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Original Article

Role of chondrogenic tissue in programmed cell death and *BMP* expression in chick limb buds

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ABSTRACT In the developing chick leg bud, massive programmed cell death occurs in the interdigital region. Previously, we reported the inhibition of cell death by separation of the interdigital region from neighboring digit cartilage. In this study, we examined the relationship between cell death and cartilaginous tissue *in vitro*. First, cell fate was observed with DiI that was used to examine cell movement in the distal tip of leg bud. Labeled cells in the prospective digital region were distributed only in the distal region as a narrow band, while cells in the prospective interdigital region expanded widely in the interdigit. In coculture of monolayer cells and a cell pellet tending to differentiate into cartilage, monolayer cells migrated into the cell pellet. These results suggested that digit cartilage tends to recruit neighboring cells into the cartilage during limb development. Next, we observed the relationship between cell death and chondrogenesis in monolayer culture. Apoptotic cell death that could be detected by TUNEL occurred in regions between cartilaginous nodules in mesenchymal cell culture. More apoptotic cell death was detected in the cell culture of leg bud mesenchyme of stage 25/26 than that of leg bud mesenchyme of stage 22 or that of stage 28. The most developed cartilaginous nodules were observed in the cell culture of stage 25/26. Finally, we observed *Bmp* expression *in vitro* and *in vivo*. *Bmp-2*, *Bmp-4* and *Bmp-7* were detected around the cartilage nodules. When the interdigit was separated from neighboring digit cartilage, *Bmp-4* expression disappeared near the cut region but remained near the digit cartilage. This correlation between cell death and cartilaginous region suggests that cartilage tissue can induce apoptotic cell death in the developing chick limb bud due to cell migration accompanying chondrogenesis and *Bmp* expression.

KEY WORDS: *Programmed cell death, Limb bud, BMP.*

Introduction

Programmed cell death is a very important phenomenon in morphogenesis (Saunders, 1966). There are certain mesenchymal cell death areas in the developing chick limb bud, namely, the anterior necrotic zone (anterior area of the limb bud), posterior necrotic zone (posterior area), and interdigital necrotic zone (Gasseling and Saunders, 1964; Hinchliffe, 1974; Saunders and Fallon, 1967). Massive cell death occurs in the interdigital zone. Digit formation starts at approximately stage 28. At this stage, soft tissue is between each digit primordium and connects the digit primordium. After stage 31, programmed cell death starts and the digits separate from each other (Saunders and Gasseling, 1962). There are some chick mutants that have malformed limbs because excessive cell death has occurred (Hinchliffe and Ede, 1973) or because normal cell death has not occurred in limb development (Cairns, 1977; Hinchliffe and Ede, 1967; Hinchliffe and Thorogood, 1974). These phenomena show that interdigital cell death is one of the important factors for completing digit formation.

It is known that interdigital cell death is influenced by surrounding tissue. When the ectoderm covering the interdigital mesenchyme is removed, interdigital cell death is inhibited and excessive cartilage mass or an ectopic part of the digit is formed (Hurle and Gañan, 1986; Hurle *et al.*, 1989, 1991). This indicates the possibility that the

Abbreviations used in this paper: BMP, bone morphogenetic protein; DiI, 1,1-dioctadecyl-3,3,3´,3´-tetramethyl-indocarbocyanine perchlorate.

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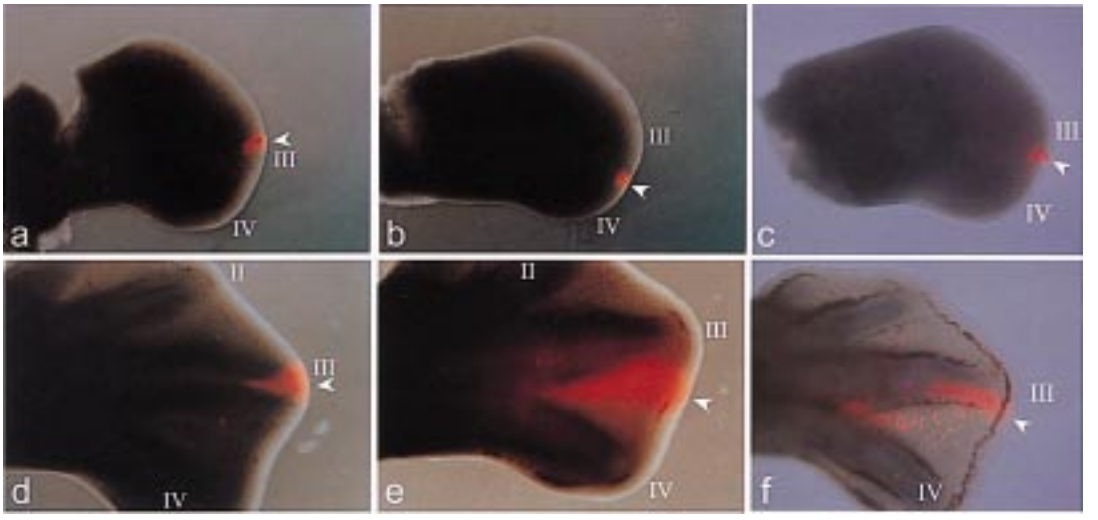

