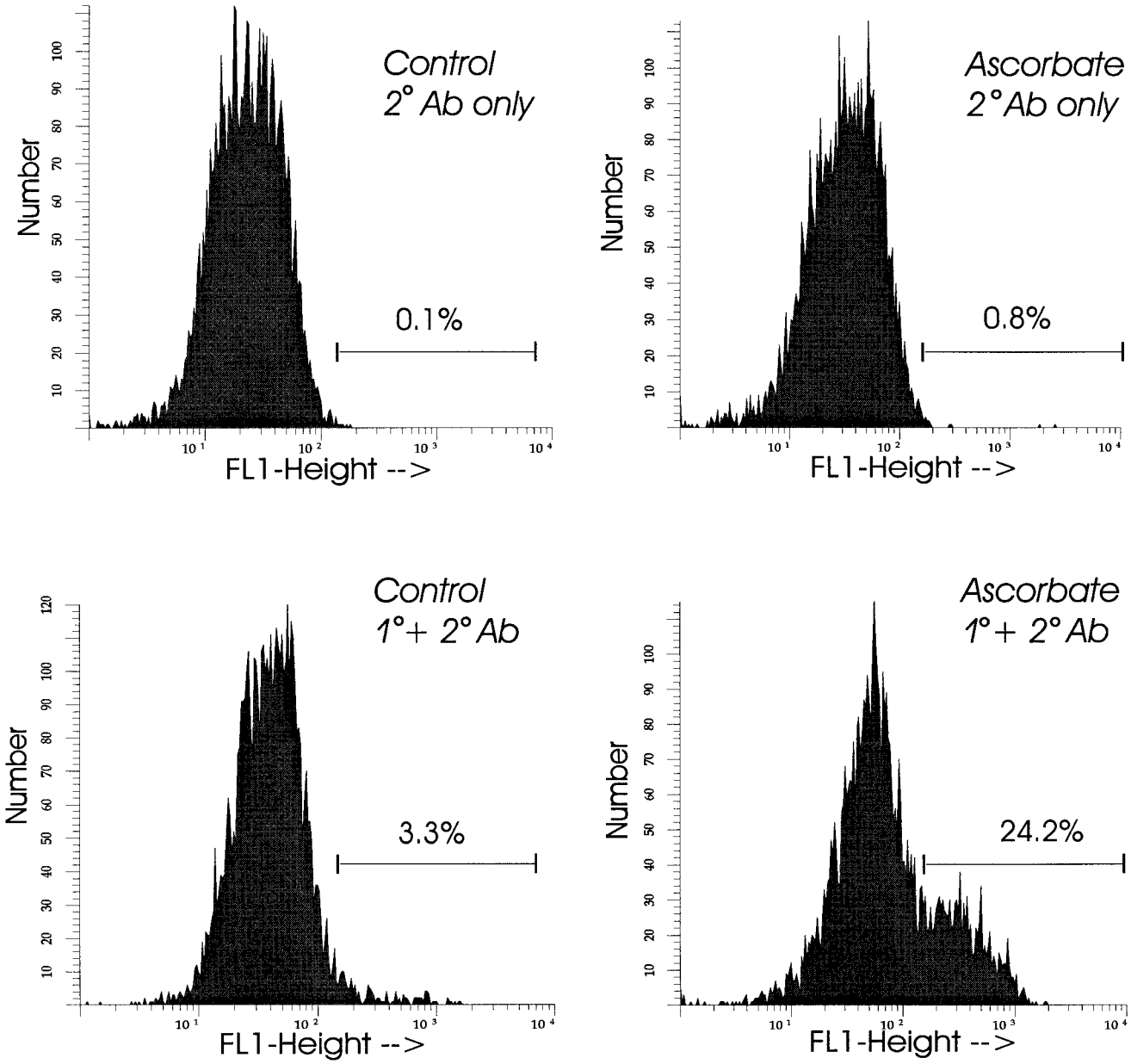

Fig. 2. Flow cytometric analysis of cephalic sternal chondrocytes labelled with bromo-deoxyuridine (BrdU). Cells were cultured with or without ascorbic acid and harvested after 24 h, with BrdU added during the last 4 h. Cells were harvested with collagenase/trypsin, washed, and reacted first with mouse antibody against BrdU, and then with goat anti-mouse second antibody. Upper panels show patterns with primary antibody omitted.

a marked effect on DNA synthesis of chondrocytes in serum-free medium; thymidine incorporation in ascorbate-treated caudal chondrocytes was 4–5× that in cultures without ascorbate (control cultures) while ascorbate caused a 3× increase with cephalic chondrocytes. Cultured skin fibroblasts from 14-day chick embryos, plated at the same density as chondrocytes, showed no effect of ascorbate on thymidine incorporation (Fig. 1). It is also noteworthy that, in contrast to fibroblasts, chondrocytes appear capable of significant proliferation under serum-free conditions even in the absence of ascorbate.

