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Identification and Characterization of Various Differentiative Growth Plate Chondrocytes From Porcine by Countercurrent Centrifugal Elutriation

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Abstract Countercurrent centrifugal elutriation was used to separate growth plate chondrocytes from porcine basing on their differences in sizes and densities. Eighteen fractions of cells with different sizes and densities were obtained. The mean cellular volumes increased progressively in each of successive fractions, and that increase was associated with specific phenotypic changes, such as biochemical differences in DNA synthesis, proteoglycan synthesis, and activities of alkaline phosphatase. Three distinct chondrocyte subpopulations with their unique characteristics were identified among the elutriated fractions. The resting chondrocytes were found to be small in size and quiescent. The hypertrophic chondrocytes were found to be large in size and metabolically active both in alkaline phosphatase and in proteoglycan productions. The proliferative chondrocytes exhibited a high DNA synthesis rate, and their sizes were found to be between those of the resting and hypertrophic chondrocytes. © 1996 Wiley-Liss, Inc.

Key words: chondrocyte, porcine, countercurrent centrifugal elutriation, cartilage, alkaline phosphatase

Cartilage is a specialized dense connective tissue which is avascular and does not contain nervous tissue. It is made up of chondrocytes embedding in a matrix which consists largely of water, collagen fibrils, and proteoglycan macromolecules. These macromolecules form a fiber-reinforced gel. In all mammals, cartilage acts as temporary skeleton in the embryo and serves as a basic structure on which bones develop. In human adults, the main function of cartilage is to provide support for soft tissues and gliding areas for joints. Moreover, cartilage is also formed during the process of fracture healing.

The characteristics of cartilage are determined by the different amounts of proteoglycan and the ratio of the different types of collagen in the matrix. As all the matrix substances are secreted by the chondrocytes, it is of value to establish an in vitro model to facilitate the study of the biochemistry of these cells. A better understanding of the differentiation process of the chondrocytes in the cartilage should be essential for improvement in the treatment of fractures and other orthopaedic diseases.

During the last decade, most works on cartilage research have been focused on the composition of the extracellular matrix and the molecular structure of its components, with little advance being made in the area of characterization of chondrocytes [reviewed by Delbruck and Gurr, 1986]. One of reasons may be due to the difficulties in isolating chondrocytes from the cartilage. Most investigators have attempted to prepare pure samples of the different zones of growth plate cartilage by microdissection. For example, Wuthier [1969] reported the method of scraping off sequential layers of calf growth plate. Kuhlman [1965] reported the method of taking microtome sections of dog and rabbit growth plates. However, the scraping technique presents problems in reproducibility and identification of layers, while the microtome method requires tedious procedures including preliminary freezing. Another technique, micropuncture, allows precise localization of the cells to be analyzed, but the potential number of analyses

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that can be performed is limited. Moreover, Ray et al. [1982] reported that the Ficoll density gradient which is a commonly used method in hematological studies cannot be used to separate chondrocytes with different cell densities. Until recently, O'Keefe et al. [1989] successfully applied a relatively new technique of countercurrent centrifugal elutriation (CCE) which is a relatively new technique to separate and identify the chondrocytes from chick growth plate according to their sizes and densities. When studying the biochemistry of these cells, they found that transforming growth factor-β exhibits a larger mitogenicity on hypertrophic cells and proliferating cells than on smaller resting cells [Rosier et al., 1989]. They further showed that alkaline phosphatase activity and type X collagen synthesis are increased in hypertrophic chondrocytes [O'Keefe et al., 1990; Gunter et al., 1990]. It is of interest to investigate whether maturationally distinct fractions of chondrocytes from species other than chick can also be separated by CCE so that the biochemistry of those cells can be elucidated. In this study, we have applied CCE to separate porcine chondrocytes on the basis of sizes and densities. We also studied the alkaline phosphatase activity and the rates of DNA synthesis and proteoglycan synthesis of these cells at various stages of differentiation.