Fig. 1. Cell fate analysis with DiI in leg buds. (a, d) *Cell fate of labeled prospective digital region 0 h (a) and 48 h (d) after labeling. Labeled cells accumulated as a narrow band in the digit III region.* **(b, e)** *Cell fate of prospective interdigital region 0 h (b) and 48 h (e) after labeling. Labeled cells expanded in the interdigital and moved toward the digital region at the proximal level.* **(c, f)** *Cell fate of prospective interdigital region nearer prospective digit III compared to the case of (b, e) 0 h (c) and 48 h (f) after labeling. Labeled cells migrated into digit III cartilage. II, digit II; III, digit III; IV, digit IV, arrowhead, marked area. II-IV, digit number; arrowhead, marked region.*

interdigital ectoderm controls interdigital cell death. Further, when the interdigital zone is surgically separated from neighboring digit cartilage before cell death starts, cell death is inhibited and an excessive cartilage mass or an ectopic part of the digit is formed same as in the case of removal of the ectoderm (Gañan *et al.*, 1994; Ros *et al.*, 1994; Omi and Ide, 1996). This indicates the possibility that digit cartilage controls the interdigital cell death.

It has recently been shown that bone morphogenetic proteins (BMPs) have an activity that mediates cell death in the limb bud. *Bmp-2*, *Bmp-4* and *Bmp-7* are expressed in the interdigital mesenchymal zone (Francis *et al.*, 1994; Luo *et al.*, 1995). When BMP signaling is inhibited by a dominant negative BMP receptor, cell death is stopped and excessive webs are formed between digits (Yokouchi *et al.*, 1996; Zou and Niswander, 1996; Zou *et al.*, 1997). Furthermore, BMP protein-soaked beads implanted in limb bud can induce ectopic mesenchymal cell death (Gañan *et al.*, 1996; Macias *et al.*, 1997). We have previously reported the possibility that interdigital cell death is controlled by the neighboring digit cartilage (Omi and Ide, 1996). In this study, we examined the influence of cartilage tissue on cell death with in an *in vitro* culture system.

Results

Fate map of the distal region of the chick leg bud

To analyze mesenchymal cell movement in the distal region of the leg bud during digit cartilage formation and interdigit formation, we traced the movement using fluorescent dye DiI. At stage 25/26, the distal tip of precartilage condensation of digit III and the distal region between precartilage condensation of digit III and that of digit IV (namely, the 3rd interdigit) were marked with DiI (Fig. 1 a,b,c). After 48 hours, at stage 30, the marked cells in precartilage condensation of digit III were distributed limitedly only in the inside of the digit III region as a narrow band (Fig. 1d), while the cells in the prospective 3rd interdigit were distributed widely in the 3rd interdigital region (Fig. 1e). Table 1 shows the area size and length along the antero-posterior axis of labeled regions. Both the numerical value of the area size and that of the length of the marked region in the 3rd interdigit were larger than those of the marked region of digit III ($p<0.05$). These results indicate that cells constituting a prospective interdigital region expand their distributions widely during limb development, while cells at the distal tip of a prospective digit condense tightly. When the prospective 3rd interdigit region was stained, the stained region was close to the digit III (7/10, Fig. 1e) or digit IV (3/10). Furthermore, when the distal region near the prospective digit III in the interdigit was labeled (Fig. 1c), the labeled region migrated into the digit III cartilage (5/10, Fig. 1f). These findings indicate that interdigital mesenchymal cells migrate to neighboring digit cartilage.

TABLE 1

AREA SIZE AND LENGTH OF DII-LABELED REGION

	prospective digit III (n=7)	prospective 3rd interdigit (n=10)
average area size (±SD) (mm^2)*	0.104 ± 0.026	0.467 ± 0.343
average length (±SD) (mm)* along A-P axis	0.332 ± 0.053	0.622 ± 0.314

* The differences in area size and average length are statistically significant ($P<0.05$).

Mesenchymal cell migration to the chondrogenic region *in vitro*

Since the above results indicate the possibility that limb mesenchymal cells are recruited into chondrogenic tissue, the cells were cultured with a cell pellet that differentiates cartilage to observe whether limb mesenchymal cells migrate into developing cartilage tissue or not (Fig. 2). It has been reported that limb mesenchymal cells tend to differentiate into cartilage when they form a pellet by centrifugation (Karasawa *et al.*, 1979). Distal mesenchymal cells of the leg bud at stage 25 stained with red fluorescent dye PKH26 were cocultured with a cell pellet stained with green fluorescent dye PKH2. After 96 hours, a confocal microscopy showed that monolayer cells were distributed in the cell pellet at a height of 40 μm from the bottom of the culture dish (Fig. 2d). Monolayer cells were not distributed at the center of the pellet, but there were more cells distributed at the margin of the pellet. Around the pellet, monolayer

cells had a ring-shaped distribution. Cells that made up the pellet did not migrate toward these surroundings (Fig. 2 e,f).