Ascorbate dose response analyses indicated that 28–56 μM ascorbate (5–10 μg/ml) added to serum-free medium yielded maximal stimulation of thymidine incorporation. This concentration is far lower than the customary level of ascorbate (280 μM) used to stimulate collagen hydroxylation and secretion in cultured cells. To estimate the proportion of cells undergoing DNA replication, chondrocytes in serum-free medium were

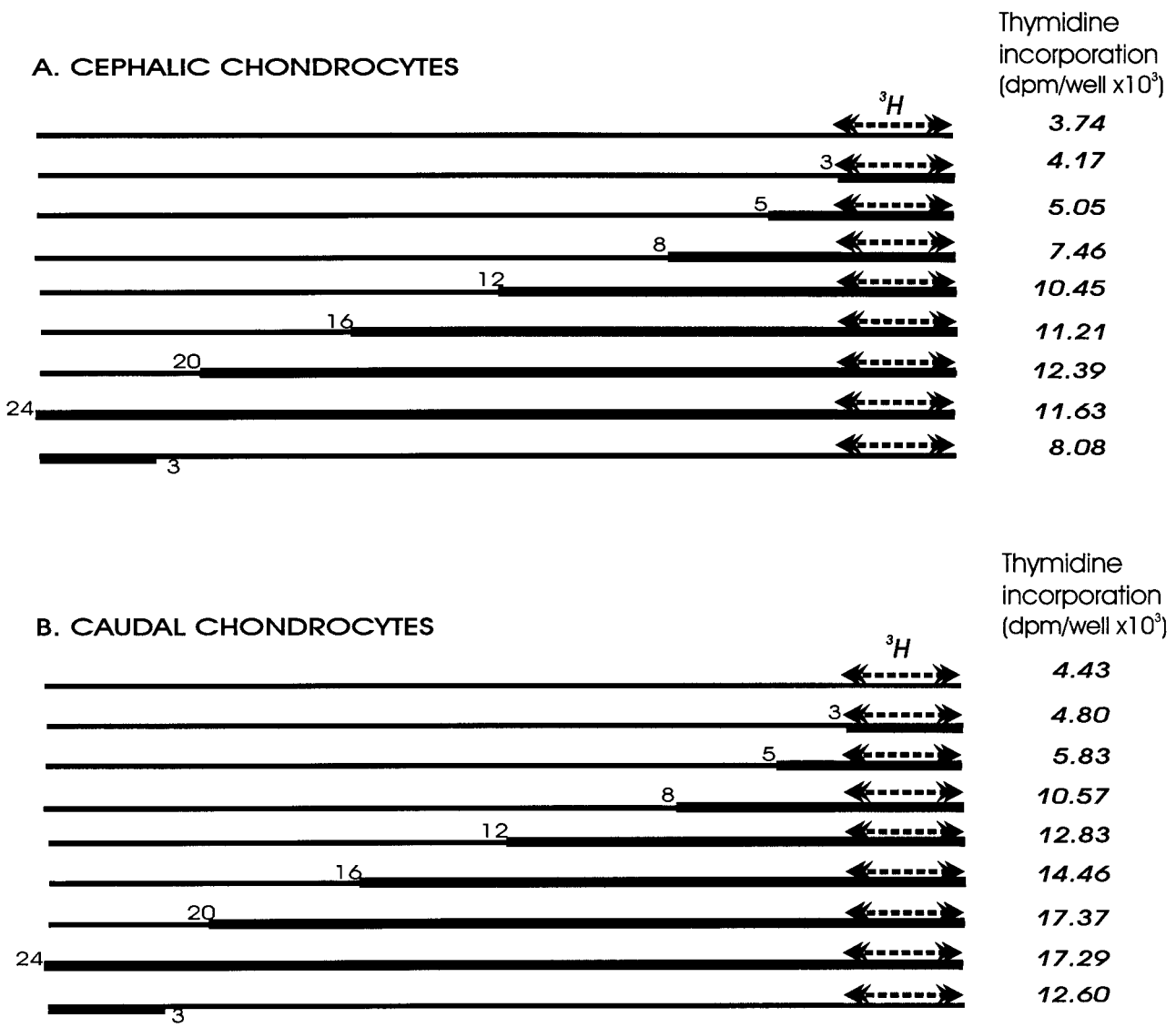

Fig. 3. **A**,**B:** Effect of varying ascorbic acid treatment on thymidine incorporation. Chondrocytes were cultured for 24 h, with thymidine added for the last 3 h (dashed lines). Length of ascorbate addition (56 μM) is indicated by heavy solid line.

labelled for 4 h with BrdU before harvesting at 24 h. Flow cytometry analysis with antibody against BrdU (Fig. 2) indicated that while approximately 3% of cells from control cultures incorporated BrdU, almost 25% of cells from ascorbate-treated cells had replicated their DNA during the 20- to 24-h labelling period.

