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Studies on the Differentiation of Chondrogenic Cells in Developing Chick Embryo I. Cellular Aggregation and Chondrogenesis

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To establish the *in vitro* culture system and quantitation for chondrogenesis, and to investigate the relationship between cell aggregation and chondrogenesis, chick limb bud mesenchymal cells of Hamburger-Hamilton stage 23/24 were micromass cultured in various cell densities. The chondrogenesis was assayed based on checking the alcian blue-stained nodule numbers, the amount of alcian blue extracted, the change in cell numbers, the rate of [^{35}S] sulfate incorporation and expression of type II collagen. Mesenchymal cells plated with an initial density of high (1×10^7 cells/ml)- and intermediates (5×10^6 cells/ml)-density were differentiated into cartilage. On the other hand, the cells of low density (2×10^6 cells/ml, 5×10^5 cells/ml) of stage 23/24 cells and the stage 18/19 cells in three kinds of cell density did not differentiate into cartilage even though the cells formed an aggregated core at the center of cultured mass. From these results and others obtained in this study, it can be stated that the stage 23/24 mesenchymal cells are likely to pass over the aggregation step and have the potentiality to differentiate into chondrocytes. Thus chondrogenesis *in vitro* can be observed when mesenchymal cells are plated over the threshold density of 5×10^6 cells/ml. Hyaluronidase (HAase) activity was relatively constant throughout the culture, suggesting that the role of HAase may not be important for the cells of stage 23/24.

KEY WORDS: Chick embryo, Chondrogenesis, Micromass culture, Cell aggregation, Proteoglycan, Hyaluronidase

The chick limb bud has been widely used as a model to study cell differentiation. Undifferentiated limb bud mesenchyme consists of at least two separate, possibly predetermined, population of progenitor cells, one derived from somatic mesoderm that gives rise exclusively to skeletal muscle, and one derived from somatopleural mesoderm that gives rise to the cartilage and connective tissue of limb (review by Solursh, 1983).

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Abbreviations: HH, Hamburger-Hamilton; GAG, glycosaminoglycan; HA, hyaluronate; HAase, hyaluronidase; CMF-HBSS, Ca^{2+}, Mg^{2+}-free Hank's balanced salt solutions; FCS, fetal calf serum; TCA, trichloroacetate

Chondrogenesis is one of the first overt cell differentiation events in development of the vertebrate embryonic limb. In the developing chick embryo limb bud, a central core of mesenchymal cells begins to aggregate at around Hamilton-Hamburger(HH) stage 22-24, resulting in an almost 50% increase in cell-packing density (Ede, 1983; Solursh, 1983). In this condensed core, chondrocytes then appear and secrete large amounts of cartilage-specific macromolecules, such as type II collagen (Dessau *et al.*, 1976; Deluca *et al.*, 1977; Shambaugh & Elmer, 1980; Kimura *et al.*, 1981; Kosher and Rodgers, 1987).

The micromass culture method established by Ahrens *et al.* (1977) has been widely used to investigate the chondrogenic process *in vitro*. Through these *in virtro* systems, it has been re-

ported that chondrogenesis is dependent on the developmental stage as well as the plating density of mesenchymal cells. For example, prechondrogenic mesenchymal cells from the earlier limb bud in embryos (at around HH stage 20) will form cartilage only when plated above 2×10^6 cells/ml density (Ahrens *et al.*, 1977; Osodoby and Caplan, 1979; Solursh and Reiter, 1980).

Increasing evidence indicates that cell-cell interactions in limb mesenchyme trigger chondrogenesis by eliciting an increase in intracellular cAMP levels, which leads to formation of cellular aggregation then to differentiation (Kosher, 1983; Solursh, 1983). Although nothing is vitually known about the nature of these cell interactions, the involvement of extracellular matrix molecules such as type I collagen and fibronectin in mesenchymal cell aggregation is suggested by their spatial and temporal pattern of expression in the developing limb (Dessau *et al.*, 1980; Kimura *et al.*, 1981). Also the extracellular hyaluronate (HA) accumulates in whole chick limb buds at prechondrogenic stages, and then disappears from the core prior to cellular condensation process (Toole, 1972). It has been postulated that a pericellular hyaluronate coat prohibits interaction between mesenchymal cells, and that the subsequent decreased synthesis of HA and/or increased secretion of hyaluronidase (HAase) cause the disintegration of this coat to allow cell aggregation followed by the onset of cartilage differentiation (Kosher *et al.*, 1981; Knudson and Toole, 1985; Oster *et al.*, 1985; Kulyk and Kosher, 1987; Toole *et al.*, 1987). Nevertheless, there are controversal observations on the regulation of turnovr of HA. By *in vivo* studies, Toole (1972) reported that HAase activity is first detected in a whole limb bud at the onset of cartilage differentiation and continues to increase during the subsequent chondrogenesis. On the other hand, Kulyk and Kosher (1987) described that HAase activity is clearly detectable in undifferentiated wing bud at the earlier stage (18/19) and remains relatively constant thereafter throughout the subsequent stages of development. They concluded that the onset of chondrogenesis is not associated with changes in the activity of HAase. Most of these studies were carried out *in vivo*.

