Original Article

Hypertrophy is not a prerequisite for type X collagen expression or mineralization of chondrocytes derived from cultured chick mandibular ectomesenchyme

SUNETRA EKANAYAKE* and BRIAN K. HALL

Department of Biology, Life Sciences Centre, Dalhousie University, Halifax, Nova Scotia, Canada

ABSTRACT Meckel's cartilage in the avian mandible is a neural crest-derived permanent cartilage. To investigate whether chondrocytes that form Meckel's cartilage can be induced to undergo maturation and mineralization by manipulating the environment, we used *in vitro* micromass culture in which young embryonic mandibular ectomesenchymal cells were maintained at a high cell density ($2x10^7$ cells/ml) and treated with ascorbic acid (AA) or with dexamethasone and ascorbic acid (DEX+AA). Chondrogenesis and chondrocyte maturation were analyzed by histological, immunohistochemical and SDS/PAGE techniques. Chick mandibular ectomesenchymal cells from Hamburger and Hamilton (*J. Morphol. 88:* 49-92, 1951) stage 21 (HH stage 21) chick embryos undergo chondrogenesis forming cartilage nodules when maintained under micromass culture conditions. These chondrocytes undergo maturation in response to AA but not DEX. Addition of AA to culture medium induced type X collagen expression by these chondrocytes. With prolonged culture, chondrocytes began to mineralize turning cartilage nodules into completely mineralized structures. There was no correlation between chondrocyte size and type X collagen expression. Small chondrocytes as well as large (hypertrophic) chondrocytes expressed type X collagen and then underwent mineralization. Co-treatment of cultures with DEX+AA caused reduction of chondrogenesis and inhibited chondrocyte maturation and mineralization seen with AA alone.

KEY WORDS: *collagen X, mineralization, Meckel's cartilage, ascorbic acid, dexamethasone*

Introduction

Chick mandible provides an ideal model for understanding mechanisms of chondrogenesis and osteogenesis of ectomesenchymal cells. Mandibular ectomesenchymal cells which are derived from the neural crest (i.e. are ectodermal in origin) give rise to a rod-like cartilaginous structure known as Meckel's cartilage and several membrane bones surrounding it. Most cartilages that appear during embryonic development undergo endochondral ossification and are replaced by bone. Only a few remain as permanent cartilages.

The main body of Meckel's cartilage in birds does not undergo endochondral ossification, but remains as a permanent cartilage (Romanoff, 1960). The main body of Meckel's cartilage in mammals studied to date also does not undergo endochondral ossification, but becomes the sphenomandibular ligament (Bhaskar *et al.*, 1953; Richany *et al.*, 1956). The mechanisms directing chondrocytes towards a certain fate, whether to remain permanently cartilaginous or to undergo endochondral ossification, are not fully understood. Such mechanisms could be intrinsic to particular cartilages or regulated by the microenvironment (Solursh *et al.*, 1986; Castagnola *et al.*, 1987).

Although much work has been done to understand endochondral ossification in mesodermally-derived cartilages such as growth plates in long bones and the sternum, endochondral ossification of ectodermally-derived cartilages has not been well studied. In growth plate cartilage during endochondral ossification, resting chondrocytes proliferate and then mature into hypertrophic chondrocytes which are by definition, much larger in size than chondrocytes. Maturing and hypertrophic chondrocytes re-organize the extracellular matrix and facilitate subsequent mineralization by synthesizing high levels of alkaline phosphatase and by secreting type X collagen while decreasing secretion of type II collagen (Capasso *et al.*, 1982; Schmid and Conrad, 1982; Chen *et al.*, 1992; Valle *et al.*, 1993). During *in vivo* development, either mineralized cartilage degenerates and is replaced by osteoblasts brought in by the circulation (Poole, 1991), or cells from mineralized

Abbreviations used in this paper: AA, ascorbic acid; ECM, extracellular matrix; DEX, dexamethasone; EDTA, ethylene diamine tetraacetic acid; ßGP, ß-glycerophosphate; FITC, fluorescein isothiocyanate; NBF, neutral buffered formaline; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; SDS, sodium dodecyle sulfate; T1, treatment 1: cultures treated with AA+ßGP; T2, treatment 2: cultures treated with DEX+AA+ßGP.

***Address for reprints:** Department of Biology, Life Sciences Centre, Dalhousie University, Halifax, Nova Scotia B3H 4J1, Canada. FAX: 902.494-3736.

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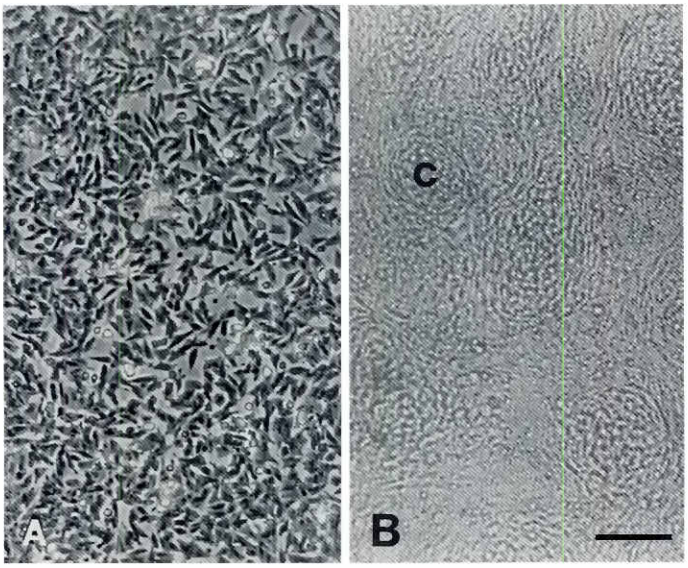


Fig. 1. Photomicrographs of live micromass cultures of mandibular ectomesenchymal cells showing initial stages of chondrogenic differentiation. (A) *The central area of a micromass, 1 h after inoculation. Cells were inoculated at a high cell density (2x10^7 cells/ml), as 10 μl drops on to the bottom of wells in 24-well tissue culture plates. Note that within 1 h, cells have adhered to tissue culture plastic and appear fibroblastic in morphology.* **(B)** *Signs of initiation of chondrogenesis in a 2 day-old control culture. Chondrogenic foci (c) appear as swirls of cells. At day 2, there were no recognizable differences between control cultures and either of the treated (T1 and T2) cultures. Bar, 92 μm for both figures.*

cartilage are released from lacunae, become osteoblasts and secrete bone matrix (Richman and Diewert, 1988; Yoshioka and Yagi, 1988). Since type X collagen is restricted to hypertrophic cartilage *in vivo*, secretion of type X collagen has been used for the past decade as a marker for chondrocyte hypertrophy both *in vivo* and *in vitro* (Gibson and Flint, 1985; Schmid and Linsenmayer, 1985a,b; Poole and Pidoux, 1989).