MATERIALS AND METHODS

Isolation of Porcine Chondrocytes

Protocol concerning animal research ethics has been approved by the relevant committee of our Institute. The method of O'Keefe et al. [1989] was followed with minor modifications. Chondrocytes were isolated from porcine between 4 and 5 weeks of age (about 10 kg). After the animal was sacrificed by injecting 10 ml of 2.5% pentobarbital directly into its heart, the rib cage was aseptically dissected and cleaned until free from soft tissue. The growth plate cartilage was taken out at the osteochondral junctions of all the ribs. The cartilage was then placed in a modified F-12 medium (magnesium-free, 0.5 mM $CaCl_2$) (Sigma, St. Louis, MO). After weighing, the cartilage was cut into 0.1 mm pieces and subjected to digestion with 0.1% trypsin (type III; Sigma) for 30 min in the modified F-12 medium at 37°C in order to break down the core and link protein in the cartilage matrix. The tissue was subsequently rinsed twice with the modified F-12 medium and subjected to 0.1% hyaluronidase (type I-S; Sigma) digestion for 1 h to cleave the proteoglycan aggregate. Overnight digestion in 0.1% collagenase (type IIA; Sigma) in a shaking water bath at 37°C was further applied to break down the collagen fibrils. One milliliter of each enzyme solution was used for every 20 mg of cartilage. After centrifugation (for 4 min at 600*g*), the cells were then filtered through a glass wool filter and washed twice with the modified F-12 medium to remove the digested matrix debris. Subsequent to counting with a hemocytometer, the chondrocytes were then separated by countercurrent centrifugal elutriation.

Labeling of Chondrocytes for Elutriation

Chondrocytes were released from resting zone cartilage far from the osteochondral junction of ribs. At the same time, thin growth plate cartilage layers at the osteochondral junction were also obtained. Part of the resting zone and growth plate cartilage was retained for histological confirmation.

Chondrocytes from the resting cartilage were labeled with L-[2,3-^{3}H] proline (100 μCi/mmol) in the presence of 0.1 mol unlabeled proline in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma) with 5% fetal bovine serum for 2 h at 37°C. These cells were rinsed twice with the modified F-12 medium containing cold proline (1 mmol) to remove radioactivity that was not associated with the cells. The labeled chondrocytes were then pooled with unlabeled total growth plate cells (i.e., cells from resting zone cartilage plus cells from thin growth plate cartilage) for countercurrent centrifugal elutriation.

In some experiments, chondrocytes released from the thin layer growth plate cartilage were labeled by the same procedures as described for the chondrocytes released from the resting cartilage.

Countercurrent Centrifugal Elutriation (CCE)

A Beckman J-6M/E centrifuge (Beckman, Palo Alto, CA), installed with a JE-5.0 elutriator and Sanderson Chamber, was thoroughly equilibrated with modified F-12 medium containing 5% fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY). The cells were exposed briefly to DNase (Sigma) to prevent cell aggregation. About 6×10^7 cells were loaded onto the rotor (radius = 10.6 cm) in 20 ml of the modified F-12 medium containing 5% FBS at 4°C. Loading was performed at a constant rotor speed of 3,000

rpm. After the cells were loaded and equilibrated in the separation chamber for 10 min, elutriation was performed at various rotor speeds from 3,000 to 1,600 rpm (see Table I). The fluid flow was maintained at a rate of 30 ml/min throughout the procedure. Cells were collected with two 50 ml sterilized centrifuge tubes (Corning, Corning, NY) at each rotor speed.

Chondrocyte Size Determination

The Coulter Counter-Channelyzer C256 system (Coulter Electronics, Hialeah, FL) was used to determine the size distribution of the separated cells in each tube. The mean cell volumes were determined by using a calibration constant derived from latex spheres of uniform size. Standard deviations for the mean volumes and diameters of the elutriation fractions were determined by analyzing the distribution curves from the Coulter Counter.

Chondrocyte Cultures

Chondrocytes in the pooled elutriation fractions were plated in 24-well culture plates (Corning) at a density of 1×10^5 cell/cm^2 in DMEM containing 5% FBS, 50 mg/ml ascorbate (Sigma).