In this report, we focused on the establishment of *in vitro* culture and assay systems of limb mesenchymal cells for the further studies to elucidate the mechanism of differentiation of chondrogenic cells. In addition, the role of hyaluronic acid in chondrogenesis has been examined.

Materials and Methods

Materials

Fertilized white Leghorn chicken eggs were obtained from Singi hatchery (Tague, Korea) and incubated at 38°C with the 60% of a relative humidity for use in this study.

Cetylpyridine chloride, chondroitin sulfate, dimethylaminobenzaldehyde, hyaluronate (HA), hyaluronidase (HAase), saccharic acid 1,4-lactone, Triton X-100 and FITC-conjugated swine anti-rabbit IgG were purchased from Sigma Chemical Co. (St. Louis, USA). Trypsin was ordered from Fluka(Buchs, Switzland); alcian blue from TAAB Lab (Emmer Green, W/G); scintillation cocktail from Packard (Downers Grove, USA); $Na_2{}^{35}SO_4$(1 mCi) from Amersham(Arlington, USA). All other chemicals were of the highest purity available. Most of the cell culture media and equipments were obtained from Flow Lab (Worth Lyde, Australia) or from GIBCO (Grand Island, USA).

Micromass culture

The micromass technique by Ahrens *et al.*(1977) was used with a slight modification as described below. Limb buds were removed from chicken embryos at Hamilton-Hamburger(HH) stage 18/19 or 23/24, cut in half and placed in Ca^{2+}, Mg^{2+}-free Hank's balanced salt solution (CMF-HBSS) at 37°C. The buds were then enzymatically dissociated in 0.1% trypsin, 0.1% collagenase in CMF-HBSS at 37°C for 30 min with a continuous, gentle shaking. The digestion was stopped by adding fetal calf serum (FCS; 5% final) and tissue clumps were dispersed by vortexing. The cell suspenion was then cleared by passing through eight layers of lens paper and cells were pelleted by centrifugation of 10-ml aliquots (230 × g, 10 min). After resuspenssion in Ham's F-12 with 10% FCS, the cells were examined for viabil-

ity (routinely >95%) based on trypan blue dye exclusion. Cell densities were then adjusted to 1.0 $\times 10^7$ (high-density), 5 $\times 10^6$ (intermdiate-density), and 2 $\times 10^6$ cells/ml (low density), and micromasses were formed by placing three drops of 20-μl of the suspension at the center of a 35-mm plastic culture dish. The cells were then allowed to attach for 1-2 hr at 37°C and 5% CO_2-95% air (a 100 μl drop of Ham's F-12 medium was placed at the edge of the culture dish to maintain humidity during incubation). After incubation, 2 ml of medium was added to each culture. The medium was completely removed and replaced daily with 1.5 ml of fresh medium. Occasionally, 1 ml of cell suspension was applied to 35-mm culture dish for the [^{35}S]sulfate incorporation and HAase activity assay.

All cultures were maintained in Ham's F-12 (GIBCO) with 10% FCS, penicillin (50 U/ml) and streptomycin (50 μ/ml).

Quantitation of chondrogenesis

(1) Nodule number: At various time intervals, cultured cells were rinsed in PBS and fixed with Kahle's fixative for 10 min and stained for sulfated glycosaminoglycans with alcian blue at pH 1.0 for 8 hr (Lev and Spicer, 1964). The scoring of alcian blue-positive cartilage nodules was used as a parameter for assaying of chondrogenesis. Nodules were counted under the inverted microscope (Leitz, Model Labovert).

(2) Amount of dye bound: The quantity of cartilage was estimated by measuring the amount of extractable dye (Pennypacker, 1983; San Antonino and Tuan, 1986). Dye was extracted in 6 M guanidine-HCl (1 ml/dish) for 8 hr at room temperature and dye concentration was estimated as absorbance at 650 nm (A_{650})

(3) Cell number counting: Plates were treated with 0.1% collagenase and 0.1% trypsin for 20 min and the cell number was counted with Coulter counter (Coulter Electronics, Model ZM).

(4) Glycosaminoglycan synthesis: The incorporation of [^{35}S]sulfate into glycosaminoglycans of the cell surface and medium was measured (Goldberg and Toole, 1984). Cultures were labelled with 5-10 μCi of $Na_2{}^{35}SO_4$ for 6 hr and cell layer and medium fraction were separated at the end of incubation period. Plates were washed 4 times with saline to remove unlabelled [^{35}S]sulfate. Cells were homogenized by sonication. To the 1 ml of cell homogenate, 1 ml of 20% TCA with 0.1 ml of carrier fetal calf serum was added and stayed overnight.

Proteins were precipitated by centrifugation (10,000 $\times$ g, 10 min) and washed 3 times with 10% TCA-0.1 M $MgSO_4$. Pellets were dissolved in 1 ml of 0.2 M Tris-HCl buffer containing 1 mg/ml of pronase, pH 7.8, and incubated for 24 hr at 55°C. After reaction was stopped by boiling, the labeled glycosaminoglycans were precipitated with 1 ml of 1% cetylpyridium chloride and 1 ml of Tris buffer (pH 7.8) in the presence of carrier chondroitin sulfate. After 1 hr of incubation at room temperature, the samples were centrifuged at 10,000 $\times$ g for 10 min. the pellets were dissolved in 0.3 ml of methanol followed by addition of 2 ml of scintillation cocktail and the radioactivity were counted with the liquid scintillation counter (Packard, Model Tricarb 1500).