Previous studies have shown that chondrocytes from normally-hypertrophic cartilage produce type X collagen when maintained *in vitro*, but that chondrocytes from permanent cartilages do not (Gibson *et al.*, 1984; Reginato *et al.*, 1986; Gerstenfeld *et al.*, 1989). However, chondrocyte hypertrophy can be manipulated by changing environmental conditions. A number of environmental factors that enhance *in vitro* expression of type X collagen by chondrocytes from normally-hypertrophic cartilage have been described by several groups. These include culture of chondrocytes in suspension (Castagnola *et al.*, 1986; Adams *et al.*, 1989), in a three dimensional matrix such as hydrated collagen gels (Solursh *et al.*, 1986) or agarose (Iwamoto *et al.*, 1989; Hunter *et al.*, 1993), exogenous addition of ascorbic acid (Hall, 1981a; Leboy *et al.*, 1989; Wu *et al.*, 1989; Gerstenfeld and Landis, 1991), retinoic acid

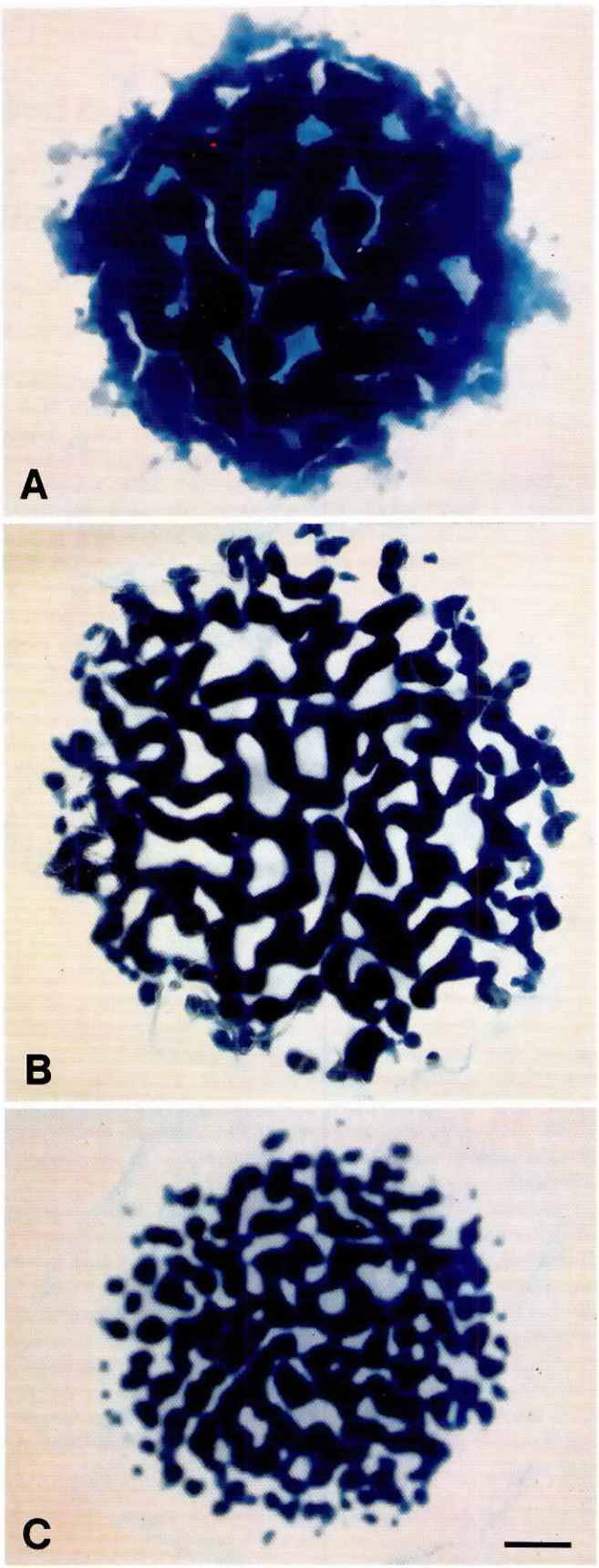


Fig. 2. The central region of 6-day-old micromass cultures stained with alcian blue at pH 1.0 to distinguish cartilage nodules. (A,B,C) *Control, T1 and T2 cultures respectively. Note that the cartilage nodules in the control culture are larger but fewer in number than nodules in T1 and T2 cultures. The diameter of the central cartilaginous region is larger in T1 than in control and T2 cultures. Bar, 0.4 mm for all the figures.*

TABLE 1

THE EFFECT OF AA AND DEX ON CHONDROGENESIS OF ECTOMESENCHYMAL CELLS IN MICROMASS CULTURE

	Control	T1 *(AA+ßGP)*	T2 *(DEX+AA+ßGP)*
Mean # nodules per mass	47 ± 3.2	160 ± 3.8	127 ± 8.0
Mean diameter of the cartilaginous area (mm)	3.20 ±0.06	3.80 ±0.09	2.86 ± 0.09

Five masses from each of the culture groups were analyzed in calculating the mean number of nodules per micromass and the average diameter of the cartilaginous area in the micromass (±values are standard deviation). One-way ANOVA ($P<0.0001$, $F= 569.41$ with 2, 12 DF) followed by the Tukey multiple Range test at $\alpha= 0.05$ indicated that the mean numbers of nodules per mass for each of the three culture groups were significantly different. Similarly, one-way ANOVA ($P<0.0001$, $F= 160.74$ with 2, 12 DF) followed by the Tukey multiple Range test at $\alpha= 0.05$ indicated that the mean diameter of the cartilaginous area for each of the three culture groups were also significantly different. Furthermore, semi-quantitative data on cartilage nodule size (data not shown) indicated that the control cultures produced larger nodules while T2 cultures produced smaller nodules than T1 cultures (see also Fig. 2A-C).

(Oettinger and Pacifici, 1990; Pacifici *et al.*, 1991b), thyroxin, insulin or serum (Bruckner *et al.*, 1989; Bohme *et al.*, 1992; Quarto *et al.*, 1992).

Interestingly, similar *in vitro* environmental conditions induce type X collagen expression in chondrocytes from permanent articular cartilage (Iwamoto *et al.*, 1989; Pacifici *et al.*, 1991a) and also in chondrocytes from the caudal portion of the sternum which normally do not undergo hypertrophy until much later in development (Solursh *et al.*, 1986; Castagnola *et al.*, 1987; Pacifici *et al.*, 1991b).

Most studies on mechanisms that determine the fate of chondrocytes have used mesodermally-derived cartilages. Only a few have used ectodermally-derived cartilage cells. A study by Richman and Diewert (1988) indicated that the main body of Meckel's cartilages of rats maintained in ocular culture transformed into fibroblasts mimicking the *in vivo* situation. However, another study indicates that mesenchymal cells from maxilla and mandible of the chick can be induced to produce type X collagen by culturing in collagen gels (Solursh *et al.*, 1986).

Ascorbic acid (vitamin C), a factor that promotes collagen X expression in mesodermally-derived chondrocytes has long been known to be involved with endochondral ossification and with collagen biosynthesis in other skeletal tissues *in vivo* (Schiltz *et al.*, 1977). Corticosteroids also have been shown to induce precocious accumulation of calcium and matrix vesicles formation in young condylar cartilage cells (Lewinson and Silbermann, 1984) and to stimulate expression of the osteoblastic phenotype in periosteal or bone marrow cells (Tenenbaum and Heersche, 1985; McCulloch and Tenenbaum, 1986; Bhargava *et al.*, 1988).

In the present study, we further extend current knowledge on chondrocyte maturation and mineralization to cells derived from the neural crest. We do this by examining whether the permanently cartilaginous cells of Meckel's cartilage of the chick can be induced to mature *in vitro* by addition of either ascorbic acid (AA) or dexamethasone and ascorbic acid (DEX+AA) and if so, whether in a pattern typical of the *in vivo* hypertrophy and mineralization seen in other cartilages. Cultures were supplemented with ß-glycerophosphate (ßGP) to facilitate mineralization. Analyses were done using histological, immunohistochemical and biochemical methods.

Our results indicate that cartilage nodules formed in micromass cultures of mandibular ectomesenchymal cells can be induced to undergo maturation and mineralization following manipulation of their environment. AA and ßGP induced type X collagen expression, which was followed by mineralization. However, type X collagen expression and chondrocyte hypertrophy occurred independently. Dexamethasone decreased chondrogenesis and inhibited AA-induced chondrocyte maturation and mineralization.

Results

The effect of AA and DEX on chondrogenesis and chondrocyte maturation in cultures of mandibular ectomesenchymal cells from HH stage 21 chick embryos were examined under long-term (up to 21 days) micromass culture conditions. Each of the three groups of cultures; control, T1 (treated with AA+ßGP) and T2 (treated with DEX+AA+ßGP) contained 24 micromasses plated as 1 micromass per well in a 24-well tissue culture plate. Similar results were obtained in three replicate experiments.