In the case of culture with distal interdigital mesenchymal cells of stage 28 and pellets consisting of cells in precartilage condensation, the same result as that for limb bud cells of stage 25 was observed after 96 hours. Monolayer culture interdigital cells were observed within the pellet (40 μm upper), while pellet cells did not migrate toward the surroundings (data not shown).

Few marked cells were observed at the center of the pellet when either stage 25 and or stage 28 cells were used. It was reported that extracellular matrices such as collagen are abundant while the cell number decreases in the center of a chondrogenic pellet (Eguchi and Okada, 1971). Thus, the cells incorporated into the pellet seem to localize in the surface of the chondrogenic pellets.

Cell death and chondrogenesis in a monolayer culture

The relationship between cell death and chondrogenesis was analyzed in a monolayer cell culture system. Distal cells of leg buds of stage 22, 25 and 28 were cultured at 8.8×10^5 cells/cm^2. Cell death was detected by the TUNEL method, and cartilage nodules were detected by Alcian blue staining (Fig. 3). When distal cells of stage 25 were cultured, TUNEL-positive signals started to be detected after 48 hours (Fig. 3e). These signals did not show a random distribution but were somewhat aggregated. After 72 hours, cartilage nodules started to be detected by Alcian blue staining (Fig. 3h), and the TUNEL signals were distributed in regions between nodules, that is, internodular regions, but not in any of the nodules. This aspect became striking after 96 hours (Fig. 3k).

In the case of a culture using distal cells of stage 22, cartilage nodules were closer to each other and tended to join each other, and the internodular regions were therefore very small. TUNEL signals were detected in internodular regions, but they were less than those in case of a cell culture of stage 25 cells after 96 hours (Fig. 3j). In the case of a culture using interdigital cells of stage 28, no cartilage nodules stained by Alcian blue were observed even after 96 hours, and only a few TUNEL signals were observed compared to those in the stage 25 cells (Fig. 3l).

Voronoi analysis was applied to the distribution of TUNEL signals and cartilage nodules in the culture of stage 25 cells, for which TUNEL signals and chondrogenesis were most remarkable (Fig. 4b). Each nodule was regarded as a dot, and the Voronoi boundaries were given as perpendicular bisectors of lines linking two dots according to the description in Materials and Methods. Voronoi analysis is a method used when analyzing an expanse of space occupied by dots, and a Voronoi boundary can be regarded as a line showing the range of the influence of each dot (Fig. 4a). Thus, it can be considered that the point of intersection of more lines is under the influence of more dots. In this case, there was a tendency for more TUNEL signals to be observed at the point of intersection of more boundaries (Fig. 4b), while less TUNEL signals were observed at the point of intersection of fewer boundaries (Fig. 4b). This suggests that cell death might tend to occur at the point surrounded by and influenced by more cartilage nodules.

***Bmp* expression in the interdigital region separated from the digit**

Bmp-2, *Bmp-4* and *Bmp-7* are known to be expressed in the interdigital region and can mediate the cell death. Since we previously reported the possibility of interdigital cell death being controlled by neighboring digit cartilage (Omi and Ide, 1996), we

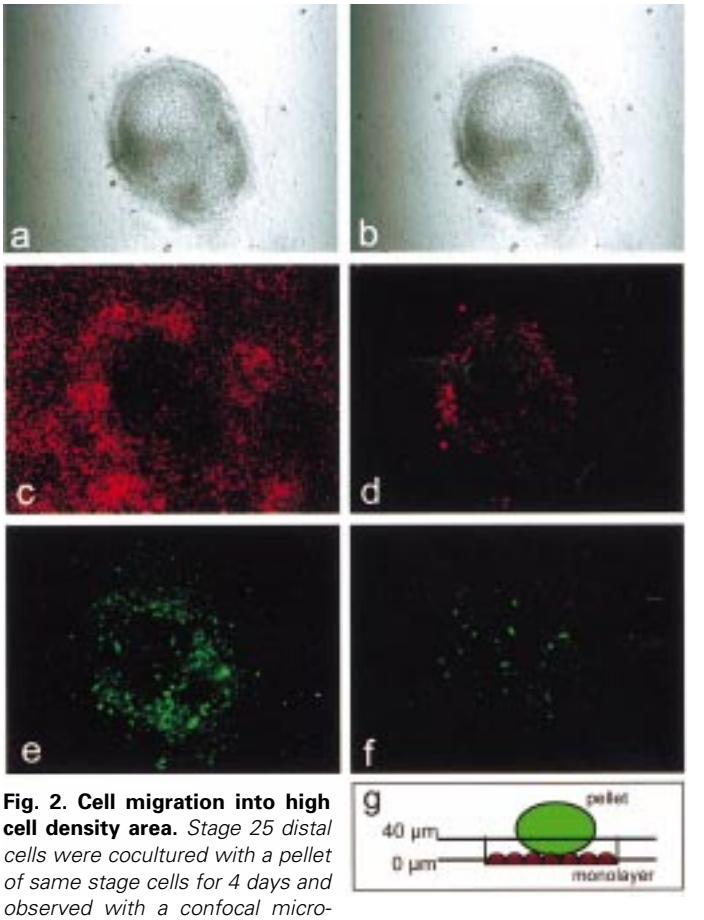

Fig. 2. Cell migration into high cell density area. *Stage 25 distal cells were cocultured with a pellet of same stage cells for 4 days and observed with a confocal microscope. (a, c, e) Sections at the bottom level. (b, d, f) Sections at the 40 μm level from the bottom. Cells in monolayer were stained with PKH26 (red) and the pellet was stained with PKH2 (green).* **(a, b)** *Bright-field.* **(c, d)** *Distribution of cells in monolayer.* **(e, f)** *Distribution of cells in the pellet. Monolayer-cultured cells (red) migrated into the inside of the pellet.* **(g)** *Schematic illustration of the coculture. Red semicircle, monolayer cells stained with PKH26; Green ellipse, pellet.*