TABLE I. Porcine Chondrocyte Elutriation Profile*

Elutriation Fraction	Rotor speeds (rpm)	Number of cells (millions)	Mean cell volume (fl)
1	2,900	0.61	767
2	2,800	0.44	810
3	2,700	0.87	832
4	2,600	2.17	853
5	2,500	4.01	886
6	2,400	6.35	927
7	2,300	7.17	981
8	2,250	5.60	1,042
9	2,200	4.00	1,196
10	2,150	3.04	1,388
11	2,100	2.75	1,569
12	2,050	2.76	1,749
13	2,000	2.09	1,878
14	1,950	1.68	2,073
15	1,900	1.49	2,326
16	1,850	0.95	2,668
17	1,800	0.56	2,796
18	1,700	0.63	2,859

*About 6×10^7 porcine chondrocytes were loaded onto the rotor (radius = 10.6 cm) of a Beckman J-6 M/E centrifuge. Elutriation was performed at various rotor speeds (in rpm) as indicated.

Assays on cultured cells were performed after the cells had settled for 9 h in culture.

Thymidine Incorporation Assay on Cultured Chondrocytes

After the elutriated cells had settled for 9 h in monolayer culture, they were then labeled with 5 μCi/ml [^{3}H]-thymidine (40 Ci/mM) (New England Nuclear, Boston, MA) in the presence of 5 μM unlabeled thymidine as carrier in DMEM containing 5% FBS for 4 h at 37°C. The medium with excess thymidine was aspirated, and the cells were washed with 1 ml 0.15 N NaCl. The cells were then lysed with 0.25 ml 0.25 N NaOH. After 20 min, attached cells were scraped out with a cell scraper. The lysed cell solution was transferred to a 5 ml plastic tube. Another 0.25 ml NaOH was used to rinse the well of the culture plate and also poured into the same tube. Alkalinity was neutralized by adding 0.5 ml of 0.25 N HCl. One milliliter of HEPES-Mg-Ca with 2.5 mg/ml bovine serum albumin, which acted as protein carrier, was also added to the tube. Deoxyribonucleic acid (DNA) was precipitated with 0.5 ml of 10 N perchloric acid at 4°C for 20 min. The precipitate was spun at 18,000g for 30 min at 4°C. After aspirating the supernatant, 0.5 ml of 0.25 N NaOH was added to resuspend the pellet. The resuspended solution was transferred to counting vials, and 4 ml of scintillation fluid was added. The radioactivity of the acid-insoluble DNA content was counted by a Beckman Liquid Scintillation Spectrometer. Standards of the radiolabeled medium were prepared for direct calculation of the incorporation of radioactive thymidine into DNA.

Alkaline Phosphatase Assay on Cultured Chondrocytes

The enzyme activity of alkaline phosphatase was by colorimetric method with p-nitrophenyl phosphate as the substrate [O'Keefe et al., 1989]. Assays were performed directly in the culture wells containing approximately 1×10^5 cells/well after the isolated chondrocytes had settled for 16 h. The culture medium was aspirated from the wells, the cells were then rinsed with 150 mM NaCl solution, and the supernatant again was aspirated. One milliliter of reaction buffer containing 0.25 M 2-methyl-2-amino propanol, 1 mM $MgCl_2$, and 2.5 mg/ml p-nitrophenyl phosphate (Sigma) at pH 10.3 was then added to the wells at 37°C to initiate the reaction. After the reaction was stopped by adding

0.5 ml 0.3 M trisodium phosphate, pH 12.3, the absorbance at 410 nm was measured using 0.15 M NaOH as blank. The activity of alkaline phosphatase was calculated from a standard curve prepared by using p-nitrophenol and expressed as micromoles per 10^6 cells per hour ($\mu M/10^6$ cells/h).