During the first 3 days of culture, there were no distinguishable differences in organization of micromasses in control and treatment groups. Within one hour of incubation, cells adhered to the culture plate and appeared fibroblastic (Fig. 1A). Cell density was much higher in the center of each micromass than at the periphery. At day 2, swirl-like prechondrogenic cell condensations were apparent in the center of the micromasses of all three culture

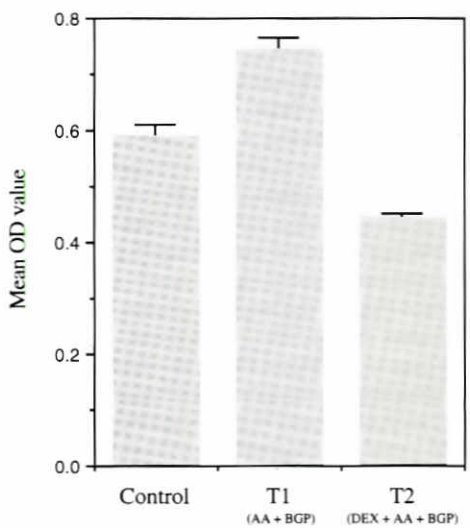


Fig. 3. The effect of AA and DEX on the production of cartilage matrix by mandibular ectomesenchymal cells undergoing chondrogenesis. *Six-day-old micromass cultures were stained with alcian blue at pH 1.0. Alcian blue bound to cartilage matrix in each micromass was extracted in guanidine hydrochloride and quantified by spectrophotometry. OD value at 650 nm for individual micromass was measured against a guanidine hydrochloride blank and the mean OD value of each treatment group was compared with that of control when determining significance (n= 6-8 per culture group). The amount of alcian blue bound to cartilage matrix was significantly higher in T1 cultures (treated with AA in the absence of DEX) and less in T2 cultures (treated with DEX+AA) than control cultures ($P<0.001$ and 0.002 respectively). Bar represents standard error.*

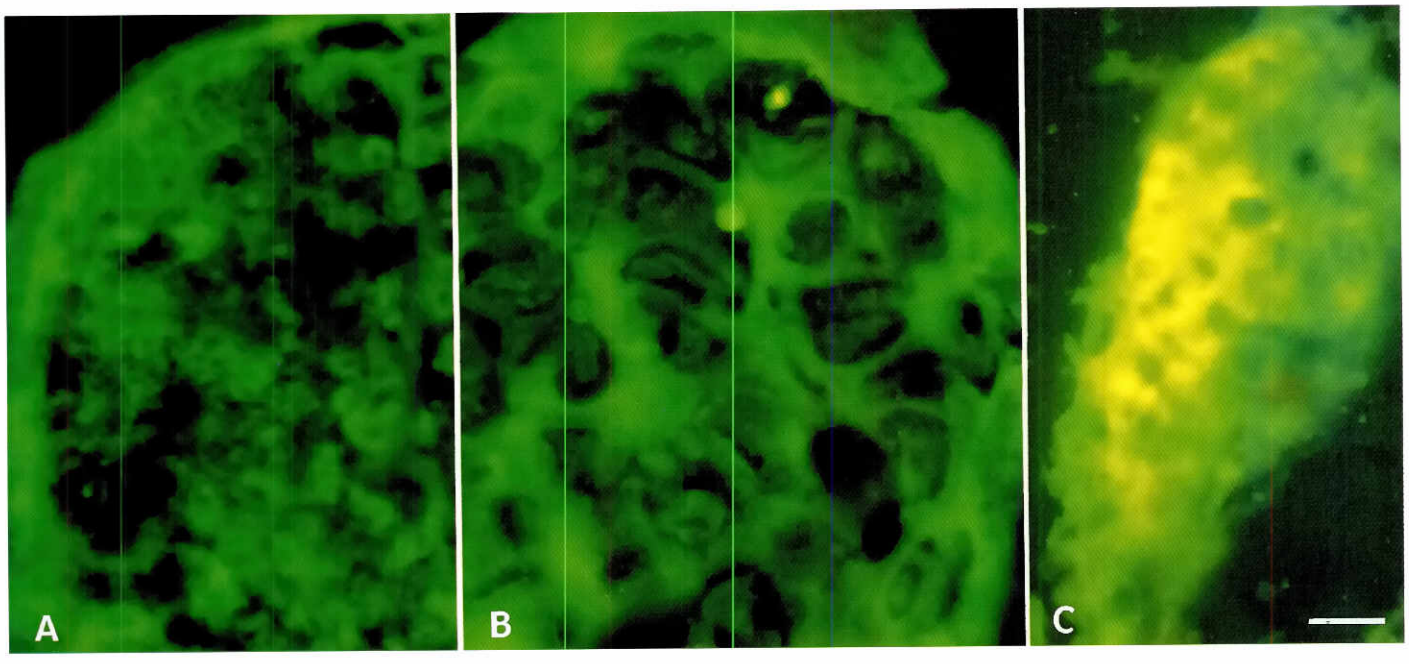


Fig. 4. Expression of collagen II by cells undergoing chondrogenesis. *Cryosections of central region of 8-day-old micromass cultures stained with anti-chicken type II collagen monoclonal antibody followed by fluorescein isothiocyanate (FITC) conjugated secondary antibody. All culture groups stained positively for type II collagen. Positive staining is uniformly distributed in the extracellular matrix.* **(A,B,C)** *Control, T1 and T2 cultures respectively. Bar, 19.5 μm for all the figures.*

groups (Fig. 1B). By day 3, cells in these chondrogenic foci appeared round or polygonal and had extracellular matrix around them that stained positively with alcian blue at pH 1.0. These cell condensations gradually developed into cartilage nodules. Cells at the periphery of the micromass appeared fibroblastic.