The length of time required for ascorbate response was determined by adding ascorbate for varying times prior to labelling for 3 h with thymidine. When ascorbate was added for a 3-h period along with thymidine (from 21 to 24 h), only a modest increase in DNA synthesis was seen; increasing the length of ascorbate exposure up to 20 h increased DNA synthesis (Fig. 3). If ascorbate was added for the first 3 h and then removed, thymidine incorporation was 70–75% of that seen with continuous ascorbate exposure (Fig. 3). Thus, an initial 3-h exposure to ascorbate is effective at stimulating DNA synthesis 18–21 hours later, but 3-h exposure to ascorbate simultaneously with thymidine labelling is much less effective. No differences in ascorbate-stimulated thymidine incorporation were observed whether chondrocytes were labelled at 12–15 h, 15–18 h, or 18–21 h after ascorbate addition (data not shown). These results suggest that ascorbate is gradually recruiting chondrocytes into the cell cycle, and by 12 h, the number of cells in S phase is approaching steady-state levels.

Proliferation during chondrocyte hypertrophy

Cephalic sternal chondrocytes from 14-day embryos are prehypertrophic; when cultured in the presence of ascorbate, these cells will decrease type II collagen synthesis and express markers of further chondrocyte differentiation including type X collagen and alkaline

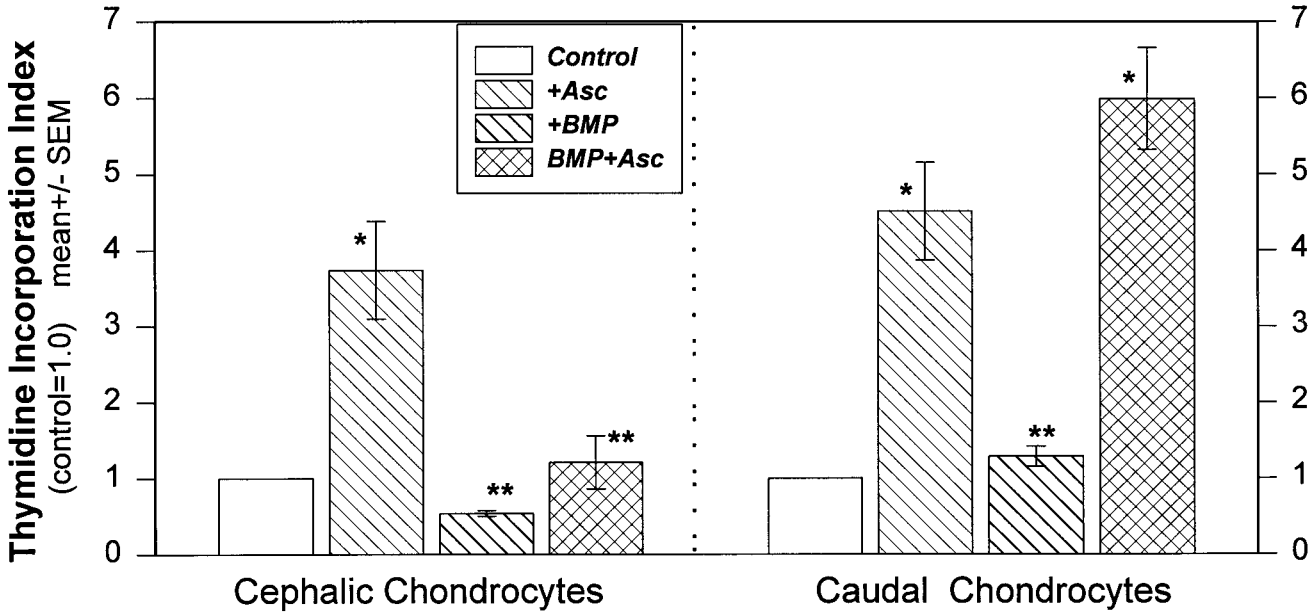

Fig. 4. Thymidine incorporation in sternal chondrocytes treated for 24 h with 56 μM ascorbic acid, 30 ng/ml rhBMP-2, or both bone morphogenetic protein (BMP) and ascorbic acid (B+A). Proliferation is expressed relative to incorporation in control cultures containing neither ascorbic acid nor BMP. Values represent the average of at least three experiments with triplicate samples; error bars indicate standard deviation. *Different from control at $P < 0.01$. **Different from ascorbic acid-treated at $P < 0.01$.

