Original Article

Sequential synthesis of cartilage and bone marker proteins during transdifferentiation of mouse Meckel's cartilage chondrocytes *in vitro*

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ABSTRACT Meckel's cartilage cells cultured *in vitro* undergo phenotypic transformation toward osteogenic cells. We examined whether these cells synthesize type X collagen and bone morphogenetic protein-2 (BMP-2). We also examined the results of Alcian blue staining and the expression of type I and type II collagen, osteocalcin and chondroitin sulfate proteoglycan (CSPG) during this transdifferentiation. Meckel's chondrocytes, isolated from day-17 mouse embryos, were inoculated at $1x10^4$/penicylinder and cultured in α-MEM for periods up to 4 weeks. Alcian blue staining and immunostaining of type II collagen and CSPG confirmed that, after cell culture for 2 weeks, the cartilaginous phenotype was expressed most intensely. Later in culture, chondrocytes underwent modification through the synthesis of bone-type proteins; nodule-forming small round cells showed ALPase activity and were immunoreactive for type I collagen and osteocalcin. Immunoreactivity for type X collagen was detected in the small round cells at the top of the nodules prior to calcification of the matrix, as well as in large hypertrophic cells. BMP-2 was also expressed first in similar small round cells after 3 weeks in culture, and it subsequently extended along the extracellular matrix in the calcified nodules. These results indicate that small round cells that are differentiating toward osteocyte-like cells from Meckel's chondrocytes express type X collagen and BMP-2 sequentially.

KEY WORDS: *Meckel's cartilage, immunohistochemistry, transformation, in vitro, mouse*

Introduction

Mammalian Meckel's cartilage includes fetal supporting tissues that develop first as mandibular hyaline cartilage. The rostral and auricular ends undergo endochondral-type osteogenesis. The entire middle portion is resorbed in the absence of obvious formation of a calcified matrix, and these cells undergo terminal cellular hypertrophy (Bhaskar *et al.*, 1953; Kaneta, 1961; Frommer and Margolies, 1971; Richman and Diewert, 1988).

In previous investigations, we found that *in vitro*, Meckel's cartilage chondrocytes undergo phenotypic conversion to osteogenic cells (Ishizeki *et al.*, 1996a,c). Cultures with polygonal cells in monolayer early in culture developed into cells with typical chondrocyte-specific features eventually expressing an osteogenic phenotype. This phenomenon was accompanied not only by morphological modifications but also by the synthesis of bone-type proteins such as type I collagen and osteocalcin. The aim of the present study was to determine whether such cells differentiate further into osteocyte-like cells after or before cellular hypertrophy. Particular attention was paid, therefore, to type X collagen, a specific marker protein for hypertrophic chondrocytes.

Type X collagen is expressed characteristically in hypertrophic cartilage (Adams *et al.*, 1989, 1991; Oettinger and Pacifici, 1990), but it may have many yet undetermined functions. In particular, we have investigated the relationship between type X collagen and matrix calcification in Meckel's cartilage. Recently, Chung *et al.* (1995) reported that the middle portion of Meckel's cartilage underwent chondrocyte hypertrophy, but no expression of type X collagen was detected. If Meckel's cartilage cells undergoing transdifferentiation have the capacity for synthesis of type X collagen and are involved routinely in matrix calcification *in vitro*, this system might provide an important clue to the relationship between type X collagen and matrix calcification, and it might help us explain why Meckel's cartilage does not undergo endochondral-type osteogenesis.

We also examined whether the transformed cells have the capacity to synthesize BMP-2, in addition to bone-type proteins

Abbreviations used in this paper: BMP, bone morphogenetic protein; CSPG, chondroitin sulfate proteoglycan; α-MEM, alpha-modified Eagle's medium; ALPase, alkaline phosphatase; PBS, phosphate-buffered saline; PBS-G, phosphate-buffered saline containing glucose.