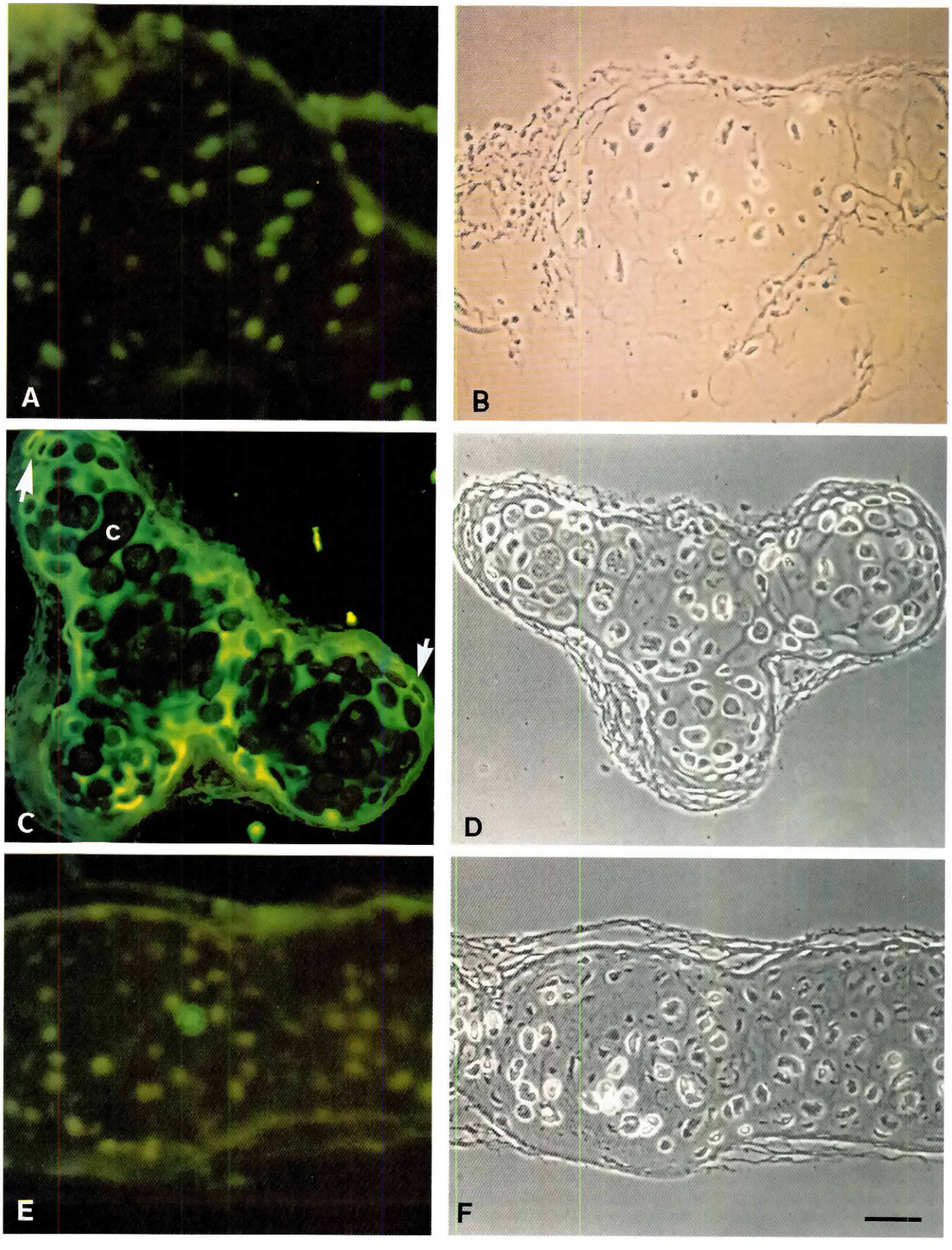
However, beginning at day 4 or 5, there were distinguishable differences in the amount of chondrogenesis between the three groups. The central cartilaginous area was larger in T1 and smaller in T2 than in control cultures (Fig. 2A-C, Table 1). The number of cartilage nodules per mass at day 6 was consistent within each treatment group but varied significantly between treatment groups (Table 1). Control cultures contained the lowest, while T1 cultures contained the highest number of cartilage nodules. The size of cartilage nodules varied within each mass. However, on average, control cultures had much larger and more spread widely nodules than the two treatment groups (Fig. 2A-C). Therefore, the three dimensionality of cartilage nodules was less discrete in control cultures than in cultures of the two treatment groups. In all cultures regardless of treatment, some adjacent nodules coalesced to form larger nodules. Such coalescence was greater in control cultures than in the two treatment groups. Due to variation in the size and degree of coalescence, a number of cartilage nodules could not be used as a quantitative measure of chondrogenesis. Therefore, spectrophotometric analysis of matrix-bound alcian blue (Lev and Spicer, 1964) was used to quantify the amount of cartilage matrix produced by micromass cultures in the different treatment groups. There were significant differences in the amount of cartilage matrix produced by micromasses in control and different treatment groups. T1 cultures produced significantly more cartilage matrix while T2 cultures produced significantly less than control cultures (Fig. 3).

These results indicate that mandibular ectomesenchymal cells can undergo chondrogenesis and form cartilage nodules even in the absence of AA, but in the presence of AA, chondrogenesis is significantly enhanced. When AA treated cultures were co-treated with DEX, chondrogenesis was reduced to a level significantly below even control cultures.

Expression of collagens

Immunohistochemical visualization of type II collagen demonstrated the presence of this cartilage-specific collagen in the matrix of chondrocytes in all three groups of cultures (Fig. 4A-C). Type II collagen appeared to be uniformly distributed in the extracellular matrix.

Fig. 5. Expression of type X collagen by cultures at day 10. *Wax sections of micromass cultures were stained with monoclonal antibody for chicken type X collagen followed by a secondary antibody conjugated with FITC.* **(A)** *Fluorescent micrograph of a cartilage nodule in control culture. Note the absence of positive antibody staining.* **(B)** *A phase contrast image of the same specimen.* **(C)** *A fluorescent micrograph of a T1 culture. This cartilage nodule was formed by the coalescence of 3 adjacent nodules. Note the presence of positive antibody staining in the pericellular matrix of chondrocytes (arrow heads) at the periphery of the coalesced nodules. More centrally located chondrocytes (c) are larger in size but do not express type X collagen at day 10.* **(D)** *A phase contrast image of the specimen shown in* **C**. **(E)** *A fluorescent micrograph of a cartilage nodule in a T2 culture. There is no positive antibody staining in the matrix.* **(F)** *A phase contrast image of the specimen shown in* **E**. *Bar, 33 μm for A, 40 μm for B, 39 μm for C and D, 38 μm for E and 45 μm for F.*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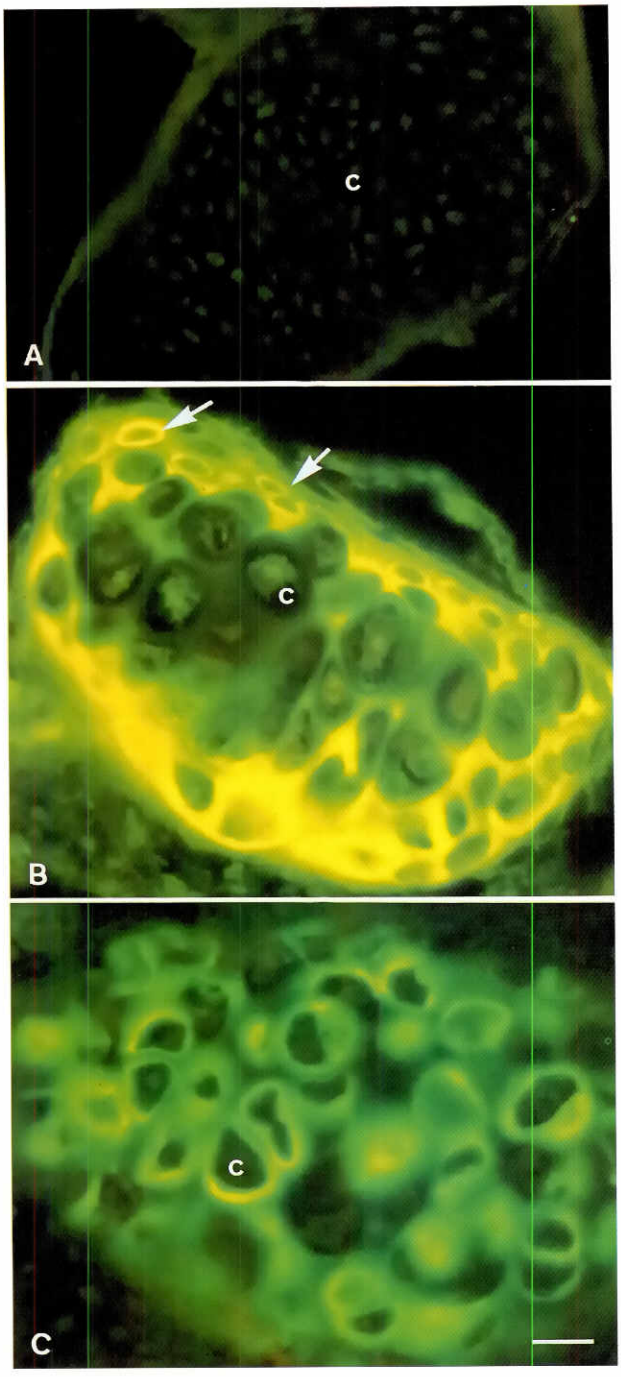

Fig. 6. Immunofluorescent localization of type X collagen expression by T1 cultures at different times in culture. (A) *A cartilage nodule in an 8-day-old culture. There is no positive antibody staining in the extracellular matrix of chondrocytes (c).* **(B)** *A high magnification fluorescent micrograph of a cartilage nodule in a 10-day-old culture. At this stage type X collagen is expressed by those chondrocytes at the periphery of cartilage nodules regardless of cell size. Type X collagen is mainly located in the pericellular matrix (arrow). More centrally located chondrocytes (c) are much larger and appear hypertrophic but do not express type X collagen.* **(C)** *A high magnification fluorescent micrograph of a cartilage nodule in a 15-day-old culture. At this stage both peripheral and central chondrocytes have type X collagen in pericellular matrix. Bar, 31 μm for A and 20 μm for B and C.*