phosphatase (Leboy et al., 1989). In contrast, caudal chondrocytes are derived from a region of the sternum which does not undergo hypertrophy and endochondral bone formation during embryonic growth, and ascorbate does not alter the phenotype of cultured caudal chondrocytes. Like ascorbate, BMP-2 also induces maturation in cephalic but not caudal chondrocytes, and addition of both ascorbate and BMP-2 (B+A) to serum-free cultures of cephalic chondrocytes produces maximal stimulation of type X collagen and alkaline phosphatase activity within 24 h (Leboy et al., 1997; Sullivan et al., 1994). To examine the relationship between chondrocyte maturation and cell proliferation, we compared the effects of ascorbate and rhBMP-2. A summary of thymidine incorporation in chondrocytes cultured with BMP, ascorbate, and BMP + ascorbate is presented in Figure 4. Treatment of either cephalic or caudal chondrocyte cultures with BMP alone did not stimulate thymidine incorporation. However, the combination of BMP + ascorbate produced markedly different effects in caudal vs. cephalic chondrocytes. Caudal chondrocytes cultured with B+A showed elevated thymidine incorporation comparable to that with ascorbate alone. In contrast, treatment of cephalic chondrocytes with B+A reduced thymidine incorporation to control levels. Therefore, the ability of BMP and ascorbate to induce maturation of prehypertrophic chondrocytes is associated with reduced DNA synthesis, while ascorbate alone results in increased DNA synthesis.

Increase in number of cells. The effects of ascorbate, BMP and B+A on cell population increase was determined by measuring cell numbers after 24, 48 and 72 h treatment (Fig. 5). In both cephalic chondrocytes (Fig. 5A) and caudal chondrocytes (Fig. 5B), ascorbate caused a significant net increase in cell numbers compared to control cultures, with caudal-derived cells showing more rapid proliferation than cephalic-derived cells (note the difference in scales between Fig. 5A and 5B). However, as suggested by the thymidine incorporation, the combination of B+A had markedly different effects in the two types of chondrocytes. Cultures of cephalic chondrocytes, which are undergoing maturation in the presence of B+A, showed increasing variability in cell numbers after exposure to B+A, and by day 4 the total cell numbers were not significantly different from those of control cultures. In contrast, caudal chondrocytes treated with B+A showed a linear increase in cell numbers, and by day 4 caudal chondrocyte cultures had significantly more cells with B+A than with any other culture condition. A calculation of population doublings per 48-hr period for both caudal and cephalic chondrocytes is presented in Table 1.

Apoptosis in ascorbate-treated cultures

Although assays of thymidine incorporation, BrdU incorporation, and cell numbers all showed the same pattern of increasing proliferation in the presence of ascorbate, the net increase in cell numbers with ascorbate was lower than that expected by thymidine and BrdU measurements of DNA synthesis. Both cephalic and caudal chondrocytes, cultured in pellet cultures, were sectioned and examined by electron microscopy. The ascorbate-treated chondrocytes had intact cytoplasmic and nuclear membranes, but many showed cell shrinkage and chromatin condensation, a morphology consistent with apoptotic cells (data not shown). We therefore examined whether chondrocyte cultures treated with ascorbate were undergoing apoptosis by flow cytometric analysis using both Hoechst dye staining and biotinylated-dUTP nick labelling (TUNEL).

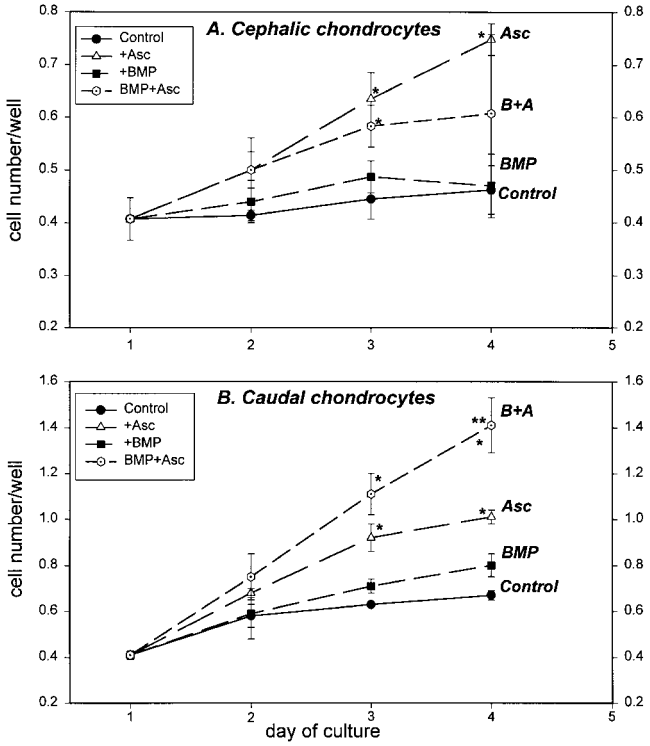

Fig. 5. **A,B:** Increase in cell numbers after exposure to ascorbic acid, BMP, or both BMP and ascorbic acid (B+A) in serum-free culture for 1–3 days. Chondrocytes were transferred from NuSerum to serum-free medium 24 h after plating (day 1). At days 2, 3, and 4, cells were harvested with collagenase/trypsin, diluted in Isoton buffer, and aliquots counted in triplicate with a Coulter Counter. Values are the average ± S.D. for three independent experiments. Probabilities were calculated using paired t statistics. *Different from control at $P < 0.001$. **Different from ascorbate-treated at $P < 0.01$.