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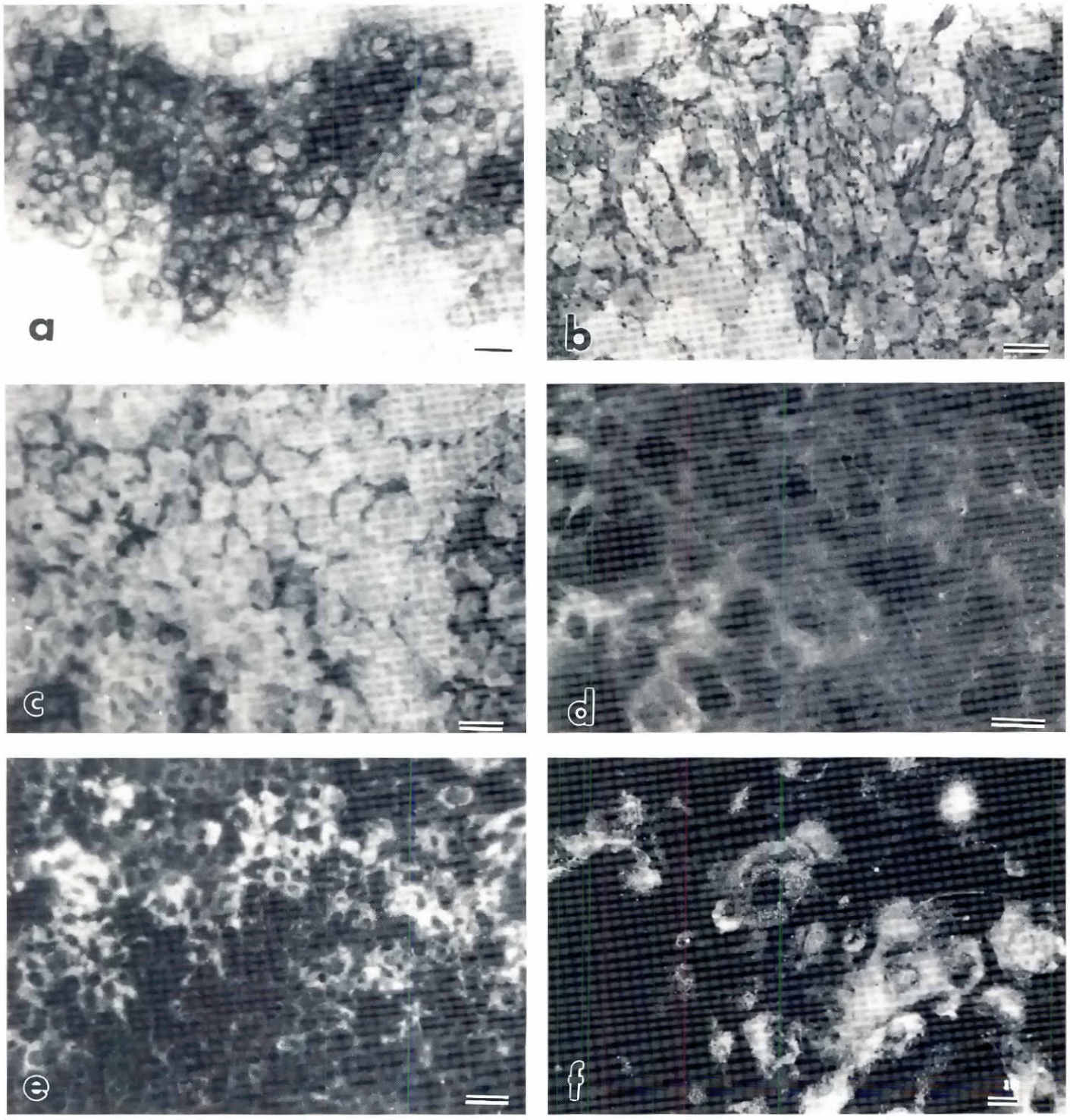

Fig. 1. Histochemical staining and immunostaining of Meckel's cartilage chondrocytes *in vitro*. **(a)** *Alcian blue-positive proteoglycan can be seen in the aggregates of chondrocytes after 2 weeks in culture. Bar, 70 μm.* **(b)** *ALPase activity is detectable on cell membranes of cells cultured for 3 weeks. Bar, 100 μm.* **(c)** *Immunoperoxidase staining shows the accumulation of the chondroitin sulfate proteoglycan-positive matrix around chondrocytic cells after 2 weeks in culture. Bar, 100 μm.* **(d)** *Immunofluorescence staining for type II collagen after 2 weeks in culture. Immunopositive fibrils are distributed widely in the extracellular matrix. Bar, 100 μm.* **(e)** *Immunofluorescence micrograph showing expression of type I collagen after 4 weeks in culture. Nodules are composed of an intensely immunoreactive calcified matrix. Bar, 50 μm.* **(f)** *Immunofluorescence localization of osteocalcin in 3-week-old cultures. Osteocalcin-positive cells that form cellular nodules are stained intracellularly. Bar, 10 μm.*