examined the gene expression of *Bmp* when the interdigital region was separated from the digital region by making an incision along the digital ray surgically. When the 3rd interdigital region was separated from digit III, *Bmp-4* expression disappeared at the incision side of the interdigit 24 hours after the operation was performed (arrow in Fig. 5b), whereas *Bmp-4* expression remained strong in the region near digit IV in the interdigit (Fig. 5b). From the location of the end of AER (asterisk in Fig. 5b), it is concluded that the incision side of the interdigit where *Bmp-4* is not expressed (arrow in Fig. 5b) is correspondent to the region where *Bmp-4* is expressed in a control limb bud, not correspondent to the most distal region where *Bmp-4* is not expressed originally. *Bmp-7* expression was down-regulated at the side of incision in the interdigit when the 3rd interdigit was separated from digit IV (Fig. 5d), but the expression was not down-regulated as dramatically as that of *Bmp-4* expression. *Bmp-7* expression remained strong in the region near digit III and at the incision side near by digit IV.

***Bmp* expression in cell culture**

Next we examined whether *Bmp*s were expressed in this monolayer culture. *Bmp* mRNA probes were applied to the cell

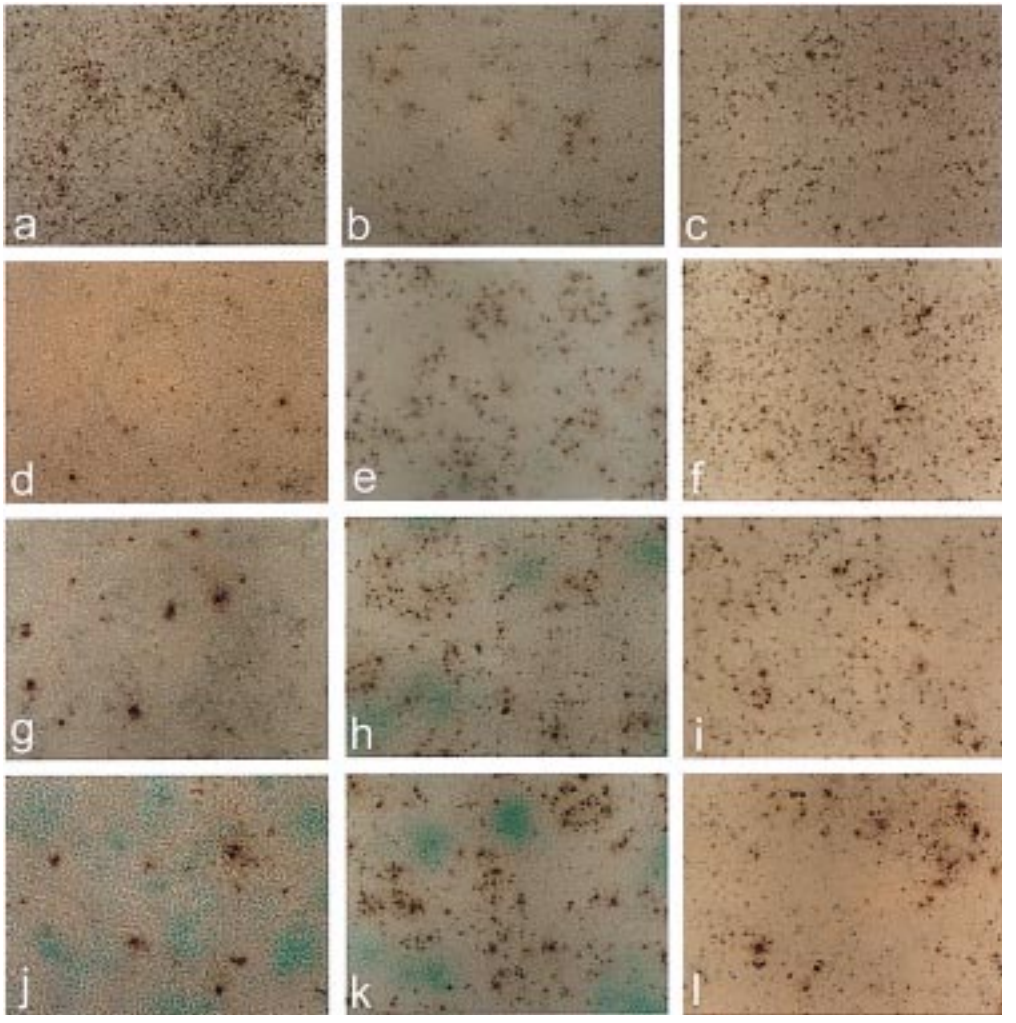

Fig. 3. Distribution of TUNEL-positive cell death (dark brown dots) and Alcian blue-positive cartilage nodules (blue) in monolayer cultures. *(a, d, g and j) Culture of stage 22 cells. (b, e, h and k) Culture of stage 25. (c, f, i and l) Culture of stage 28.* **(a, b and c)** *After 1 day.* **(d, e and f)** *After 2 days.* **(g, h and i)** *After 3 days.* **(j, k and l)** *After 4 days. In the culture of stage 25 cells, Alcian blue-positive cartilage nodules were formed at day 3 (h) and TUNEL-positive signals appeared from day 2 (e). TUNEL signals were observed only in the internodular region at day 3 (h) and day 4 (k). In the culture of stage 22 cells, nodules fused each other and consequently the internodular region decreased, and TUNEL-signals were less apparent than in stage 25 cells (g, j). In the culture of stage 28 cells, Alcian blue-positive nodules were not observed and TUNEL signals appeared sparsely (l).*

culture of limb bud mesenchyme of stage 25. *Bmp-2* (Fig. 6 a,b), *Bmp-4* (Fig. 6 e,f) and *Bmp-7* (Fig. 6 i,j) were all expressed around the chondrogenic nodules in day 4 cultures.

Discussion

In this study, we showed cell migration toward cartilage tissue and correlation between chondrogenesis and cell death in the developing chick limb bud *in vitro* and *in vivo*.

Cell fates in the autopod