Both assay systems indicated that ascorbate promotes not only proliferation but also cell death within 24 h. Representative flow cytometric analyses of TUNEL and 7-AAD/Hoechst-stained cephalic chondrocytes are shown in Figure 6. In Figure 6A, apoptotic cells show increased incorporation of biotinylated d-UTP. The 7-AAD/Hoechst analysis of apoptotic cells distinguishes Hoechst fluorescence in intact cells from fluorescence of cells which are necrotic and have become permeabilized by staining with 7-AAD which cannot penetrate cells with intact membranes. In Figure 6B, cells in the lower right quadrant which have bright Hoechst fluorescence but dim 7-AAD stain are considered apoptotic. A summary of results with both Hoechst dye/7-AAD and TUNEL assays is shown in Figure 7. Ascorbate caused increased apoptosis of both cephalic and caudal chondrocytes in NuSerum, while chick skin fibroblasts were unaffected by ascorbate (Fig. 7A). Assessment of BMP effects on apoptosis were done with chondrocytes transferred to serum-free media (Fig. 7B). The effect of ascorbate was similar to that seen in NuSerum, and BMP had no significant effect on apoptosis in either cephalic or caudal sternal chondrocytes. However, the combination of BMP plus ascorbate produced different effects depending on whether chondrocytes were prehypertrophic cephalic sternal chondrocytes or nonprehypertrophic caudal chondrocytes. With cephalic chondrocytes, apoptosis in B+A cultures was as high as with ascorbate alone. In contrast, caudal chondrocyte cultures with B+A showed significantly fewer apoptotic cells than those with ascorbate alone. Thus, caudal chondrocytes are stimulated to proliferate under B+A conditions, but fewer of the cells undergo apoptosis (Table 2), leading to the increased population doublings seen in B+A-treated caudal chondrocytes (Table 1).

DISCUSSION

The addition of ascorbate to connective tissue cells, at 25–50 μg/ml (0.14–0.28 mM), is generally considered to be necessary for synthesis of a collagen-rich extracellular matrix, but not for cell growth. However, there are numerous reports of ascorbate effects on proliferation, with tumor cells generally showing increased proliferation and many other cells displaying ascorbate toxicity (Alcaín and Burón, 1994; Brigelius-Flohe and Flohe, 1996). In 1984, Benya et al. reported that rabbit cartilage slices showed a marked increase in DNA synthesis if the tissue was transferred to low serum media followed by ascorbate supplementation. Similarly, there is evidence that addition of 25–50 μg/ml ascorbate to osteoblast-like MC3T3-E1 cells promotes DNA synthesis and increased cell numbers (Harada et al., 1991; Quarles et al., 1992). The present study demonstrates that lower levels of ascorbate (30–50 μM) are capable of markedly stimulating DNA synthesis as well as apoptosis in chick embryo chondrocytes cultured under serum-free conditions. Prolonged exposure of articular chondrocytes to higher doses of ascorbate (0.28 mM) are reported to cause increased cell numbers associated with dedifferentiation to a fibroblastic phenotype (Hering et al., 1994); however, the culture conditions described here caused no apparent dedifferentiation, and chondrocytes continued to make type II or type X collagen without detectable type I collagen mRNA (Leboy et al., 1997).

While the ability of chondrocytes to survive in serum-free culture has been noted previously (Glaser and Conrad, 1984; Tschan et al., 1990; Rosselot et al., 1992; Ishizaki et al., 1994), our data indicate that they are also capable of proliferation, even in the absence of ascorbate. The thymidine incorporation of both cephalic and caudal chondrocytes in serum-free medium without ascorbate was 45–55% of that seen with parallel cultures in NuSerum. However, chondrocytes from the caudal region of sternae, which are often classified as "proliferative chondrocytes," consistently showed more DNA synthesis (Fig. 1) and shorter population doubling times (Table 1) than prehypertrophic chondrocytes from cephalic sternae, regardless of culture condition. When ascorbate was added to serum-free culture, prehypertrophic cephalic chondrocytes had DNA synthesis rates comparable to that seen with NuSerum-containing medium, and caudal chondrocytes in serum-free medium + ascorbate showed DNA synthesis levels almost twice than that seen with NuSerum (Fig. 1). The serum requirement of primary chick skin fibroblasts was markedly different; the fibroblasts showed higher thymidine incorporation than chondrocytes in NuSerum, but little thymidine incorporation in serum-free medium, with or without ascorbate. Our results indicate not only that chondrocytes can survive and