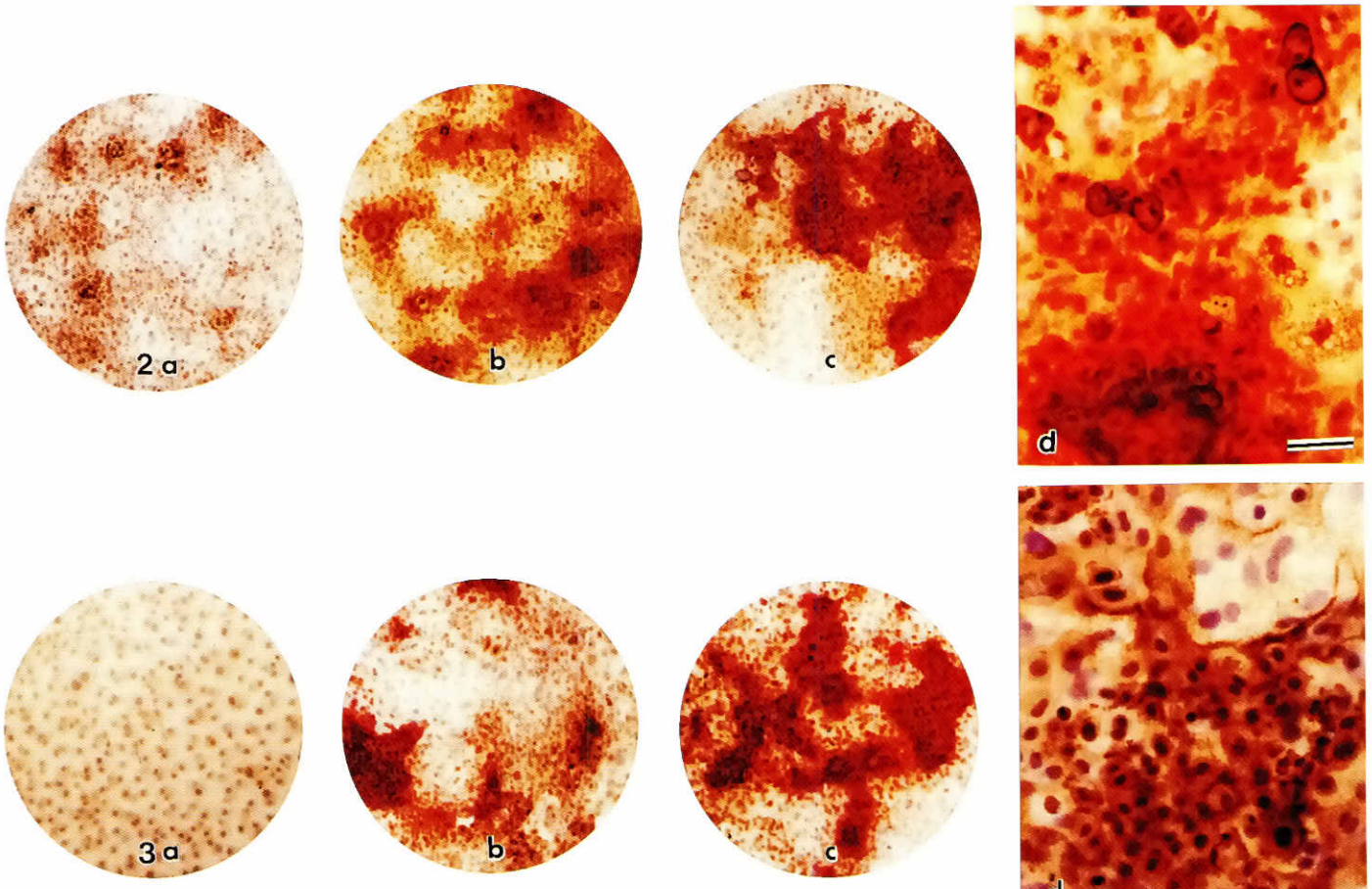

Fig. 2. Immunodetection of type X collagen after 2 weeks (a, x40**), 3 weeks (b,** x40**), and 4 weeks (c,** x40**) in culture.** *Immunoreactivity is evident in the nodule-forming cells. Violet areas show the calcifying matrix of the nodules.* **(d)** *Micrograph of cells after 3 weeks in culture, showing intense positive reaction in the cellular elements of the scattered large round cells and small round cells in the nodules. Bar, 50μm.*

Fig. 3. Immunoperoxidase staining for BMP-2 after 2 weeks (a, x50**), 3 weeks (b,** x40**) and 4 weeks (c,** x40**) in culture.** *Note that the size of immunopositive areas increases gradually with calcification of nodules.* **(d)** *Light micrograph showing positive staining on the extracellular calcified matrix within 3 weeks. Bar, 50 μm.*

such as osteocalcin and type I collagen. BMPs, with the exception of BMP-1, are members of the transforming growth factor-beta (TGF-ß) superfamily (Wozney *et al.*, 1988; Celeste *et al.*, 1990; Wang *et al.*, 1993), and there is some evidence that BMP-2, which is abundant in bone matrix, regulates many morphogenetic events during embryogenesis (Wang *et al.*, 1990; Chen *et al.*, 1991; Hiraki *et al.*, 1991; Yamaguchi *et al.*, 1991).

We report here that type X collagen is expressed *in vitro* in Meckel's cartilage in small round cells in association with synthesis of BMP-2, as well as in hypertrophic cells.

Results

Histochemistry

Alcian blue staining and ALPase activity

In specimens cultured for 2 weeks and stained with Alcian blue, positive reactions appeared in nodule-forming areas (Fig. 1a). Strong staining accompanied the formation of the cartilaginous matrix by multilayered nodules but staining subsequently decreased in intensity prior to intensive calcification of the matrix.

ALPase activity was first detected on the cell membranes of a small number of stratified cells and extended to the periphery of nodules within 3-4 weeks in culture (Fig. 1b).

Immunostaining

CSPG and type II collagen

Immunostaining for CSPG was more intense on the extracellular matrix around aggregates of chondrocytes than in monolayered areas (Fig. 1c). The pattern resembled that of Alcian blue staining, and immunostaining was intense at the stages when cells had a typical chondrocytic phenotype.