Sulfate Incorporation Assay on Cultured Chondrocytes

In this assay, cell cultures were exposed to 12 $\mu Ci/ml$ of $^{35}SO_4^=$ sodium salt (DuPont NEN, Boston, MA) in DMEM, which contained 0.814 mM carrier sulfate, for 4 h at 37°C. After incubation, the medium was transferred into a small plastic tube. Cells were lysed by adding 0.25 ml of 0.25 N NaOH to each well and left for 20 min. Cells were then scraped out with a cell scraper and were transferred to the same plastic tube. Another 0.25 ml NaOH was used to rinse the well of the culture plate and transferred to the tube. After adding 0.75 ml of 0.15 N NaCl, the solution was dialyzed against phosphate buffered saline, pH 7.4, using 12,000–14,000 MW dialysis tubing (Spectrum Medical, Los Angeles, CA) in order to remove unincorporated radiolabeled sulfate. Phosphate buffered saline was changed twice a day. After 3 days, 0.75 ml of the sample inside the dialysis tubing was placed in a scintillation vial to which 4 ml of scintillation fluid was added. The radioactivity of $^{35}SO_4^=$ incorporated macromolecules was quantified by a Beckman scintillation spectrometer. Data are expressed as nanomoles of sulfate incorporation per 10^6 cells per hour (nmol/10^6 cells/h).

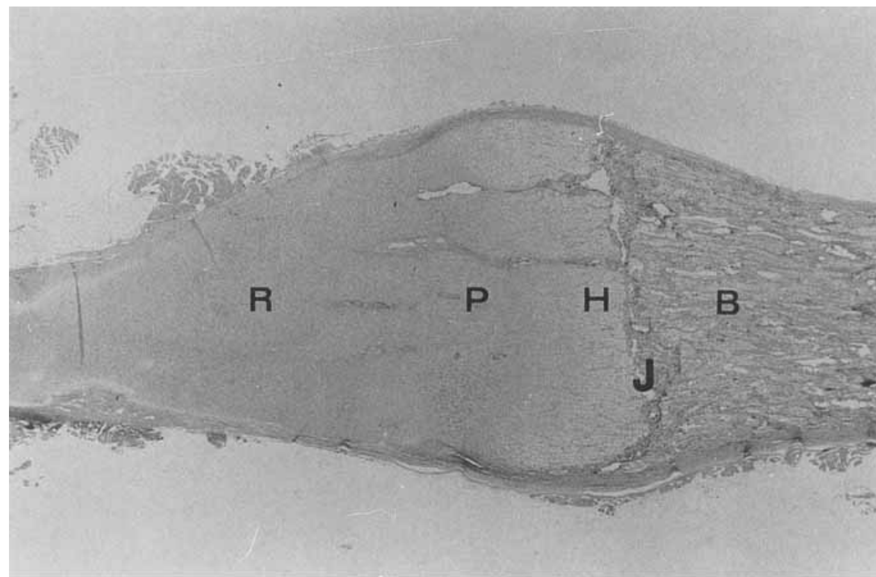
RESULTS

Porcine rib is an ideal model to study endochondral ossification since its growth plate chondrocytes differentiate in a very regular manner, as demonstrated in Figure 1, which shows the histological section of porcine rib growth plate. The cartilage far from the osteochondral junction is the resting zone cartilage containing homogeneous small resting chondrocytes. At about 1 mm from the junction, unidirectional chondrocyte divisions occur, and stacks of proliferative chondrocytes can be seen. At the front of these stacks facing the junction, chondrocytes become hypertrophic, and the largest chondrocytes are located adjacent to the bone (Fig. 1a,b). Figure 2 shows the histological sections of a growth plate slice for chondrocyte isolation. A smooth mineralized and vascularized zone separates the bone and cartilage tissue. When force was applied to both ends of the rib, the cartilage tissue containing the entire growth plate detached easily from the mineralized bone tissue. A thin slice of growth plate cartilage, approximately 1.5 mm thick, containing all the hypertrophic and proliferative chondrocytes, part of the resting chondrocytes, and some red blood cells which were attached on the fracture site (Fig. 2a) could then be dissected. At the same time, pure resting chondrocytes were obtained by dissecting tissue from the resting zone cartilage (Fig. 2b).