TABLE 1. Population doublings of chondrocytes cultured in serum-free medium

		Doublings/48-hr period[1]			
		Control	Ascorbate	BMP	B + A
Caudal chondrocytes	day 1–3	0.63 ± 0.09	1.16 ± 0.04*	0.79 ± 0.12	1.44 ± 0.07*
Cephalic chondrocytes	day 1–3	0.22 ± 0.14	0.64 ± 0.16*	0.27 ± 0.18	0.54 ± 0.23
	day 2–4	0.19 ± 0.15	0.64 ± 0.13*	0.18 ± 0.15	0.13 ± 0.33**

[1]Calculated as $N_H/N_S = 2^X$, where N_H is numbers of harvested cells, N_S is numbers of starting cells, and X = population doublings (Cristofalo and Phillips, 1989).
*Significantly higher than control at $P < 0.01$.
**Significantly lower than ascorbate at $P < 0.01$.

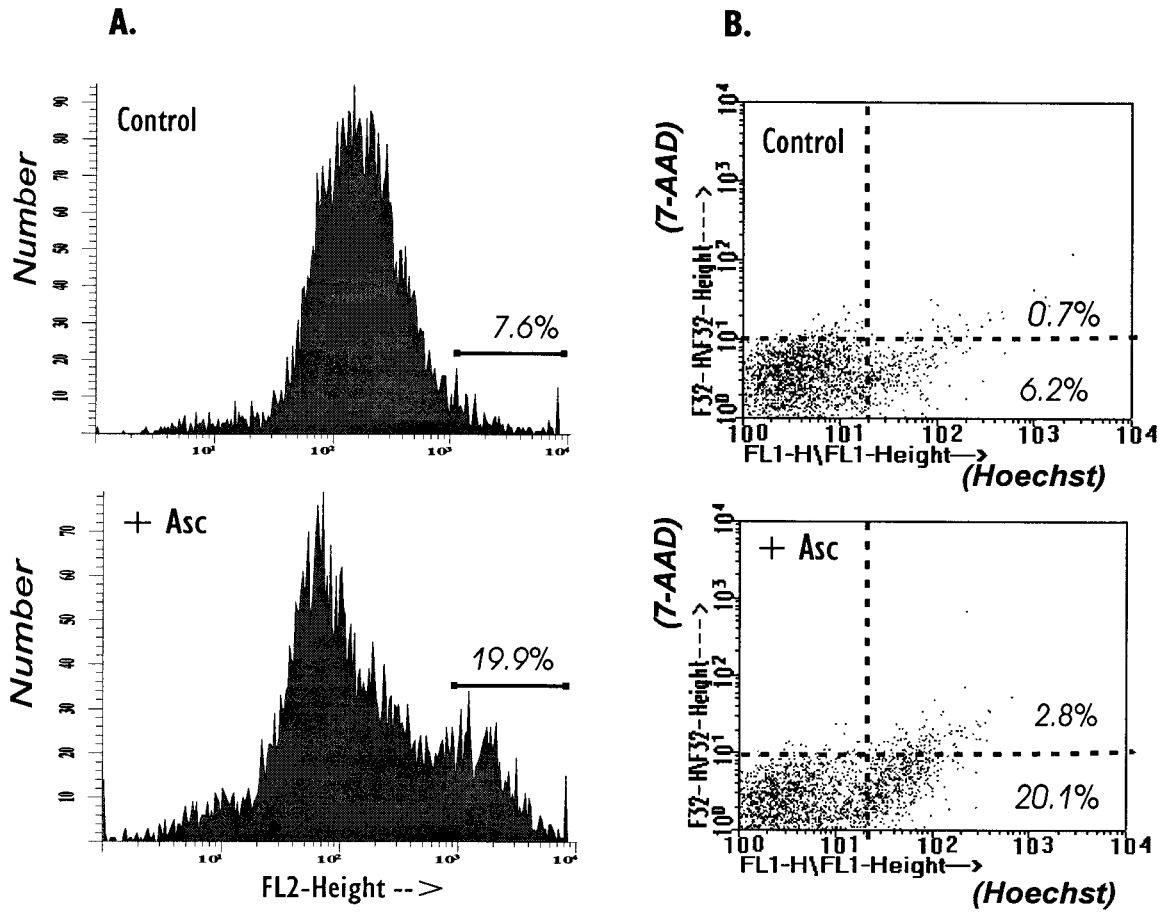

Fig. 6. Flow cytometric analyses of chondrocyte apoptosis with TUNEL and 7-amino-actinomycin D (7-AAD)/Hoechst 33258 assays. **A:** terminal uridine nucleotide end-labelling (TUNEL) assay. Biotinylated dUTP binding to termini of DNA results in increased staining of apoptotic cells containing fragmented DNA. Percent apoptotic cells is determined by comparison to cells exposed to biotin-dUTP in the absence of DNA polymerase. The upper panel displays cephalic chondrocytes without ascorbate; lower panel displays cephalic chondrocytes cultured 24 h with ascorbate. **B:** 7-AADand Hoechst 33258 staining. 7-AAD will only enter cells which have lost membrane integrity, while Hoechst dye will enter all cells. Therefore, the upper right quadrant includes necrotic cells which have high levels of both 7-AAD and Hoechst staining, while the lower right quadrant contains apoptosic cells with high levels of Hoechst staining but little 7-AAD. The upper panel displays results with control cephalic chondrocytes, and the lower panel displays results with ascorbate-treated cephalic chondrocytes.