We observed cell fate in the interdigital zone and the digital zone at a relatively late stage using fluorescent dye DiI. Cell fate of developing limb mesenchyme has been investigated in previous studies (Stark and Searls, 1973; Hinchliffe *et al*., 1984; Bowen *et al*., 1989; Vargesson *et al*., 1997); however, in those studies mesenchymal cell fate was observed at a relatively early stage but not at relatively late stage. In the present study, we studied mesenchymal cell fate, especially in the interdigital region at a relatively late stage, and we found that the cells in the region between digit III and digit IV expanded their distribution and that some of them migrated into the digit cartilage, whereas cells at the tip of digit III assembled in chondrogenic digit III as a narrow band.

Condensation is at first necessary for chondrogenesis in the developing limb bud (Abbot and Holtzer, 1966; Thorogood and Hinchliffe, 1975). It is also known that formation of cartilaginous nodules occurs in a high cell density culture *in vitro* (Umansky, 1966; Caplan, 1970; Karasawa *et al*., 1979). The DiI narrow band consisting of stained distal tip cells in prospective digit III is likely to have resulted from the cell condensation to form the cartilage element of digit III. There are two possible mechanisms of cell condensation. One possibility is an increase in the number of cells due to a local increase in activity of cell proliferation, and another is a localized concentration of cells by cell migration. According to the results of studies using autoradiography, there was no increase in the activity of cell proliferation accompanying chondrogenesis in the limb bud mesenchyme (Janners and Searls, 1970; Ede, 1983). Furthermore, it was reported that cell density increases as the mitotic index decreases in the central region of limbs at the beginning of chondrogenesis (Summerbell and Wolpert, 1972; Ede, 1983), and that cell proliferation was detected in undifferentiated mesodermal cells but not in cartilaginous nodules in micromass culture of limb mesoderm (Jung and Tsonis, 1998). Our data together with previously reported data suggest that cell condensation preceding chondrogenesis is due to localized cell migration from the surroundings rather than to an increase in cell

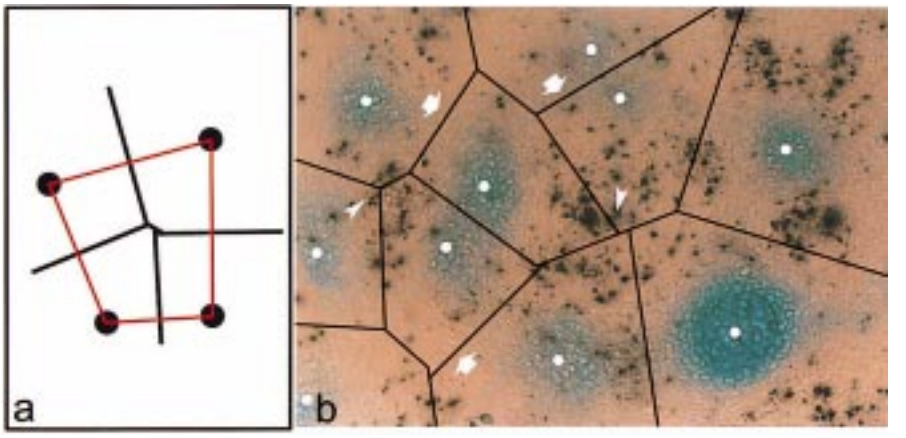

Fig. 4. (a) *Schematic representation of a Voronoi tessellation analysis. Each boundary line (black) is detected as a perpendicular bisector of a line (red) linking two points (black).* **(b)** *Voronoi analysis for culture of stage 25 cells for 4 days. White dots represent centers of each nodule and black lines represent boundaries. In a region that has more boundaries (arrowheads), that is, a region influenced by more nodules, there are more TUNEL signals, while in a region with only one boundary (arrows), only a few TUNEL signals are observed.*

number by proliferation. In the labeling study using DiI, the narrow, not spreading, band of DiI-labeled cells in the digital region showed that the mitotic index does not increase in the chondrogenic region, indicating the possibility that cell density become high in the digital region and low in the interdigital region.