The cultures reached confluence and showed intense immunostaining for type II collagen. By two weeks in culture the extracellular matrix was densely filled with type II collagen in a well-organized fibrous-mat pattern (Fig. 1d). However, significant immunostaining was not detected on the nodules in 4-week-old cultures.

Type I collagen and osteocalcin

Immunoreactivity for type I collagen was closely associated with the calcified matrix that surrounded small round cells and extended along the areas of nodules after 4 weeks in culture (Fig. 1e).

Products of immunostaining for osteocalcin appeared intracellularly in nodule-forming cells that had been cultured for 3 weeks (Fig. 1f), and strong expression was seen during the calcification stages (data not shown). These patterns of expression were similar to those in a previous report (Ishizeki *et al.*, 1996c).

Type X collagen and BMP-2

Light microscopy analysis revealed immunoreactivity specific for type X collagen along the nodule-forming cells at 2-4 weeks in culture. Positive reactivity was first recognized on some cellular clusters after 2 weeks in culture (Fig. 2a). By 3-4 weeks, numerous large round cells with chondrocyte hypertrophy and small round cells appeared on the well-organized nodules, and both types of cells were positively stained for type X collagen (Fig. 2b,c). In particular, detailed microscopic analysis revealed that this immunoreactivity was located in the pericellular matrix and in intracellular elements of the small round cells, as well as the large round cells (Fig. 2d).

Sequential examination revealed no immunoreactivity for BMP-2 early in culture (Fig. 3a), but after an additional week slight expression of BMP-2 was detected first on the extracellular matrix at the top of nodules (Fig. 3b), subsequently extending along the calcified matrix. With intense calcification of the matrix throughout the fourth week in culture, immunopositive areas became extensive (Fig. 3c). Detailed analysis of BMP-2 expression revealed that, prior to calcification, the small round cells were stained intracellularly and this reactivity extended gradually over the calcified nodular matrix (Fig. 3d).