Immunohistochemistry

Chick myosin and type II collagen prurified from chick breast muscle and sternal cartilage, respectively, were used for immunogen, and the antisera against chick myosin and type II collagen were raised in rabbits by conventional method. The detailed purification and characterization of these immunogen and antibodies were described elsewhere (Kang *et al.*, 1990).

For the immunofluorescene, cultures were fixed with 95% ethanol and rinsed with 0.01 M Tris--buffered saline (pH 7.5). Rabbit anti-type II collagen antiserum was added to the sample and incubated for 30 min. The samples were stained with FITC-conjugated swine anti-rabbit IgG and observed under the fluorescenece miscroscope (Nikon, Model Labophot).

Determination of hyaluronidase activity

Cells were harvested and sonicated in cold formate extraction buffer (0.1 M sodium formate/0.15 M NaCl/0.1% Triton X-100, pH 3.7) containing 2.5 mM saccharic acid 1,4-lactone, an inhibitor of exoglycosidase activity. Aliquots of sonicate were removed for protein determination (Hartree, 1972) and the remainder of the sonicate

was centrifuged at 10,000 × g for 5 min. Aliquots of the supernatant were incubated at 37°C for 18 hr in the presence of exogenous HA substrate (Sigma grade I HA; 100 μg HA per 125 μl reaction mixture). HAase activity was measured by assaying the formation of reducing terminal N-acetylglucosamine-containing oligosaccharides as described by Kulyk and Kosher(1987). Briefly, the 125 μl reaction mixtures were evaporated to dryness, and the residue was dissolved in 35 μl of 0.125 M potassium tetraborate and heated in a boiling water bath for exactly 3 min. After cooling, the samples were supplied with 165 μl of diluted dimethylaminobenzaldehyde reagent and incubated at 37°C for 20 min. At the end of incubation, samples were cooled in an ice bath and centrifuges at 10,000 × g for 5 min., and the absorbance of the supernatants was measured at 585 nm (Hitachi, Model U-2000). One unit of HAase is defined as micrograms of terminal N-acetylglucosamine released per 18 hr at 37°C.

Results

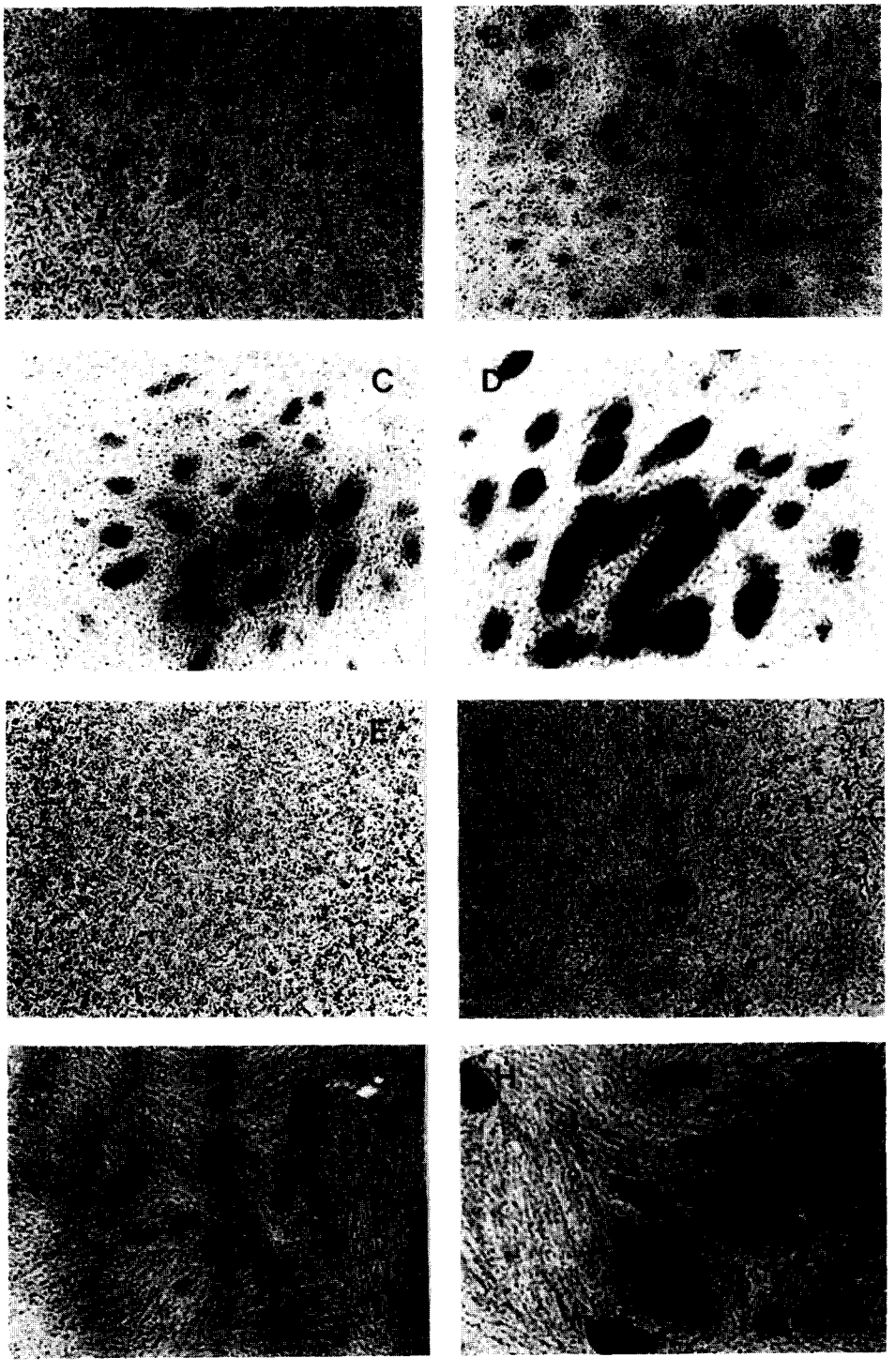
Limb bud mesenchymal cells isolated from HH stage 23/24 chicken embryos exhibited distinct phenotypic changes when cultured as a micromass *in vitro* (Fig. 1). Culture cells seeded at an initial plating density of 1×10^7 cells/ml (high-density cultures) began to form cartilage nodules between day 2 and day 3, and the number of nodules was increased up to 15-18/dish by day 3 in cultures (Fig. 1B). With further development, nodules were fused together to become larger ones (Fig. 1C and D). In the intermediate-density (5×10^6 cells/ml) cultures (Fig. 1E-H), nodules appeared slowly compared with those in the high-density cultures, however, the overall patterns of morphological change were similar to those in high-density cultures. On the other hand, the cells in low-density (2×10^6 cells/ml) cultures did not make any nodules throughout the culture periods (Fig. 1I-L). As shown in Fig. 2, the alcian blue-stained nodules are formed by the condensation of cells, therefore, these serial events resulting in the increase the number of formation of cartilage nodules is indicative of chondrogenesis *in vitro* (Fig. 1).