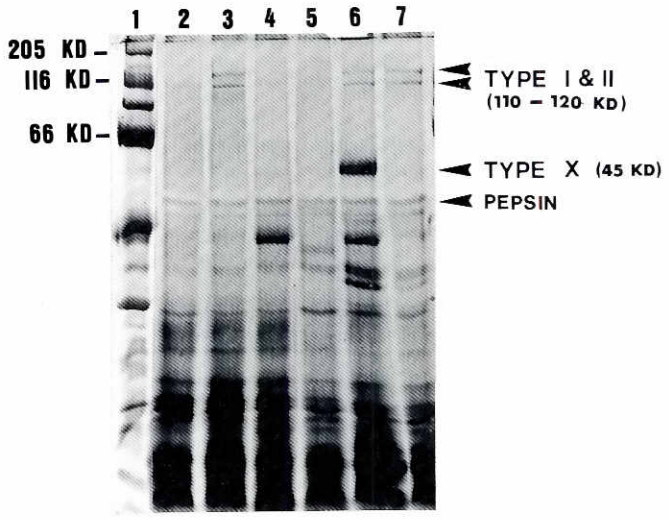

Fig. 7. SDS/PAGE of collagens extracted by pepsin digestion of control (lanes 2,5), T1 (lanes 3,6) and T2 (lanes 4, 7) cultures. *Collagens were extracted from cultures at two different time intervals; after 8 days of culture* **(lanes 2,3,4)** *and after 13 days of culture* **(lanes 5,6,7)**. *Samples were analyzed in a 8% gel and stained with Coomassie blue followed by silver stain. Note that type X collagen [α_1(X)], a 45 kDa protein, is present only in lane 5, T1 cultures, cultured for 13 days. Type I and II collagens (110-120 kDa) are also present in varying amounts in all the samples.* **Lane 1** *contains molecular weight markers.*

Type X collagen was absent in control and T2 cultures throughout the culture period. However, type X collagen was observed in T1 cultures, beginning at day 10 of culture (Figs. 5A-F, 6A,B, 10). Type X collagen was located pericellularly, as a brightly fluorescing layer just outside the cell membrane. Initially, type X collagen was present mostly around cells at the periphery of each cartilage nodule regardless of cell size. Although chondrocytes occupying the center of each nodule appeared much larger, they did not express type X collagen at day 10 (Figs. 5C, 6B). In older cultures, collagen X was present throughout the nodule (Fig. 6C). This pattern of type X collagen expression was seen in three replicate experiments and indicated a lack of correlation between cell size and expression of type X collagen, suggesting that type X collagen expression and chondrocyte hypertrophy may be independent processes.

Immunohistochemical observations were confirmed by analysis of cell cultures and the respective culture medium by SDS/PAGE (Fig. 7). Cell cultures at two different ages (culture days 8 and 13) were analyzed. At day 8, collagen type X (Mr= 45 kDa) was absent from all the cultures. Collagens with high molecular weights (Mr= 110-120 kDa) were present. These may represent chains of collagen I (from peripheral fibroblasts) and II (from chondrocytes). However, at day 13, type X collagen was present in T1 cultures, but not in either control or T2 culture groups. Interestingly, T2 cultures were given DEX in addition to AA and ßGP. As observed by both immunohistochemical and SDS/PAGE analysis, co-treatment with DEX abolished the AA and ßGP-induced expression of type X collagen.

Mineralization of cultures

Mineralization was absent in both control and T2 cultures during the whole culture period of 21 days. However, in T1 cultures,

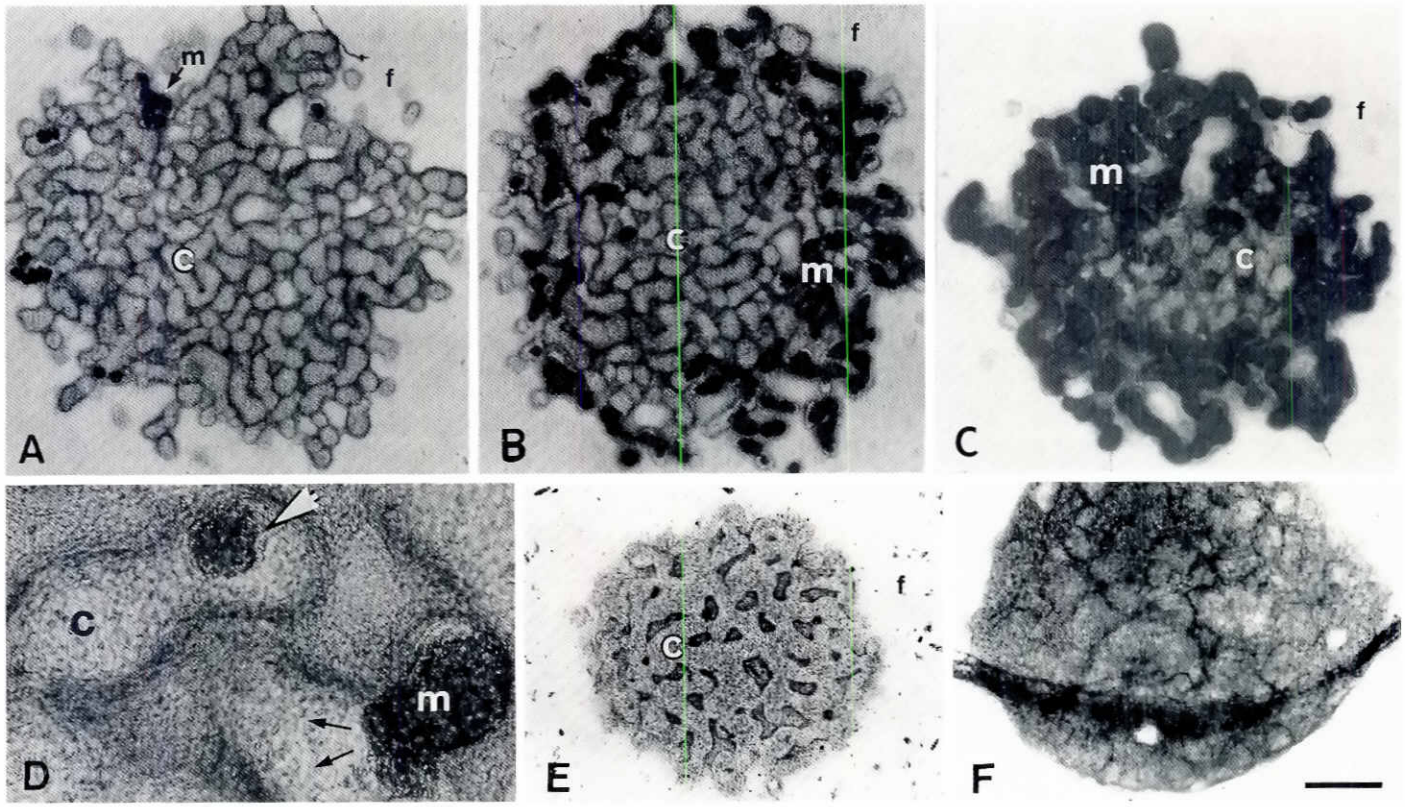

Fig. 8. Photomicrographs of live micromass cultures showing the presence or absence of mineralization in cartilage nodules. *Only the central cartilaginous region of each micromass is shown.* **(A)** *A T1 culture showing initiation of mineralization (m) at day 12 of culture. Note that only a few cartilage nodules located at the periphery of the cartilaginous mass have begun mineralization. The rest is unmineralized (c).* **(B)** *A 14-day-old T1 culture showing the progression of mineralization (m) in cartilage nodules. Cartilage nodules (c) located at the center of the micromass are unmineralized.* **(C)** *A 20-day-old T1 culture showing heavy mineralization (m). Only a few cartilage nodules (c) at the center are unmineralized.* **(D)** *A high magnification photomicrograph of a specimen similar to* **A** *above showing a nodule initiating mineralization (large arrow) and a mineralizing nodule (m) at a more advanced stage (two small arrows indicate the direction of spreading of mineralization). Mineralization is initiated at a focal point and spread throughout the nodule.* **(E)** *A 20-day-old T2 culture. All the cartilage nodules (c) are unmineralized. Note that the cartilaginous area is much smaller in* **E** *(culture treated with DEX+AA+ßGP) than in* **A,B** *and* **C** *(cultures treated with AA+ßGP only).* **(F)** *A 20-day-old control culture. There is no evidence of mineralization. The micromass is disintegrating and has began to peel off from the culture dish. In all the cultures, the area surrounding the central cartilaginous region and the internodular space is occupied by fibroblasts (f). Bar, 90 μm for D and 0.65 mm for all the other figures.*