thrive without serum, but that serum may contain factors which restrict chondrocyte proliferation or entry into the cell cycle. Alini et al. (1996) have previously suggested that serum also contains factors which inhibit chondrocyte hypertrophy.

Based on the population doubling data in Table 1, it can be calculated that caudal sternal chondrocytes in serum-free culture double their number every 40 h with ascorbate, and every 33 h with B+A. These numbers are consistent with estimates for in vivo cell cycle times of 30–50 h for chondrocytes in the proliferative zone of rat growth plate labelled with BrdU (Wilsman et al., 1996). The data for cephalic sternal chondrocytes indicate a doubling time of approximately 75 h with ascorbate and 90 h with B+A from day 1 to day 3, but doubling times of over 300 h for B+A from days 2–4. By

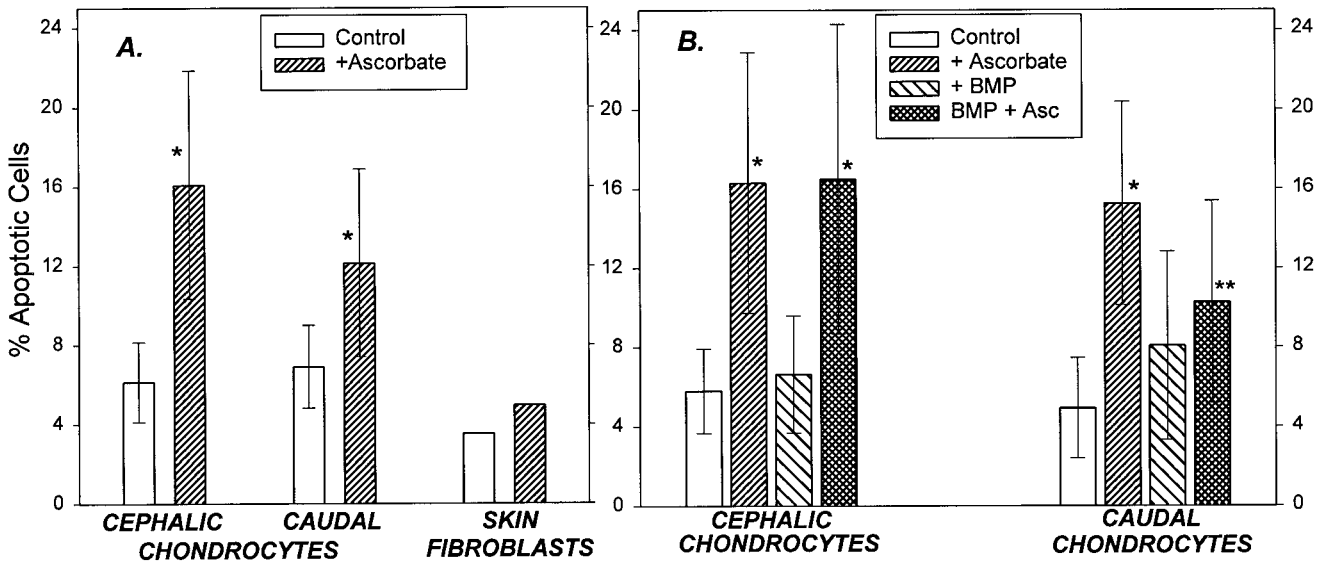

Fig. 7. Proportion of apoptotic cells seen in NuSerum (A) or serum-free medium (B). **A:** Cells cultured for 24 h in NuSerum with or without ascorbate. Chondrocyte values are the average ± S.D. for three independent experiments with flow cytometry done on duplicate samples. Fibroblast values are from one experiment. **B:** Cells cultured for 24 h in serum-free medium with no additions (control), 56 μM ascorbic acid, 30 ng/ml BMP-2 or both BMP and ascorbic acid. Values are the average ± S.D. of six independent experiments, three assayed by the TUNEL procedure and three assayed after 7-AAD/Hoechst staining. Probabilities were calculated using paired t statistics. *Different from control at $P < 0.05$. **Different from ascorbate-treated at $P < 0.05$.