Since the production of sulfated glycosaminoglycans (GAGs) is a hallmark of chondrogenesis(Hunter and Caplan, 1983), quantitation of alcian blue bound to sulfated GAGs was carried out by dye exclusion method as described in 'Materials and Methods'. The dye concentration (A_{650}/dish) in hgih-density culture decreases slightly at day 2, and then increased drastically at day 3, followed by gradual increase (Fig. 3A). In the intermediate-density culture, the dye concentration appeared similar pattern to that of high-density culture, but showed narrow change. On the contrary, the dye concentration in low-density culture did not change throughout the culture periods. The pattern of cell numbers in three kind of density cultures appeared similar to that of dye concentration (Fig. 3B).

In order to analyze the extent of cartilage differentiation, the rate of [^{35}S]sulfate incorporation into cell layer macromolecules was measured. Increased synthesis of sulfated GAGs was evident in high-and intermediate-density cultures throuhout the entire culture periods (Fig. 4). On the other hand, [^{35}S]sulfate incorporation into cell layer macromolecules in low-density culture could not be detected.

To confirm the above results immunohistochemistry was carried out using anti-chick type II collagen and anti-chick myosin light (L)-chain. Only in high- and intermediate-density cultures the cells were stained with antibodies against type II collagen after day 3 in cultures, of which the cartilage nodules showed intense fluorescence, whereas the surrounding cells showed no staining (Fig. 5A). Within the nodule, the chondrocytes exhibited intense cytoplasmic fluorescence. In contrast, all the cultures stained with anti-chick myosin L-chain showed no fluorescence throughout the culture periods (Fig. 5B).