expression of type X collagen was followed by cartilage matrix mineralization (Fig. 8A-E). By day 12, signs of mineralization were apparent in live cultures as small black spots in some of the peripheral cartilage nodules (Fig. 8A,D). With further time in culture, mineralization gradually spread throughout these nodules and to other adjacent nodules in the micromass (Fig. 8B). By day 20, the whole cartilaginous area in these cultures was mineralized to near completion (Fig. 8C). Wax sections of these cultures stained by Von Kossa's silver deposition method, which blackens the mineralized matrix by deposition of silver, demonstrated the presence of mineralized cartilage matrix only in T1 cultures (Fig. 9A-E).

The summary of histological, immunofluorescent and SDS/PAGE analysis of cultures is diagrammatically shown in Fig. 10 to illustrate the chronological order of events that occurred during chondrogenic maturation induced by AA and ßGP.

Even though type X collagen expression was followed by mineralization, it did not follow the pattern of type X collagen expression. Type X collagen was initially expressed by chondrocytes located at the periphery of each nodule, but mineralization was initiated by a group of chondrocytes at a particular focal point within the nodule (Figs. 8D, 9C; compare these with Figs. 5C, 6B) and then spread throughout the nodule (Fig. 9D,E).

The pattern of type X collagen expression and mineralization as revealed by immunohistochemical and histological analysis, as well as morphological observations of live cultures are collectively shown diagrammatically in Fig. 11. Not only did type X collagen not correlate with hypertrophy, its initial expression did not spatially correlate with initiation of mineralization either. On the other hand, cartilage matrix mineralization occurred only in those cultures expressing type X collagen.

Discussion

All the cartilages in the body except those in the craniofacial region are derived from embryonic mesoderm. Craniofacial cartilages are derived from the neural crest, an ectodermal structure. During development, most cartilages are replaced by bone in a process of endochondral ossification which involves maturation of chondrocytes leading to chondrocyte hypertrophy, mineralization

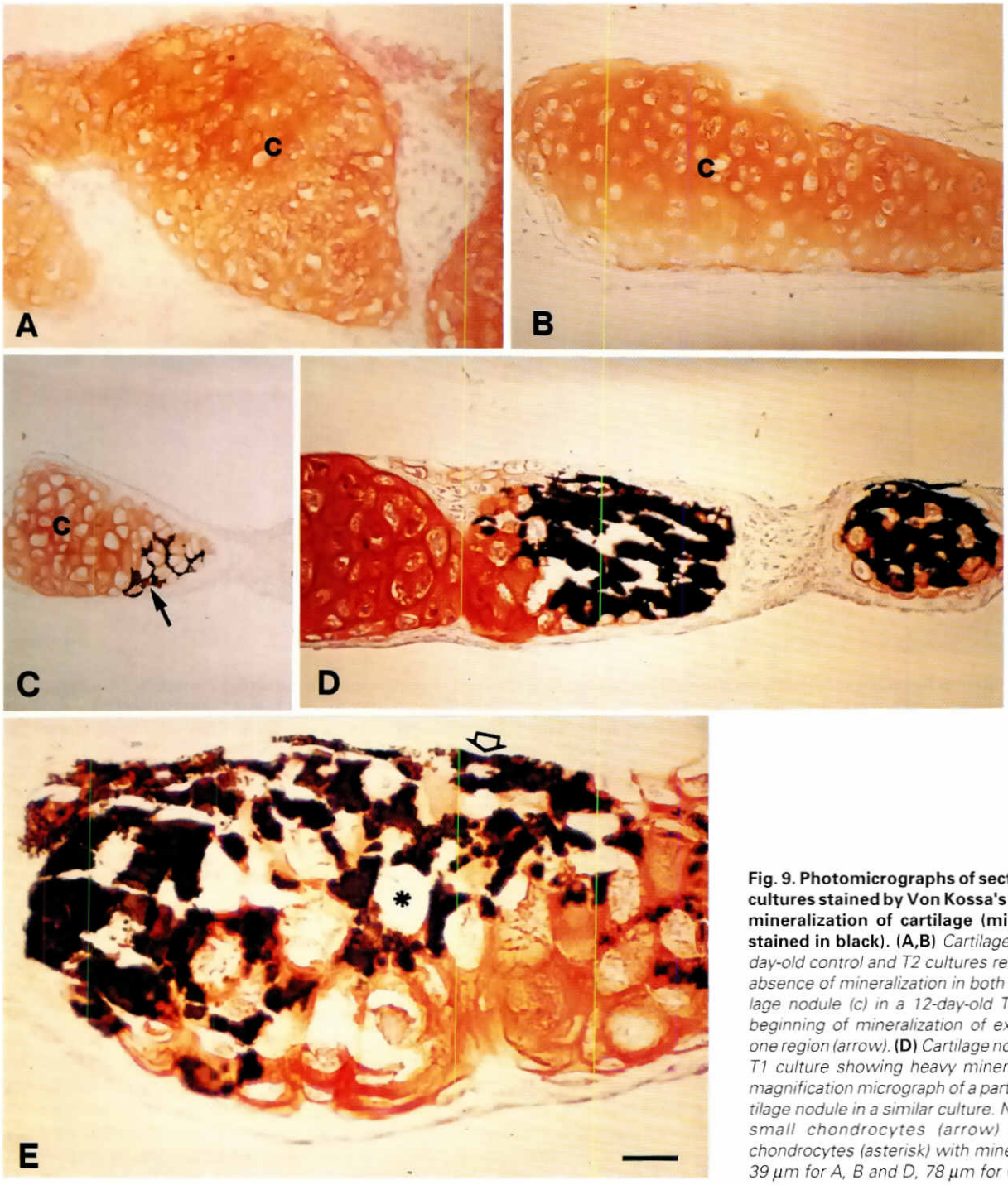

Fig. 9. Photomicrographs of sections of micromass cultures stained by Von Kossa's method to confirm mineralization of cartilage (mineral deposits are stained in black). (A,B) *Cartilage nodules (c) in a 14-day-old control and T2 cultures respectively. Note the absence of mineralization in both cultures.* **(C)** *A cartilage nodule (c) in a 12-day-old T1 culture. Note the beginning of mineralization of extracellular matrix in one region (arrow).* **(D)** *Cartilage nodules in a 14-day-old T1 culture showing heavy mineralization.* **(E)** *A high magnification micrograph of a partially mineralized cartilage nodule in a similar culture. Note the presence of small chondrocytes (arrow) as well as large chondrocytes (asterisk) with mineralized matrix . Bar, 39 μm for A, B and D, 78 μm for C and 16 μm for E.*

and replacement of cartilage by bone. In contrast, a few cartilages that appear during embryonic development maintain their chondrocytes at a resting stage and remain permanently cartilaginous throughout adult life.

Mechanisms that determine the fate of chondrocytes either to remain permanently cartilaginous or to undergo further maturation followed by endochondral ossification are not well understood. These mechanisms could be either intrinsic to particular cartilages or environmentally regulated (Solursh *et al.*, 1986; Castagnola *et al.*, 1987; Eavey *et al.*, 1988). In the present study we examined ectomesenchymal cells that give rise to Meckel's cartilage (permanently cartilaginous) of the chick for their ability to undergo chondrogenesis and further chondrocyte maturation under the influence of AA and DEX.