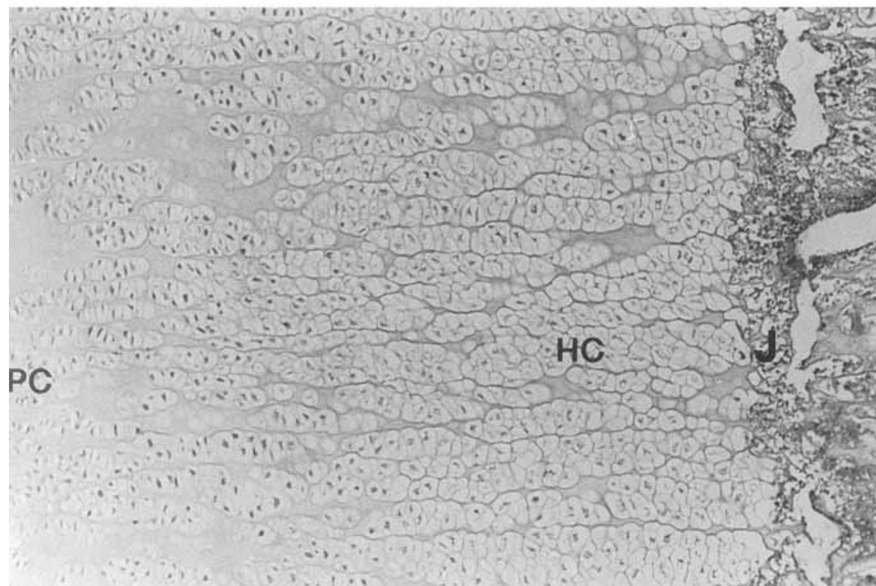
After sequential enzymatic digestion, chondrocytes released from the growth zone cartilage appeared heterogeneous, while chondrocytes released from the resting zone cartilage were homogenous in size and appearance (results not shown). Typically, the yields of resting and growth plate chondrocytes were about 5 and 6 × 10^7 cells per gram of cartilage, respectively. The viability of both types of released chondrocytes was 85%.

When isolated growth plate chondrocytes were loaded into the Beckman JE5 countercurrent centrifugal elutriation (CCE) system (Table I), 18 elutriated cell fractions were collected with sterilized tubes. The cells obtained in each fraction were spherical and quite homogeneous in size (results not shown). The recovery rate of the elutriation procedures was 80%. The mean cellular volume and the total cell number of each fraction were analyzed. The first elutriated fraction contained the smallest cells, and the cell sizes increased steadily in the proceeding fractions. The mean cell volume of the smallest chondrocyte fraction is 767 femtoliter (fl), and that of the largest is 2,859 fl. The cell distribution of chondrocytes of the elutriated fractions is also listed on Table I. The cell numbers rose dramatically in the first seven fractions and then decreased sharply at the next two fractions. From the tenth fraction, it declined steadily until the end fraction. From a plot of the cell numbers vs. the mean cell volumes, a clear picture of the cell size distribution for the elutriated chondrocytes emerged (Fig. 3). Cells with volumes ranging from 800–1,200 fl represented nearly half of the total elutriated growth plate chondrocyte population.

In order to identify the resting and hypertrophic chondrocyte subpopulations from the total elutriated growth plate chondrocyte fractions, the cell sizes of chondrocytes released from the



a.



b.

Fig. 1. Histological sections of pig rib growth plate. The growth plate region has been enlarged. **a:** Bone (*B*) and cartilage are connected by the osteochondral junction (*J*). The hypertrophic zone (*H*), proliferative zone (*P*), and resting zone (*R*) are arranged in a very regular manner. H&E, ×10. **b:** From the high power diagram of the osteochondral junction (J), the hypertrophic chondrocytes (*HC*) and proliferative chondrocytes (*PC*) can be clearly observed. H & E, ×63.

resting zone cartilage and chondrocytes released from the thin growth plate cartilage were determined. The minimum mean cell volume of released resting zone cartilage chondrocytes was not less than 212 fl and the maximum not larger than 1518 fl (Fig. 4a). For the chondrocytes released from growth plated cartilage, two populations were detected (Fig. 4b). The mean and maximum cell volumes of the first population of cells were 32 fl and 171 fl, respectively, which were confirmed to be red blood cells by light microscopy. The second population had cell sizes