Stage 18/19 mesenchymal cells were also micromass cultured and stained with alcian blue. The cells began to aggregate from day 2 in cultures and this aggregation was increased throughout cultures periods (Fig. 6). While the cells of 23/24 stage formed condensed cores in many points, the cells of 18/19 stage made only one aggregated core at the center of cultured mass. Moreover, this core was not stained with alcian blue, indicating that the 18/19 stage cells did not become cartil-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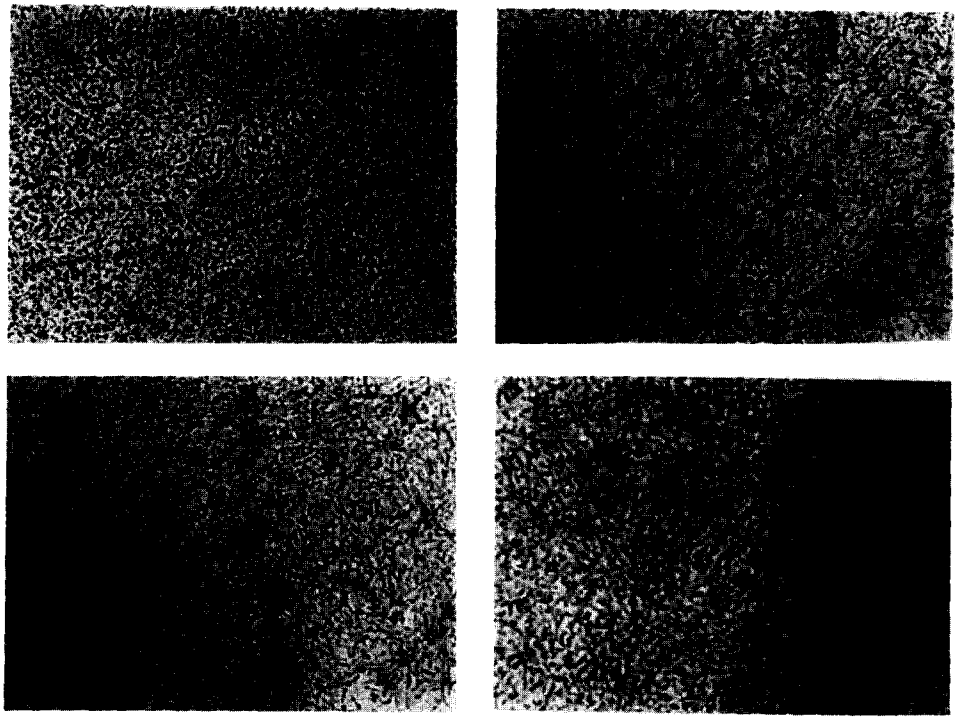

Fig. 1. Chondrogenesis of stage 23/24 limb bud mesenchymal cells. Ten μl of limb bud mesenchymal cells at HH-stage 23/24 was plated at high-density (1×10^7 cells/ml, A, B, C, D), intermediate-density (5×10^6 cells/ml, E, F, G, H), or low-density (2×10^6 cells/ml, I, J, K, L). Cells were stained with alcian blue after 1 day (A, E, I), 3 days (B, F, J), 4 days (C, G, K), and 5 days (D, H, L) in cultures. All the photomicrographs were taken at $\times$ 25.

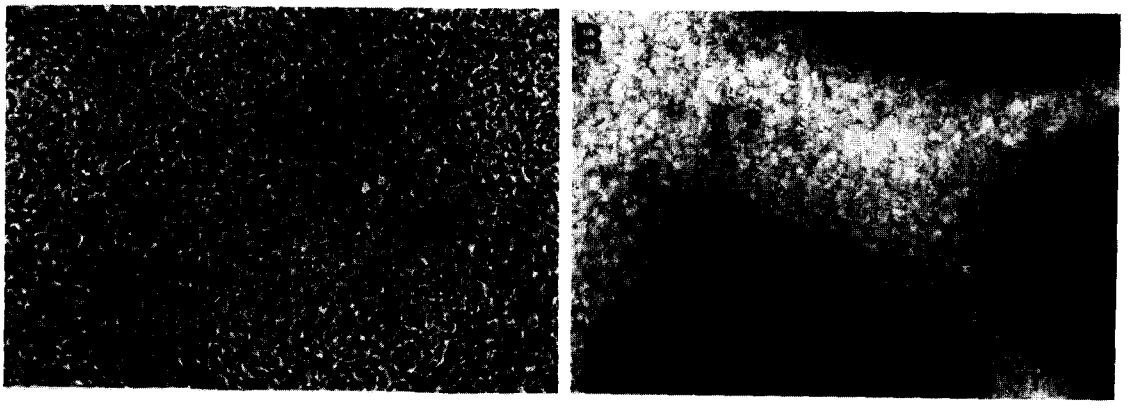

Fig. 2. Phase contrast image of cell condensation (A) and nodules stained with alcian blue (B). Limb mesenchymal cells (HH-stage 23/24) were plated at high density (1×10^7 cells/ml). The arrows indicate the location of condensation of cells (A) and cartilage nodule (B). Photomicrographs were taken at $\times$ 200.

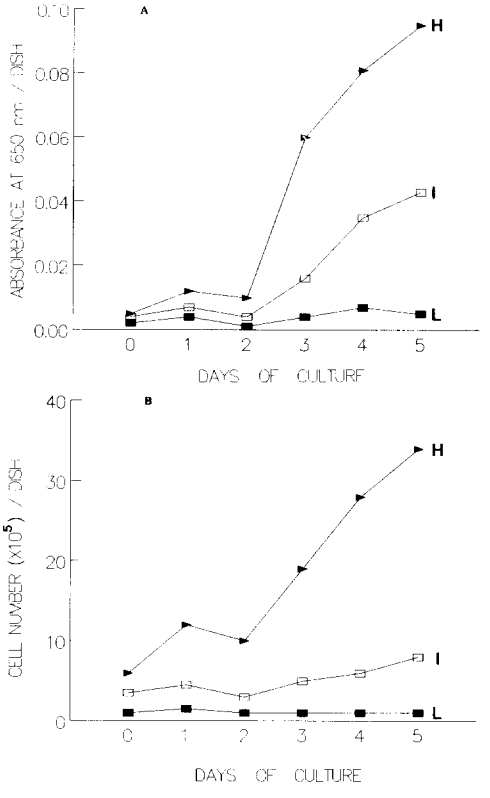

Fig. 3. (A) the amount of dye bound to sulfated glycosaminoglycan in low (2×10^6 cells/ml; L)-, intermediate (5×10^6 cells/ml I) and high (1×10^7 cells/ml; H)-density cultures. alcian blue bound to sulfated glycosaminoglycan in each culture was extracted in 6 M guanidine-HCl for 8 hr, and the dye concentration was estimated as A_{650}. (B) The counting of cell numbers in each kinds of cell density cultures. Each culture plate was treated with 0.1% collagenase and 0.1% trypsin for 20 min and the cell number was counted. Each point represents the mean of three determinations.