We confirmed the movement toward the chondrogenic region *in vitro*. Monolayer culture cells were observed inside the pellet, and these tended to differentiate into cartilage. This phenomenon was also observed when the interdigital mesoderm and digital mesoderm of a leg bud of stage 28 was cultured. However, cell migration from the pellet to the area surrounding the pellet was not observed. These results show that the chondrogenic region recruit surrounding cells into the inside during the chondrogenesis.

This culture system can be regarded as a model indicating the relationship between digits and interdigits of the limb. Namely, from the present result and the labeling study with DiI, there is a possibility that cells in the prospective interdigital region migrate toward a neighboring prospective digital region accompanying cell condensation for cartilage formation. If cell density increases at one spot due to cell migration, not due to an increase in cell proliferation, then cell density may decrease in the area surrounding this. It was reported that there are comparatively wide gaps among cells in the interdigital zone (Kelley, 1970), which is consistent with the results of our study and our hypothesis. We speculate that mesenchymal cells in the interdigital zone migrate to the cartilage digital region accompanying the process of the digit cartilage condensation and that cell density consequently decreases in the interdigital zone.

Chondrogenesis and cell death *in vitro*

It is known that mesenchymal cells of chick limbs at different stages show different chondrogenic aspects in monolayer culture (Cottrill *et al.*, 1987). In the case of cell culture using limb bud mesenchymal cells of stage 23 or less, cartilage nodules connect with each other and the total area size of the chondrogenic region is larger, and consequently the area size of internodules is small. At stage 25/26, each nodule separated from each other but degree of staining by Alcian blue was somewhat stronger. TUNEL positive signals (apoptotic signals) were observed in internodular regions but never on the nodules. At stage 22, the internodular regions were small and there were very few apoptotic signals. Furthermore, there were fewer apoptotic signals in culture with interdigital mesenchyme cells of stage 28 than with mesenchymal cells of stage 25/26, the stage at which no cartilaginous nodules were observed. These results suggest that there is a correlation between chondrogenesis and apoptosis of limb bud mesenchymal cells. Apoptotic signals in culture of stage 28 cells might occur as a result of the determination of interdigital cell death. Voronoi analysis showed that more apoptotic cell death occurred at intersections of more boundaries. Each boundary can be regarded as a region that is influenced by two spots, namely, two nodules. It is thought that regions where there are more intersections are influenced by more spots (nodules). This result also suggests that mesenchymal apoptotic cell death tends to be affected at least by cartilage tissues.

We discussed above the cell movement in the mesenchyme results in changes in cell density in cell culture and in developing autopod. Newman (1977) proposed a hypothesis that chondrogenesis occurs by the increase in cell density and that cell death in the

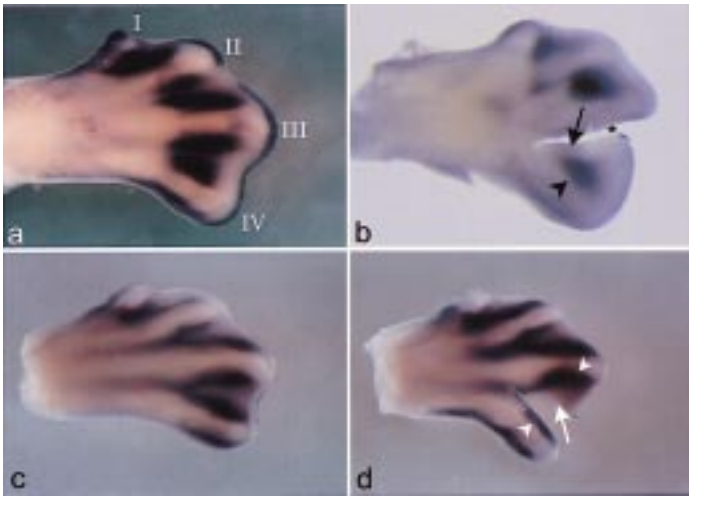

Fig. 5. *Bmp* expression after the separation of the interdigit from the digital region. (a) *Bmp-4 expression in a normal leg bud at stage 30.* **(b)** *Bmp-4 expression in an operated leg bud in which the 3rd interdigit was separated from digit III. The expression at the side of the incision was inhibited in the 3rd interdigit (arrow), while the expression at the side of digit IV was retained (arrowhead). The asterisk indicates the end of AER.* **(c)** *Bmp-7 expression in a normal leg bud at stage 30.* **(d)** *Bmp-7 expression in an operated leg bud in which the 3rd interdigital region was separated from digit IV. The expression at the side of the incision was downregulated in the 3rd interdigit (arrow), while the expression at the side of digit III and in the region nearby digit IV was retained (arrowheads). I-IV, digit number.*