Figure 4 shows the expression of cartilage- and bone-type proteins during culture of Meckel's cartilage chondrocytes as evaluated by weekly histological analysis. Marker proteins of cartilage except for type X collagen were expressed until 2 weeks in culture. With further differentiation, bone proteins were predominantly synthesized.

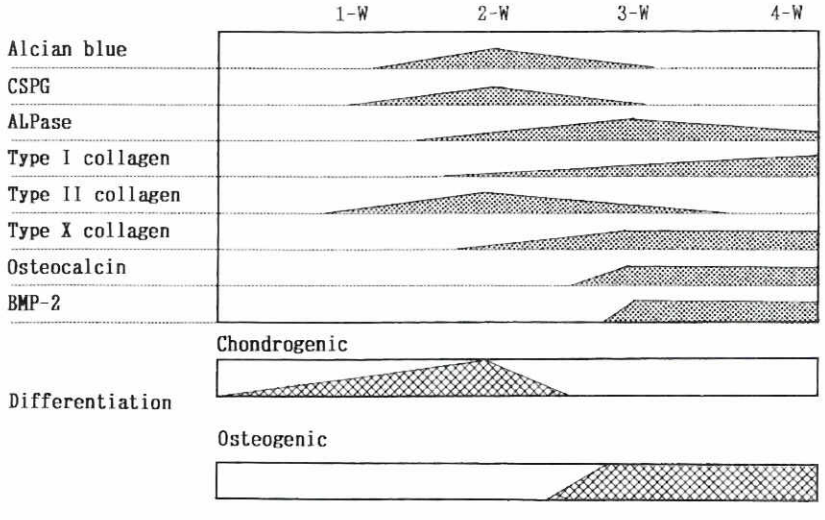

Fig. 4. The expression of cartilage- and bone-type proteins in cultured Meckel's cartilage chondrocytes. *Cells switch from expressing a cartilaginous phenotype during the early culture period to synthesizing bone-type proteins.*

Electron microscopy

The ultrastructural analysis of the matrix vesicles that appeared in nodule-forming areas early in culture revealed that they were retained in the collagenous fibrils and some contained fine crystalline structures with initial calcification (Fig. 5, inset).

Small round cells were surrounded by a calcified matrix that formed during the fourth week in culture, and they contained heterochromatin-rich oval nuclei, glycogen aggregates, some mitochondria, a few dense bodies, some rough endoplasmic reticulum, and relatively well-developed Golgi apparatus composed of several cisternae and condensing vacuoles. The pericellular space contained a few banded collagen fibrils. It was relatively wide and was traversed by elongated cytoplasmic processes that penetrated the calcified matrix (Fig. 5). Such cells were located mainly at the top of nodules, as was the case for cells that were immunopositive for type X collagen and BMP-2.

Discussion

Meckel's cartilage chondrocytes undergo phenotypic conversion to osteogenic cells both *in vitro* and *in vivo* (Ishizeki *et al.*, 1992, 1994, 1996b). In the present study, we confirmed previous reports (Ishizeki *et al.*, 1996b,c) that this phenomenon is accompanied not only by morphological modifications, but also by the synthesis of bone-type proteins. The present study further showed that small round cells that are differentiating osteocyte-like cells from Meckel's chondrocytes express type X collagen and BMP-2 sequentially.

We anticipated that type X collagen would be synthesized in our cultures by large typical hypertrophic cells. However, the synthesis of type X collagen extended over the small round cells, in addition to the large round cells. Positive reactivity in large round cells was a reasonable finding since they expressed a hypertrophic phenotype. We had identified as small round cells those cells in which the chondrocyte phenotype was no longer apparent and which, instead, had acquired osteogenic features (Ishizeki *et al.*, 1996b). In fact, these cells were numerous late in the culture period, and they were closely associated with calcification of the matrix. As reported by Richany *et al.* (1956), the middle region of intact Meckel's cartilage does not undergo endochondral-type osteogenesis, even when cartilage hypertrophy occurs. If type X collagen is expressed only under *in vitro* conditions and if its expression is accompanied routinely by calcification, it is now worth examining the relationship between type X collagen and matrix calcification in Meckel's cartilage.