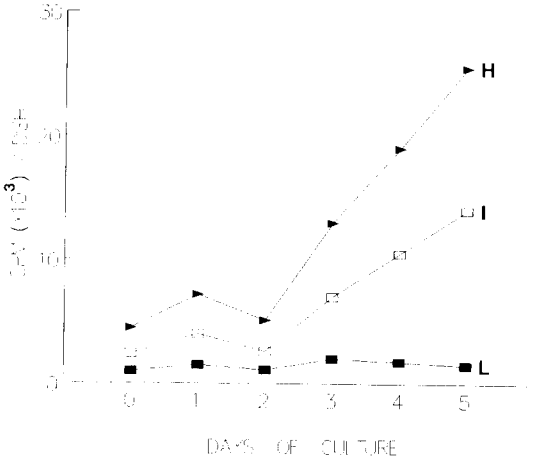

Fig. 4. The rate of [^{35}S]sulfate incorporation into cell layer macromolecules in low (L)-, intermediate (I)- and high (H)-density cultures. Each point represents the mean of three determinations.

age. The amount of alcian blue bound to cells layer was appeared low in all density cultures throughout the culture periods. Furthermore, the [^{35}S]sulfate incorporation was not detected in all cultures and no type II collagen expression could be observed (data omitted).

There were little change in the hyaluronidase (HAase) activity throughout the culture periods in all density cultures (Table 1).

Table 1. Hyaluronidase activities in low- and intermediate- and high-density cultures.

	Days of Culture				
	1	2	3	4	5
low-density culture	15.6 ± 2.2	13.4 ± 1.7	12.7 ± 1.9	13.1 ± 2.1	14.4 ± 4.8
intermediate-density culture	16.0 ± 3.0	15.3 ± 3.1	16.2 ± 2.8	17.3 ± 0.8	16.2 ± 7.8
high-density culture	16.3 ± 2.6	16.1 ± 1.9	17.5 ± 1.4	171. ± 1.0	15.8 ± 4.3

HAase activity is expressed as units per mg of protein. One unit of HAase is defined as micrograms of terminal N-acetylglucosamine released for 18 hr at 37°C. Each value is the mean ± SE of three determinations.

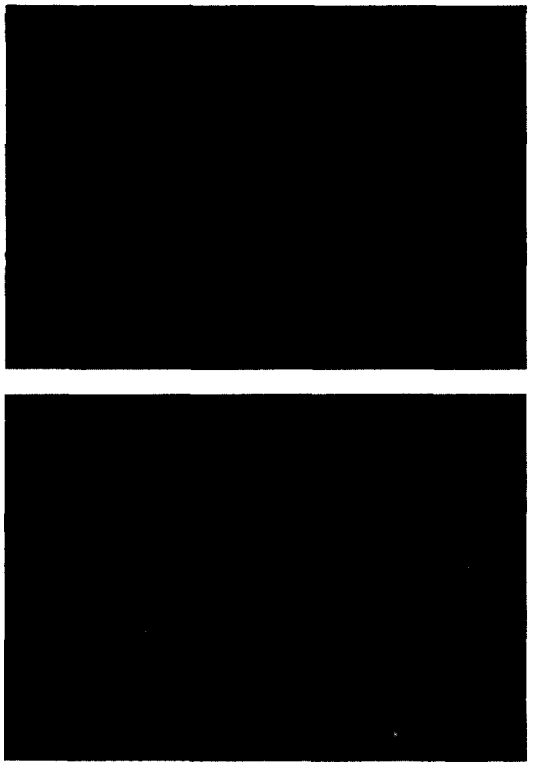

Fig. 5. Indirect immunofluorescence of day-3 cultured cells at HH-stage 23/24 in intermediate-density culture stained with rabbit anti-chick collagen type II (A) and rabbit anti-chick myosin L-chain (B) antibody, followed by FITC-conjugated swine anti-rabbit IgG. The arrows indicate the cartilage nodules showing intense fluorescence.

Discussion

It has been reported that mesenchymal cells aggregate to become condensed, followed by differentiation into cartilage cells accompanied with the synthesis of type II collagen and sulfated glycosmainoglycans (Solursh, 1983). The importance of cell aggregation for the differentiation of mesenchymal cells into cartillage *in vivo* was emphasized by many investigators (Dessau *et al.*, 1980; Solursh and Reiter, 1980; Kimura *et al.*, 1981; Kosher and Rodgers, 1987). It was also reported that the potential for chondrogenesis *in vitro* was a density-dependent event (Ahrens *et al.*, 1977; Osdoby and Caplan, 1979). San Antonio and Tuan (1986) showed that Ca^{2+} promoted cell-cell aggregation phase of chondrogenesis in normally non-chondrogenic, low density cultures. However. these workers took the presence of alcian blue stained nodules as a parameter for chondrogenesis.