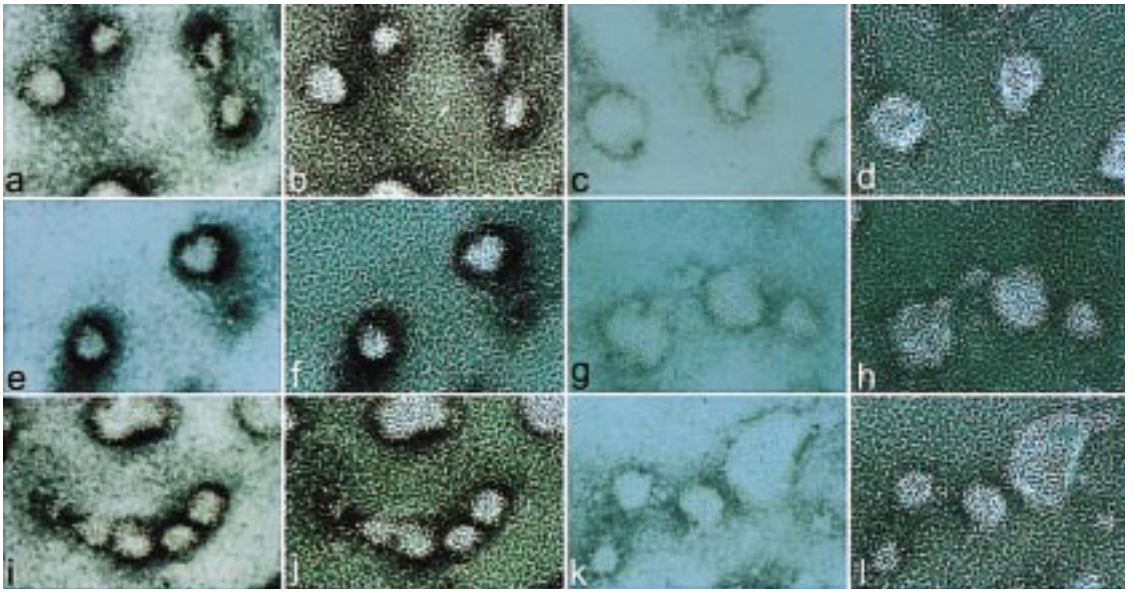

Fig. 6. *Bmp* expression in cell culture. (a, b, c and d) *In situ hybridization with Bmp-2 antisense mRNA probe (a) and with a sense probe (d). (b) and (d) are phase-contrast images, respectively.* **(e, f, g and h)** *In situ hybridization with Bmp-4 antisense mRNA probe (e) and with sense probe (g). (f) and (h) are phase-contrast images, respectively.* **(i, j, k and l)** *In situ hybridization with Bmp-7 antisense mRNA probe (i) and with a sense probe (k). (j) and (l) are phase-contrast images, respectively.*

interdigital region occurs by decrease in cell density. The present results support this hypothesis. However, low cell density culture of chick limb bud mesenchyme (1.0×10^5 cells/cm^2) did not induce significant cell death (data not shown). This result suggests that a decrease in cell density may be one requirement but not a sufficient condition. In addition, the presence of cartilage factor(s) may be necessary for cell death.

In the present study, *Bmp* expression was found in the culture and at the interdigit region separated from neighboring digit cartilage. BMPs (BMP-2, BMP-4 or BMP-7) are dominant candidates as apoptotic signal mediating-molecules in neural crest development (Graham *et al.*, 1994) and in limb development (Gañan *et al.*, 1996; Yokouchi *et al.*, 1996; Zou and Niswander, 1996; Macias *et al.*, 1997). In this paper, we reported *Bmp*s expression in the monolayer cell culture and at the interdigit separated from neighboring digit cartilage. *Bmp-2*, *Bmp-4*, and *Bmp-7* were all expressed around the chondrogenic nodules. Furthermore, when the interdigit was separated from neighboring digit cartilage, *Bmp-4* expression disappeared and *Bmp-7* expression was down-regulated on the incision side of the interdigit, while their expressions were maintained on the side near digit cartilage in the interdigit and on the other incision side near digit cartilage. We previously reported the possibility that interdigital cell death may be controlled by neighboring digit cartilage (Omi and Ide, 1996). These results suggest that digit cartilage may be able to induce or control *Bmp* expression and that it may control the interdigital cell death through controlling *Bmp* expressions.

BMPs have multiple functions, namely, they can induce chondrogenesis and they can mediate apoptosis for limb mesenchymal cells. BMP can accelerate apoptosis of mesenchymal cells but they can not induce apoptosis in the condensing area in limb buds (Macias *et al.*, 1997). It is possible that the decrease of cell density in the interdigital region increases the competence for cell death-inducing activity of BMPs. This idea may be able to explain unsolved problems about interdigital cell death, for example, region- and stage-restricted interdigital cell death pattern despite wide *Bmp* expression.