Type X collagen is synthesized only by chondrocytes in the hypertrophic cartilage zone (Schmid and Conrad, 1982; Schmid and Linsenmayer, 1985; Reichenberger *et al.*, 1991), and it has been shown to be associated with type II collagen fibrils as fine filamentous mats (Poole and Pidoux, 1989; Schmid *et al.*, 1990; Kwan *et al.*, 1991). Although the proposed functions of type X collagen are based upon its restricted localization

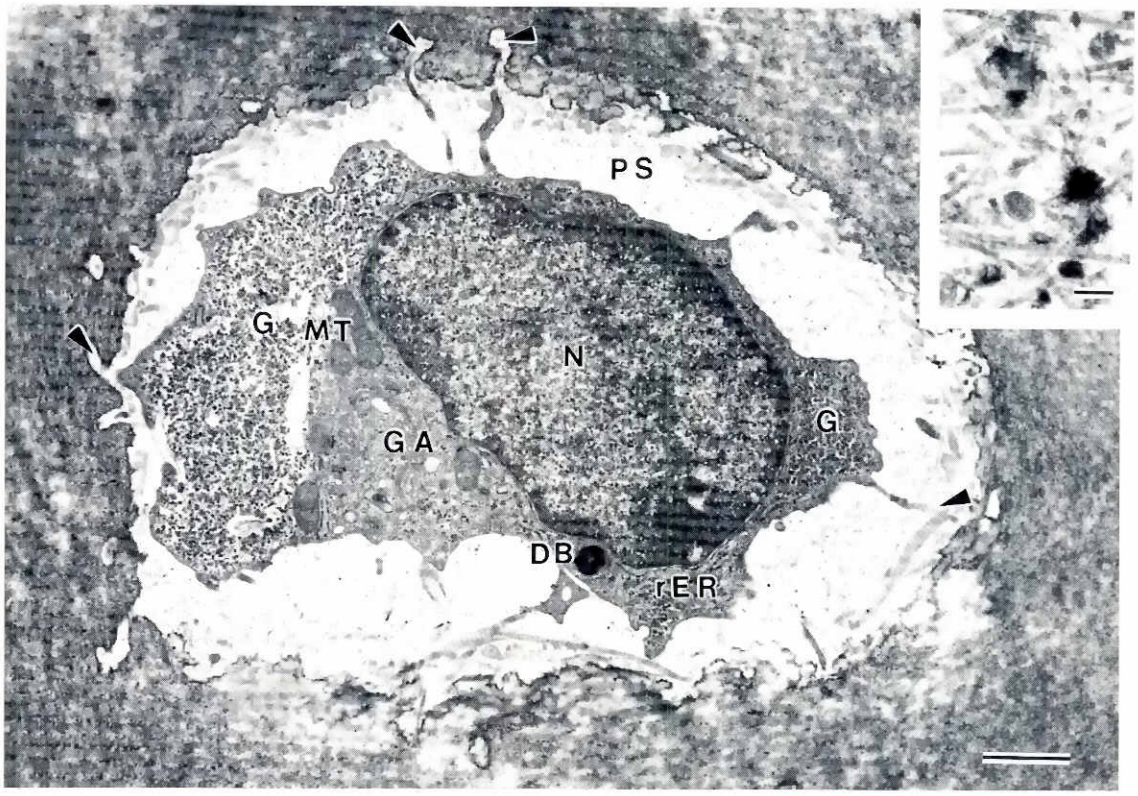

Fig. 5. Electron micrograph of small round cell in a calcified nodule after 4 weeks in culture. *The cell has retained relatively abundant organelles, such as glycogen aggregates (G), Golgi apparatus (GA), mitochondria (MT), a few dense bodies (DB), and rough endoplasmic reticulum (rER). Several elongated cytoplasmic processes (arrowheads) protrude into the calcified matrix throughout the pericellular space (PS). Such cells are identified as presumptive type X collagen-synthesizing and BMP-2-synthesizing cells. Bar, 1 μm.* **(Inset)** *Fine crystalline structures that are evidence of initial calcification associated with matrix vesicles. Bar, 0.2 μm.*