day 2, cephalic sternal chondrocytes cultured with B+A are differentiating into hypertrophic chondrocytes with high expression of type X collagen and alkaline phosphatase; in contrast, caudal chondrocytes cultured for up to 1 week with B+A show no type X collagen or alkaline phosphatase expression (Leboy et al., 1997). It is therefore not surprising that B+A treatment of caudal chondrocytes permits the high proliferation rate characteristic of ascorbate, while the same treatment of cephalic chondrocytes leads to diminished cell proliferation associated with differentiation.

Population doubling data (Table 1) suggest that caudal chondrocytes with ascorbate double their cell number about twice as fast as those without ascorbate. However, ascorbate increases thymidine incorporation into DNA 4–6×; thus, it appears that not all DNA synthesis is leading to increased cell numbers. A similar but smaller discrepancy exists when DNA synthesis and population doubling is compared with cephalic sternal chondrocytes; ascorbate increases thymidine incorporation 3.5–4×, but increases cell numbers slightly less than 3×. Estimates relying on BrdU labelling of cephalic chondrocytes exposed to ascorbate for 20 h indicated that 23% of the cells were synthesizing DNA during a 4-h labelling period (Fig. 2). Extrapolating from these data, it might be expected that the cell population would double in <24 h, rather than the approximately 75 h found. One plausible explanation for the discrepancy is that serum deprivation synchronized the cell population, and labelling occurred during a period in which a high proportion of cells were entering S phase. However, experiments in which labelling times were varied indicated that high labelling rates were not caused by cell synchronization, and suggested that apoptosis was occurring in the presence of ascorbate.

Increased apoptosis was observed with both serum-free and NuSerum-containing cultures of ascorbate-treated chondrocytes (Figs. 6 and 7), but not with ascorbate-treated skin fibroblasts (Fig. 7A). It is noteworthy that, even without ascorbate, both types of chondrocytes showed relatively high levels of apoptosis (4–8%) with NuSerum as well as with serum-free culture. The combination of B+A, which leads to rapid hypertrophy with cephalic sternal chondrocytes (Venezian and Leboy, 1995; Leboy et al., 1997), produces apoptosis levels in cephalic chondrocytes comparable to those seen with ascorbate alone. This is consistent with recent findings that hypertrophic chondrocytes undergo apoptosis (Gibson et al., 1995; Hatori et al., 1995; Roach and Erenpreisa, 1996; Amling et al., 1997). Caudal sternal chondrocytes, which do not undergo hypertrophy after B+A treatment (Leboy et al., 1997), show diminished apoptosis compared to treatment with ascorbate alone (Fig. 7B). Thus, BMP appears to have a protective effect in the nonhypertrophic chondrocytes. These results also suggest that, although BMP does not induce hypertrophy in caudal chondrocytes, the cells have some functional BMP receptors.

Ascorbate-induced apoptosis has been reported for several cell types including many tumor cell lines (Alcaín and Burón, 1994; Sakagami et al., 1996) and human neuroectodermal cells (De Laurenzi et al., 1995). The combination of increased cell proliferation and increased cell death seen with ascorbate-treated chondrocytes has also been reported for tumor cells (Alcaín and Burón, 1994; Brigelius-Flohe and Flohe, 1996). Unlike chondrocytes, tumor cells would not be

TABLE 2. Comparative effects of ascorbate and ascorbate + BMP on DNA synthesis and apoptosis in sternal chondrocytes

		Effect relative to control = 1.0	
	Assay	Ascorbate	BMP + ascorbate
Cephalic chondrocytes	thymidine (n = 8)	3.74 ± 1.63	1.12 ± 0.50
	apoptosis (n = 8)	2.68 ± 0.64	2.45 ± 1.11
Caudal chondrocytes	thymidine (n = 3)	4.83 ± 1.69	5.92 ± 0.69
	apoptosis (n = 7)	3.34 ± 1.65	1.74 ± 0.86

expected to elaborate a collagen-rich matrix; thus, ascorbate-induced changes in cell-matrix interactions would be unlikely. Preliminary studies with 3,4-dehydroproline, which inhibits collagen hydroxylation and secretion, indicate that ascorbate continues to stimulate chondrocyte proliferation under conditions in which collagen secretion is inhibited. Thus, it is unlikely that matrix production is the major factor in ascorbate regulation of chondrocyte growth.

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