We attempted to identify the state of differentiation in details by assaying the amount of extracted alcian blue bound to sulfated GAGs, the change in cell numbers, the rate of [^{35}S]sulfate incorporation into progeoglycan, and expression of type II collagen by immunohistochemistry. In this study, the cells in high (1×10^7 cells/ml)- and intermediate-density (5×10^6 cells/ml) cultures showed condensed cores (cartilage nodules) as judged by morphological patterns (Fig. 1, A-H and Fig. 2). Also the amount of alcian blue bound to sulfated GAGs, the cell numbers and the rate of [^{35}S] sulfate incorporation into cell layer macromolecules were increased throughout the culture periods (Figs. 3 and 4). Besides these cells presented positive responses against anti-type II collagen antibody by indirect immunofluorescene (Fig. 5). However, in low-density culture, no nodules were appeared (Fig. 1, I-L) and the amount of dye concentration, cell numbers and the rate of [^{35}S] sulfate incorporation were remained constant throughout culture periods. Also these culture were not stained with anti-type II collagen. In the reduced cell density cultures (5×10^5 cells/ml), similar results were obtained. The stage 18/19 mesenchymal cells did not differentiate into cartilage even though they aggregated into the center of the cultures (Fig. 6). Therefore it can be said that the HH stage 23/24 mesenchymal cells have the potentiality to differentiate into chondrocytes, and the threshold value of initial plating density for *in vitro* culture system was 5×10^6 cells/ml, below which chondrogenesis did not occur.

Thorogood and Hinchliffe (1975) showed that an increase in mesenchymal cell number per unit area occures in the central chondrogenic locus prior to matrix formation at stage 24 cell cultures. Since our cells in high- and intermediate-density cultures showed increasing cartilage nodules at day 3 (Fig. 1) and the cells proliferated drastically between day 2 and day 3 (Fig. 3B), stage 23/24