in the hypertrophic cartilage, its direct participation in the calcification process has not been verified. In the present study, if type X collagen had been involved directly in matrix calcification, calcification should have occurred universally on the interterritorial matrix that was occupied by considerable amounts of type II collagen. However, calcification was initiated in matrix vesicles, and subsequent processes showed no association with a particular type of collagen fibrils. This process of initiation of calcification is consistent with those reported previously in cartilage and bone (Anderson, 1967; Bonucci, 1967). Chung *et al.* (1995) reported the expression of type X collagen in areas that were undergoing endochondral ossification, such as the anterior and posterior portions of Meckel's cartilage, while the middle portion did not express type X collagen. Our results showed that, since chondrocytes isolated from the midportion of Meckel's cartilage expressed type X collagen *in vitro*, synthesis of type X collagen was stimulated *in vitro*.

Certain hypertrophic chondrocytes differentiate even further after the expression of type X collagen (Descalzi-Cancedda *et al.*, 1992; Galotto *et al.*, 1994). As shown in Figure 4, analysis of the cartilage- and bone-marker proteins in the present study indicated that Meckel's chondrocytes progressively expressed the osteogenic phenotype after achieving terminal chondrocytic differentiation. However, among the proteins that we examined as cartilage markers, type X collagen extended to small round cells with a chondro-osteogenic phenotype. This evidence indicates that, since type X collagen was synthesized continuously by hypertrophic large round cells with further differentiation, its expression extended to small round cells at the calcifying stages. Although the expression of type X collagen in small round cells does not correspond with that seen in hypertrophic chondrocytes *in vivo*, Pacifici *et al.* (1991) and Ekanayake and Hall (1994) showed that cellular hypertrophy and synthesis of type X collagen in chondrocytes *in vitro* are not always related. As indicated by some investigators (Strauss *et al.*, 1990; Kirsch *et al.*, 1992; Gentili *et al.*, 1993; Claassen and Kirsch, 1994; Roach *et al.*, 1995), not only synthesis of type X collagen but also the simultaneous synthesis of chondro-osseous-type proteins is likely in cells that are differentiating from chondrocytes.

Bone morphogenetic protein (BMP), which was first identified by Urist (1965) and Urist and Strates (1971), is a bone-inductive factor which is distributed extensively in the extracellular matrix of the bone. Six subfamilies of these proteins, including BMP-2, have been confirmed to date (Celeste *et al.*, 1990; Wang *et al.*, 1993). Several of these BMPs are intimately involved in the proliferation and differentiation of osteoblasts (Wang *et al.*, 1990; Hiraki *et al.*, 1991; Yamaguchi *et al.*, 1991), and they are reliable as bone marker proteins. In the present study, immunoreactivity for BMP-2 was strongest in areas of the calcified extracellular matrix. This pattern of localization was consistent with the distribution of BMP-2 in the extracellular matrix. The nodule-forming small round cells that acquired the osteogenic phenotype appeared to be the source of this protein.

In summary, our study demonstrated histochemically and immunocytochemically that cartilage- and bone-type proteins, in particular type X collagen and BMP-2, were expressed sequentially during the culture of Meckel's chondrocytes. Although the morphology of the small round cells that synthesized type X collagen did not always correspond to that of hypertrophic chondrocytes reported *in vivo*, it appeared that these cells retained functions similar to those of intact hypertrophic chondrocytes. It is now necessary to inves-

tigate whether type X collagen in small round cells is analogous in terms of function to that in intact hypertrophic chondrocytes.

Materials and Methods

Cell culture

The middle portions of Meckel's cartilage bars from day-17 mouse embryos were prepared as described previously (Ishizeki *et al.*, 1992) and placed in phosphate-buffered saline (PBS; pH 7.2) containing 0.2% glucose (PBS-G).