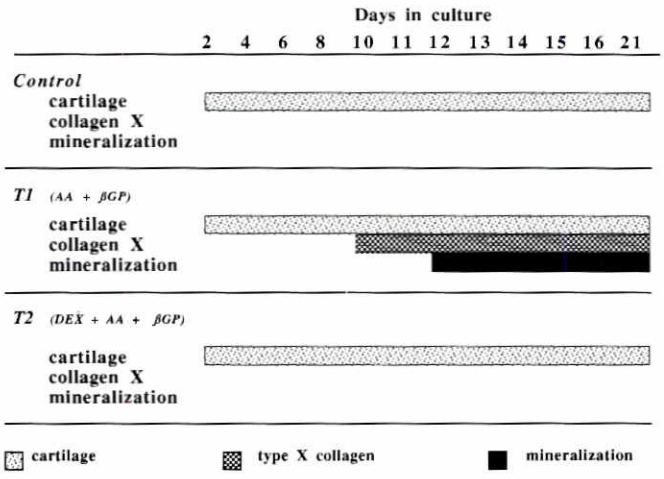

Fig. 10. The summary of histological, immunofluorescent and SDS/PAGE analyses of micromass cultures. *Cartilage (as determined by the presence of round cells with alcian blue-staining extracellular matrix and type II collagen) is present in all culture groups starting at day 2, but type X collagen and matrix mineralization are present only in T1, type X collagen appearing at day 10 and mineralization at day 12.*

Chondrogenic differentiation

Mandibular ectomesenchymal cells cultured as micromasses in control and treated media underwent chondrogenesis as evident by rounded cells secreting extracellular matrix which stained positively with alcian blue at low pH and which also contained type II collagen. In all three culture groups, cells undergoing chondrogenesis produced cartilage nodules. However, there were differences in the amount of chondrogenesis and the ability to undergo chondrocyte maturation depending on the treatment used.

The effect of AA

Ascorbic acid significantly enhanced chondrogenesis. Cultures treated with AA in the absence of DEX produced more cartilage nodules than control or DEX-treated cultures. Spectrophotometric analysis of sulfated proteoglycans in the cartilage matrix also indicated that these cultures were producing significantly higher levels of cartilage matrix than control or DEX-treated cultures.

With further time in culture, these cultures produced type X collagen as a pericellular matrix layer around chondrocytes. Type X collagen was not observed in control or DEX-treated cultures. During *in vivo* development, type X collagen is produced only by chondrocytes undergoing maturation leading to hypertrophy (Poole, 1991). Our results show that environmental conditions can induce maturation of chondrocytes that form Meckel's cartilage of the chick.

Ascorbic acid has been previously shown to enhance chondrogenesis and cartilage nodule formation (Levenson, 1969; Bounelis and Daniel, 1983). It also enhances hypertrophy of normally-hypertrophic chondrocytes from vertebrae (Gerstenfeld and Landis, 1991), sternum (Leboy *et al.*, 1989) and growth plate (Wu *et al.*, 1989) and induces hypertrophy of normally non-hypertrophic chondrocytes from articular permanent cartilage (Pacifici *et al.*, 1991a). Exact mechanisms of the action of AA are not known. However it is known that AA plays a role in collagen biosynthesis (Berg and Prockop, 1973; Olsen, 1981).

In our cultures, chondrocytes expressing type X collagen were initially located at the periphery of cartilage nodules, even though a large number of these cells were smaller in size than those chondrocytes located in the center of nodules. This feature was seen in all the cultures treated with AA in the absence of DEX. With further time in culture, chondrocytes in the center of cartilage nodules also expressed collagen X. Ascorbic acid in the culture medium may be more easily accessible to peripheral chondrocytes than central chondrocytes in the same nodule, so that they respond to AA earlier than more centrally located chondrocytes.

The size of chondrocytes did not appear to be a factor affecting expression of type X collagen. Pacifici *et al.* (1991b) also have indicated lack of recognizable size difference between type X collagen producing and non-producing chondrocytes. However, during *in vivo* endochondral ossification, chondrocyte size increases 5-10 fold as they become hypertrophic (Buckwalter *et al.*, 1986; Hunziker *et al.*, 1987). During this process chondrocytes begins to express type X collagen (Poole, 1991). Therefore, it is commonly accepted that type X collagen is a product of chondrocytes undergoing hypertrophy and it has been widely used as a marker for chondrocyte hypertrophy (Poole, 1991). Our results suggest that chondrocyte hypertrophy is not required for collagen X expression and that these can be two separate events.

Expression of collagen X in cultures treated with AA in the absence of DEX was followed by mineralization as evident by mineral deposits in live cultures and Von Kossa's staining (Page, 1982). Mineralization was first observed 2 days after the first appearance of type X collagen. ß-Glycerophosphate added to the culture medium may have facilitated cartilage mineralization. ß-Glycerophosphate has been previously shown to enhance mineralization only in chondrocytes undergoing maturation (Coe *et al.*, 1992).

The pattern of mineralization did not follow the pattern of initial expression of type X collagen in cartilage nodules. Type X collagen was initially expressed by peripheral chondrocytes in nodules.

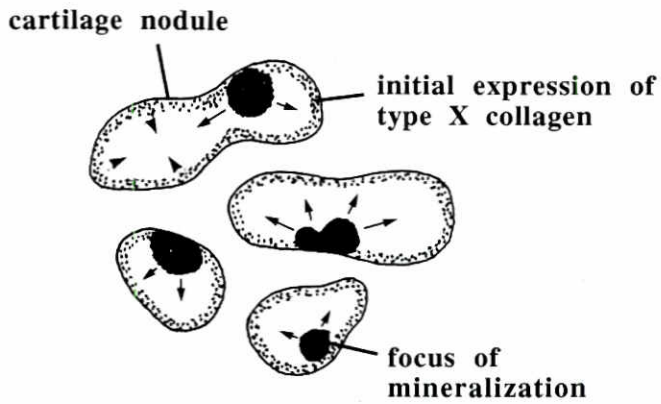

Fig. 11. A diagrammatic illustration of cartilage nodules in cultures treated with AA+ßGP in the absence of DEX to show that initiation of mineralization does not follow the pattern of type X collagen expression. *Type X collagen is initially expressed by chondrocytes located at the periphery of each nodule (dotted area) and then spreads to more centrally located chondrocytes (arrow heads). Mineralization initiates at a particular focus, and gradually spreads throughout the nodule as indicated by the arrows. Mineralization occurs only in cultures that express type X collagen, appearing 2 days after the initial expression of type X collagen.*

However, mineralization was initiated in a focal point and gradually spread throughout the nodule.

The role of type X collagen in cartilage is not known. It has been previously suggested that type X collagen may facilitate the removal of cartilage matrix during endochondral ossification (Schmid and Linsenmayer, 1987), play a role in calcification (Schmid and Linsenmayer, 1987) or function as a scaffold to prevent collapse of the cartilage matrix as proteoglycans and type II collagen are degraded (Upholt and Olsen, 1991). In our study, because 1) only those cultures expressing type X collagen undergo mineralization, and 2) type X collagen expression is followed by mineralization, type X collagen may be involved in some aspect of mineralization.

The effect of DEX

Unlike AA, DEX did not enhance chondrogenesis. When cultures were co-treated with AA and DEX, chondrogenesis was significantly reduced to a level below that of control cultures and cultures treated with AA alone as evident by alcian blue spectrophotometry. Chondrogenesis-inhibitory (Barrett *et al.*, 1966; Tessler and Salmon, 1975; Silbermann *et al.*, 1987) as well as promoting actions (Bellows *et al.*, 1989; Grigoriadis *et al.*, 1989) of glucocorticoids have been reported before. In addition, our cultures treated with DEX did not express type X collagen or matrix mineralization at any time during culture. Lewinson and Silbermann (1984) have previously reported a mineralization-promoting action of corticosteroids. Under the culture conditions used in the present study, DEX inhibited the chondrocyte maturation and mineralization induced by AA. Differing actions of glucocorticoids on chondrogenesis and mineralization no doubt have several explanations such as the complex nature of action of glucocorticoids, the influence of other molecules in the microenvironment, or the stage of development of particular cartilages.