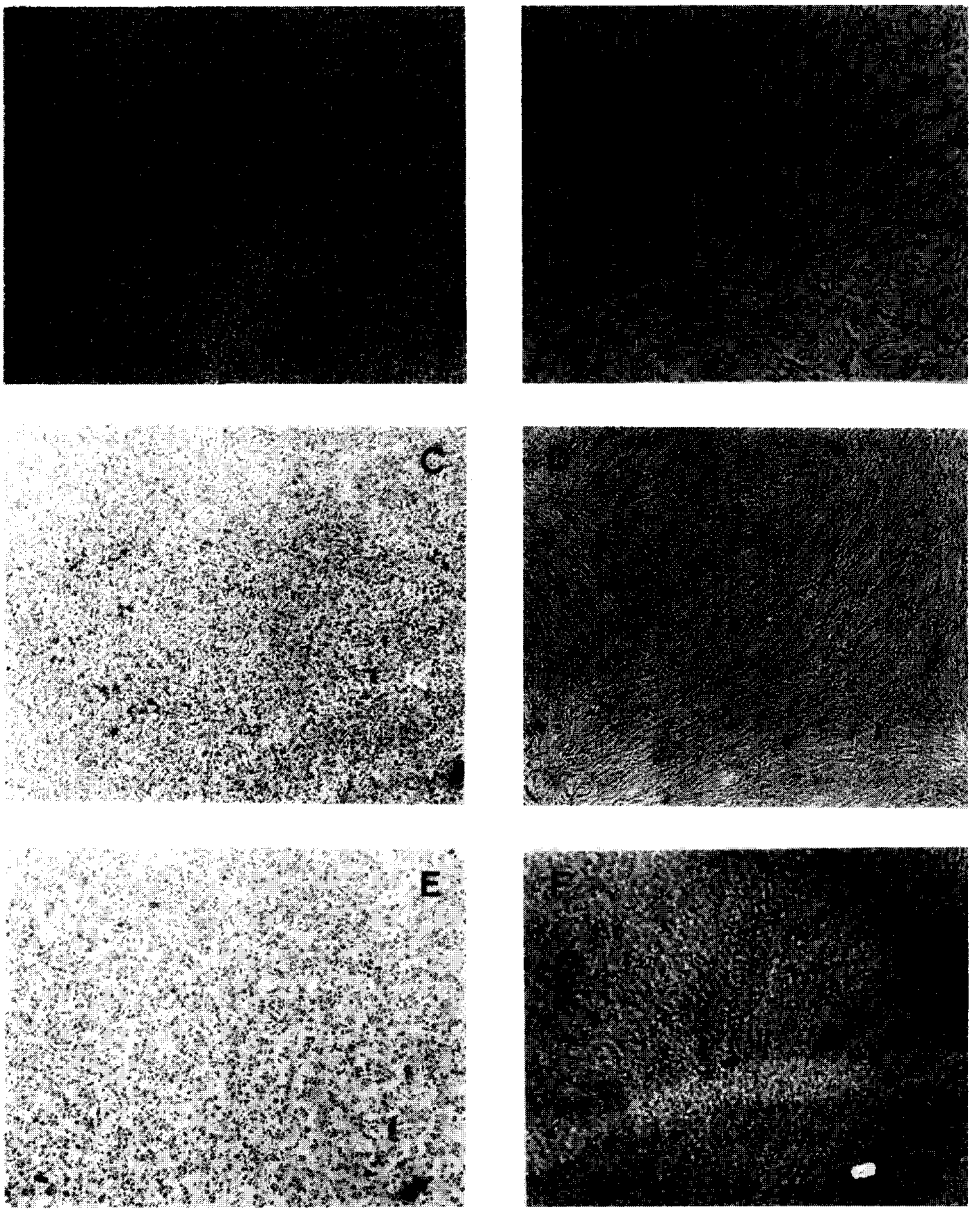

Fig. 6. Cultures of stage 18/19 limb bud mesenchymal cells. Twenty - μl of limb bud mesenchymal cells at HH-stage 18/19 were plated at high-density (1×10^7 cells/ml, A, B), intermediate-density (5×10^6 cells/ml, C, D) or low-density (2×10^6 cells/ml, E, F). Cells were stained with alcian blue after 1 day (A, C, E), and 5 days (B, D, F) in cultures.

mesenchymal cells in the present study are likely to pass over the aggregation step resulting in condensation. It is noteworthy that *in vitro* culture system undifferentiated mesenchymal cells differentiated between day 2 and 3 in culture (Figs. 1 and 3), so the chondrogenic analyses in this system should be carried out within day 3 in culture.

The interactions between cell and extracellular factors should be also considered. Cartilage differentiation is influenced by epithelia (Solursh *et al.*, 1981) or extracellular matrix such as hyaluronic acid (Solursh *et al.*, 1980; Kujawa and Caplan, 1986; Kujawa *et al.*, 1986; Knudson and Toole, 1987) and heparan sulfate (San Antonio *et al.*, 1987). Hyaluronic acid (HA) is believed to affect the plasma membrane-associated features of mesenchymal cells to influence the mesenchyme cells to express chondrogenic potency. HA concentration in the limb core decrease before the mesenchymal condensation and hyaluronidase might play a role in reducing the HA (Toole, 1972). In the present study, the hyaluronidase activity kept constant throughout the culture of 23/24 mesenchymal cell (Table 1). This result can be explained in two way. The one is that the decrease of HA during chondrogenesis is caused by a decrease of synthesis of new HA, while the degradaton rate is constant. The other one is that hyaluronidase is related to the HA reduction. Logically decrease of HA should be occured before the cell condensation, therefore, the activity of HAase in the stage 23/24 mesenchymal cell which might have passed the aggregation step does not need to be increased, which still remains to be further elucidated.

From our experimental results investigated so far, it can be suggested that stage 23/24 mesenchymal cells have already acquired the ability to differentiate into cartilage cells, and initial plating density of 1×10^7 cells/ml of HH stage 23/24 are proper to persue chondrogenesis. With this established culture system and the quantitation procedures, the morphogenesis of chondrogenic cells, chondrogenic materials and chondrogenic gene expression are scheduled to investigate, some of which are in progress.

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발생계배 연골세포의 분화기구에 대한 연구 I. 세포응집과 분화와의 관계

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연골세포 분화기구 연구의 기초단계로서 미세 세포배양법을 정립하였으며, 세포의 응집성도와 연골분화의 관계를 조사하기 위하여 계배 limb bud 간충직세포를 여러가지 농도로 micromass 배양하면서 세포농도에 따른 세포증식정도와 proteoglycan에 결합된 alcian blue의 양 및 [^{35}S]sulfate의 sulfated proteoglycan에 표지되는 속도를 측정하고, 면역조직화학법을 이용하여 type II collagen의 발현을 관찰하였다. 각 농도별로 배양한 Hamburger-Hamilton (HH) stage 23 / 24 간충직세포 중 5×10^6 cells / ml 이상의 농도로 배양한 세포는 연골세포로 분화하였으나, 저농도 (5×10^5 cells / ml, 2×10^6 cells / ml)로 배양한 세포는 분화하지 않았다. 반면에, stage 18/19 간충직세포는 고농도 (1×10^7 cells / ml) 또는 중간농도 (5×10^6 cells / ml)로 배양시 배양 가운데로 응집핵이 형성되는데도 불구하고 연골화가 되지 않았다. 이러한 결과들로 보아 stage 18/19 간충직세포는 분화단계 중에서 동일세포들끼리 응집되는 단계이며 stage 23/24 간충직세포는 이 단계를 지나 분화능을 갖는 세포응축의 단계인 것으로 생각된다. 본 연구 결과 세포응집 및 분화능은 간충직세포의 발생시기에 따라 다르며, 분화능을 갖기 위해서는 세포응집이 선행조건이고, 그 적정 미세 배양 농도는 $5-10 \times 10^6$ cells / ml임을 알았다. 한편, hyaluronidase의 활성은 stage 23/24세포 배양 전 과정에서 비교적 일정한 것으로 나타나 이 시기의 세포분화에는 별로 중요하지 않는 것으로 보인다.