Materials and Methods

Cell marking

Fertilized White Leghorn chicken eggs were incubated at 38°C and staged after Hamburger and Hamilton (1951). For cell marking, we used the lipophilic dye DiI (1,1-dioctadecyl-3,3,3´,3´-tetramethyl-indocarbocyanine perchlorate) following a protocol described by Stern and Holland (1993). Briefly, DiI (0.5% in 100% ethanol as a stock solution) was diluted 1:9 with 0.3 M sucrose containing 0.1% Nile blue sulfate that was used in confirmation of the labeled location. The DiI solution was injected into limb bud tissue using a pulled glass needle. For analysis, embryos were sacrificed, fixed in 4% paraformaldehyde (PFA) and observed using fluorescence microscopy.

Cell culture

The distal regions of limb bud of stage 22, 25 and 28 were dissected out, and mesodermal cells were isolated by the method described previously (Hattori and Ide, 1984). Stainless columns were attached to a plastic culture dish (Falcon 3001 or 3002), and the isolated mesodermal cell suspension in F12 medium containing 1% fetal calf serum (FCS) was plated into each column. After 3 hours, culture medium was added into each well, the columns were removed, and the cultures were maintained at 37°C in 5% CO_2.

In the case of culture with pellets, mesodermal cells were suspended in the F12 medium containing 10% FCS, centrifuged at 150 x g for 5 minutes, and incubated at 37°C for 1 hour to promote cell adhesion. The pellets were dropped on monolayered cell sheets. Pellets were stained with PKH26 (ZYNAXIS Cell Science, Inc.), and monolayered cells were stained with PKH2. They were observed under a confocal laser microscope (OLYMPUS).

Detection of cell death in cell culture

We used the TUNEL method (Gavrieli *et al.*, 1992) for detection of fragmented DNA in apoptotic cells using an *in situ* apoptosis detection kit (TaKaRa) according to the protocol.

Detection of chondrogenesis in cell culture

For analysis of chondrogenesis, cultured cells were fixed with 4% PFA and stained with 0.1% Alcian blue.

Voronoi analysis

We used Voronoi tessellation for analysis of the distributions of cartilage nodules and TUNEL-positive apoptotic signals. Voronoi analysis is a well-known mathematical concept (Okabe *et al.*, 1992). The analysis gives

boundaries that exist between the regions in which each nodule can have an influence; that is, it gives boundaries of the 'territory' of each nodule. Voronoi's boundary is given as a perpendicular bisector of a line linking two nodules (Fig. 4a).

In situ hybridization of cultured cells

Cloned DNA of *Bmp-2*, *Bmp-4* and *Bmp-7* were kindly provided by Dr. Tsutomu Nohno. RNA probes were transcribed by previously described methods (Nohno *et al.*, 1991; Riddle *et al.*, 1993) *In situ* hybridization of cultured cells were performed by methods described by Banker and Goslin, (1991), Hirota *et al.* (1992) and Dirks *et al.* (1998). Briefly, cultured cells were fixed with 4% PFA, and dehydrated and rehydrated by Ethanol/PBS. They were treated with 2 μg/ml Proteinase K, refixed with 4% PFA, and treated with 2 mg/ml glycine/PBS. They were incubated with a prehybridization mixture (50% deionized formamide, 10 mM Tris-HCl pH 7.6, 200 μg/ml yeast tRNA, 1 x Denhardt's solution, 10% dextran sulfate, 600 mM NaCl, 1 mM EDTA, pH 8) at 50°C for 3 hrs, then incubated with the prehybridization mixture containing an mRNA probe at 50°C for 16 hrs. Next, they were washed with solution A (50% formamide, 2x SSC, pH 7), solution B (10 mM Tris-HCl, pH 8, 500 mM NaCl, 1 mM EDTA) and then treated with 20 μg/ml RNaseA, 2x SSC and 0.2x SSC. They were treated with a blocking reagent solution to which was applied an anti-digoxigenin Fab fragments alkaline phosphatase conjugate (Boehringer-Mannheim) for 1 h. NBT and BCIP were used for coloring reaction.

Operations

Leg buds of stage 28 were used for the operations. Interdigital regions were separated from neighboring digit cartilage by making incisions along the margin of the digital ray with two tungsten needles. After the operations, embryos were allowed to develop further at 38°C. When the embryos reached stage 30, they were sacrificed for whole-mount *in situ* hybridization.

Whole-mount *in situ* hybridization

We used the method of Wilkinson (1992) with a slight modification. Embryos were fixed in 4% PFA at 4°C, washed with PBT (PBS with 0.1% Tween 20), dehydrated in methanol, bleached in 5% hydrogen peroxide in methanol, and stored at -20°C. They were then rehydrated and washed with PBT, incubated in 10 μg/ml Proteinase K, washed with 2 mg/ml glycine in PBT, rewashed with PBT, refixed in 4% PFA / 0.2% glutaraldehyde in PBT, washed with PBT, and incubated in a prehybridization mix (50% formamide, 5x SSC, pH 5.0, 1% SDS, 50 μg/ml heparin, 50 μg/ml *Escherichia coli* tRNA) at 70°C. Anti-digoxigenin Fab alkaline phosphatase conjugate (Boehringer-Mannheim) was used for staining.

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