Our results indicate that cartilage nodules formed in cultures of chick mandibular ectomesenchymal cells can be induced to undergo maturation and mineralization by manipulating their environment. Ascorbic acid and ßGP induced type X collagen expression followed by mineralization of cartilage matrix. Hypertrophy was not required for the expression of type X collagen and mineralization of chondrocytes. Dexamethasone decreased chondrogenesis and inhibited chondrocyte maturation and mineralization effect of AA.

Materials and Methods

Mandibular mesenchymal cells

Neural crest-derived ectomesenchyme in the chick mandible at HH stage 21 was used in the present study. At this stage, mandibular ectomesenchyme contains chondrogenic cells which give rise to future Meckel's cartilage (Ekanayake and Hall, 1994). Mandibular ectomesenchyme also gives rise to membrane bones at a later stage *in vivo*, but it requires an osteo-inductive epithelial-mesenchymal-type interaction with the adjacent mandibular epithelium. This interaction is not completed *in vivo* until HH stage 24 (Tyler and Hall, 1977; Hall, 1981b). Therefore, mandibular ectomesenchyme at HH stage 21 is a suitable model to study chondrocyte maturation and does not contain cells undergoing intramembranous ossification.

Mandibular processes (first visceral arches) were collected and treated with a mixture of trypsin and pancreatin (13:2 ratio) at a concentration of 1.5% in Tyrode's solution at 4°C for 1 h and 15 min to facilitate removal of the epithelia. The epithelium was manually removed from each mandibular process under a dissecting microscope. The remaining mesenchyme were pooled and vigorously mixed in a small volume of culture medium (400-600 µl) with a flame-drawn pasture pipette to prepare a single cell suspension.

Cell culture

Cell number in the suspension was counted using a hemacytometer. Cell density was adjusted to $2x10^7$ cells per ml and plated as 10 µl drops onto the bottoms of 24-well tissue culture plates (Falcon) for the formation of micromasses. Plates were incubated in a tissue culture incubator at 37°C for 1 h to allow cells to attach to the culture plate and then flooded with culture medium at 1 ml per well. The medium consisted of a mixture of Ham's F12 and BGJb (3:1 ratio) and 10% fetal bovine serum (all from Gibco).

Treatment with AA and DEX

Cultures were divided into three groups each containing 24 micromass cultures, one per well, in a 24-well tissue culture plate. One group was kept as the control, the second group (T1) was treated with AA (150 µg/ml) and the third group (T2) was treated with AA (150 µg/ml) and DEX (10^{-7}M). The two treatment groups were also given ßGP (10 mM) to facilitate mineralization (Tenenbaum and Heersche, 1982). Treatment with all reagents was started 24 h after initial plating and continued throughout culture. Media were changed daily. Cultures were examined regularly and photographed under an inverted microscope. Cells were kept up to 21 days in culture. The experiment was repeated twice.

Histology

Micromass cultures were fixed in neutral buffered formalin (NBF) and stained as whole-mounts with alcian blue at pH 1.0 overnight to distinguish cartilage nodules. Some formalin-fixed specimens were embedded in paraplast, sectioned at 6 µm and stained following Von Kossa's method (Page, 1982) to distinguish mineralized tissue.

Immunohistochemistry

Both cryosections and wax sections were used for immunohistochemical staining. For cryosectioning, unfixed cultures were quick-frozen in O.C.T. compound (Tissue-Tek 4583, Miles Scientific Division, Naperville, IL, USA) and sectioned (6 µm) in a cryostat. Sections were fixed in 100% acetone for 10 min, air dried and stored at -20°C until use. Acetone-fixed sections were mostly used for collagen II staining. For wax embedding, specimens were fixed in NBF at 4°C, dehydrated in ethanol at 4°C, embedded in X-tra (low melting point) paraplast and sectioned. Mineralized cultures were demineralized prior to wax embedding following Bourque *et al.* (1993). Briefly, specimens were fixed overnight at 4°C in a freshly prepared periodate-lysine-paraformaldehyde fixative (2% para-formaldehyde containing 0.075M lysine and 0.01M sodium periodate), washed in phosphate buffered saline (PBS), decalcified in a solution of ethylenediamine tetraacetic acid (EDTA, 0.5M) and glycerol (15%) at pH 7.3 for 4-6 days at 4°C, washed in PBS, dehydrated in ethanol, embedded in X-tra paraplast and sectioned. Wax sections were dewaxed in Histoclear and rehydrated in a series of ethanols prior to antibody staining.

Antibody staining

Cryosections and rehydrated wax sections were rinsed in PBS and digested for 30 min at 37°C with bovine testicular hyaluronidase (0.5 mg/ml in 10 mM potassium and sodium phosphate buffer; Jacenko and Tuan, 1987). They were then incubated with monoclonal antibodies to either chicken type II or X collagen raised in mice (donated by Dr. T. Linsenmayer, Tufts University, Boston, MA, USA) for 2 h at room temperature followed by fluorescein-conjugated rabbit antimouse IgG (Sigma) for 30 min at room temperature and mounted in Vectarsheild (Dimensions Lab.).

SDS/PAGE for collagens

Collagens were extracted from micromass cultures at two different stages; after 8 days of culture (4 days prior to the appearance of mineralization in cartilage nodules), and after 13 days of culture (one day after the first sign of mineralization). Cultures that were used for SDS/PAGE were given ß-aminopropionitrile fumarate (100 µg/ml, Sigma) during the last 24 h of culture to prevent cross-linking of collagen fibers. Medium was collected and frozen until use. Micromasses were scraped from the bottom of the plates, cut into small pieces in 100 µl of 0.5 M acetic acid, 0.2 M sodium chloride, and treated with pepsin (200 µg/ml, Worthington) in a

microcentrifuge vial at 4°C for 18 h. Samples were neutralized by addition of 5N sodium hydroxide and centrifuged briefly to remove insoluble materials. Supernatant was frozen until use. Collagens in culture medium were precipitated with ammonium sulfate (176 mg/ml) for 18 h at 4°C with gentle stirring. Precipitate was collected by centrifugation, resuspended in 0.5 N acetic acid and digested with pepsin (200 μg/ml) for 18 h at 4°C. These pepsin-digested samples were pooled with pepsin-digested extracts from respective cell cultures, dialyzed against 0.01% SDS, 2 mM EDTA and lyophilized.

Lyophilized samples were dissolved in sample buffer containing 10 mM Tris. hydrochloride (pH 8.0), 1 mM EDTA, 1% SDS, 10% glycerol and analyzed by SDS/PAGE (Laemmli, 1970; Sambrook *et al.*, 1989). The concentration of acrylamide was 8%. Protein bands were visualized by Coomassie brilliant blue R-250 followed by silver staining (Sigma).

Quantitative analysis of cartilage matrix production by alcian blue extraction

Micromass cultures in 24-well plates (6-day-old, 6-8 micromasses per treatment group) were fixed in Kahle's fixative for 30 min at 4°C, washed with 3% acetic acid at pH 1.0 and stained overnight with 1% alcian blue 8GX in 0.1% HCl at pH 1.0 (Lev and Spicer, 1964). Cultures were washed with 0.1 N HCl to remove unbound stain. Matrix bound alcian blue stain was extracted from each micromass culture with 300 μl of 4 M guanidine hydrochloride at 4°C for 16 h. 250 μl of extract from each culture was removed to a 96-well microtiter plate and the absorbance at 650 nm was read against a guanidine hydrochloride blank using a Dynatech MR5000 microplate reader.

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