Chondrocytes were isolated as described in our previous report (Ishizeki *et al.*, 1996c). Briefly, specimens were rinsed with PBS-G, and then suspensions of single cells were obtained by repeated pipetting after enzymatic digestion. Cells were sedimented by centrifugation at 1,800 rpm for 3 min and then inoculated at a density of $1x10^4$/200 µl of medium per 0.28-cm^2 penicylinder. The complete medium consisted of α-MEM (Flow Laboratories, Irvine, UK) supplemented with 10% fetal bovine serum (Mitsubishi Kasei Co., Tokyo, Japan), 0.1 mM L-ascorbic phosphate magnesium salt (Wako Pure Chemical Industries, Ltd, Osaka, Japan), 5 mM ß-glycerophosphate (Wako) and 60 µg/ml kanamycin (Banyu Pharmaceutical Co., Ltd., Tokyo, Japan). The medium was changed every other day and the cells were cultured under 5% CO_2 in air at 37°C for up to 4 weeks.

Histological analysis

Samples for histological analysis, which included histochemistry, immunostaining and electron microscopy, were harvested at weekly intervals. The findings from these analyses, apart from electron microscopy, were converted to arbitrarily defined values (see Fig. 4). The photographs show the most characteristic stages during the culture period.

Histochemistry

Alcian blue staining and detection of alkaline phosphatase (ALPase) activity

For analysis of proteoglycans, cells from 2-week-old cultures were fixed in 4% paraformaldehyde for 2-4 h. After washing with PBS, they were stained with 1% alcian blue (pH 1.0) for 5-8 h at room temperature.

ALPase activity was detected by Gomori's method (Gomori, 1939) with ß-glycerophosphate as substrate.

Immunostaining

For indirect immunoperoxidase staining of chondroitin sulfate proteoglycan (CSPG), type X collagen and BMP-2, cultures were fixed with ethanol and acetic acid (99:1, v/v) for 2 h at room temperature. After the samples had been treated with 0.1% hyaluronidase (Type I-S; Sigma Chemical Company, St. Louis, MO, USA), endogenous peroxidase activity was eliminated by incubation with methanol containing 0.3% H_2O_2 for 30 min. Cells were then stained with various antibodies, which included rabbit anti-bovine CSPG (Bioline Diagnostici, Torino, Italy), rabbit anti-rat type X collagen (MAP) antibody (1:150; LSL Co., Ltd., Tokyo, Japan), and rabbit anti-human bone morphogenetic protein-2 (BMP-2; 1:100, Austral Biologicals, San Ramon, CA, USA) for 2 h at room temperature. Secondary immunoreactions were performed for 1 h at room temperature with peroxidase-conjugated anti-rabbit IgG (Cappel; Organon Teknika Corporation, West Chester, PA, USA), diluted 1:100 with PBS. Immunostaining was visualized by treatment with 3,3'-diaminobenzidine (Merck, Darmstadt, Germany) in Tris-HCl buffer (pH 7.4) that contained 3% H_2O_2 for 10 min, and cells were counterstained with hematoxylin. Finally, specimens were mounted in glycerol:PBS (9:1, v/v). The specificity of the immunoreactivity was checked by using normal rabbit serum instead of the primary antibodies and also by omitting the primary antibodies.

Type I and II collagens and osteocalcin were examined by indirect immunofluorescence staining. The cells were fixed as described above and incubated with rabbit anti-rat type I collagen antibody (Advance Co., Ltd., Tokyo, Japan) diluted 1:400 with PBS, with rabbit anti-bovine type II collagen antibody (Advance) diluted 1:150 with PBS, or with rabbit anti-mouse osteocalcin (Ishizeki *et al.*, 1996c) for 45 min at 37°C. Subsequently, they were incubated for 45 min with fluorescein isothiocyanate-conjugated anti-rabbit IgG (Cappel; diluted 1:100) at 37°C. Control specimens were processed in the same way, with omission of the primary antibodies. After further rinsing, the specimens were mounted in glycerol:PBS (9:1, v/v) and examined by immunofluorescence confocal laser scanning microscopy (LSM-GB 200; Olympus, Tokyo, Japan).

Electron microscopy

The cultured specimens were fixed in 2.5% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.4), post-fixed in 1% OsO_4 solution that contained 1.5% potassium ferrocyanide (Sigma; Farnum and Wilsman, 1983), dehydrated through a graded ethanol series, and embedded in Epon 812. Ultrathin sections were cut with a diamond knife from undecalcified specimens and were observed with a transmission electron microscope (H-7100; Hitachi, Japan) after counterstaining with uranyl acetate and lead citrate.

Acknowledgments

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