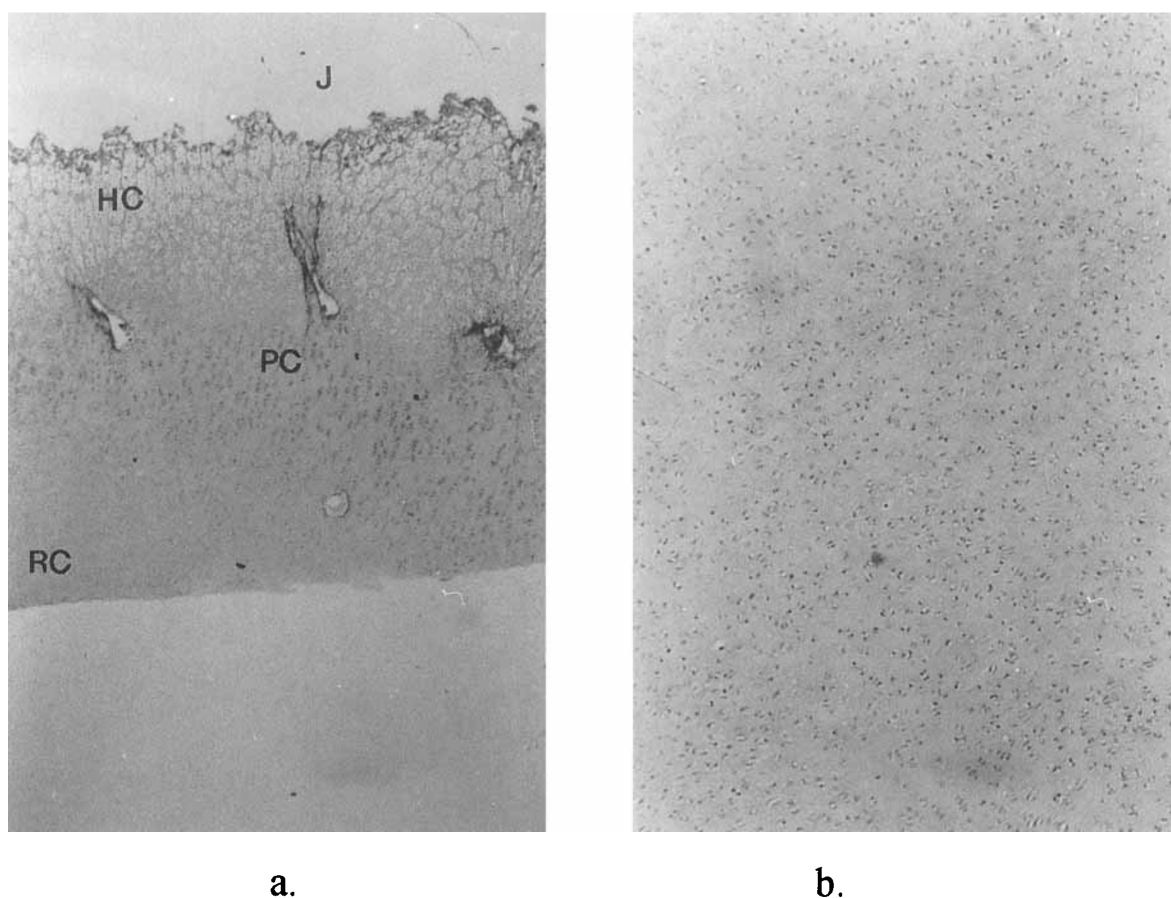


Fig. 2. Histological sections of growth plate slice for chondrocyte isolation. **a:** A growth plate slice, dissected from pig rib for elutriation, showing hypertrophic chondrocytes (*HC*), proliferative chondrocytes (*PC*), and part of the resting chondrocytes (*RC*). Some red blood cells were observed at the edge of the osteochondral junction (*J*). H&E. ×25. **b:** Resting zone cartilage for resting chondrocyte release. H&E. ×25.

ranging from 1,583–3,652 fl and represents the hypertrophic subpopulation (also see Fig. 6).

To get further evidence to confirm that elutriated chondrocyte fractions with cell volume smaller than 1,500 fl are the resting chondrocyte subpopulation, cells released from resting zone cartilage were labeled with tritiated proline. After pooling this radioactive labeled resting chondrocytes with the total growth plate chondrocytes, the cells were subjected to CCE. Radioactivities of the various elutriated fractions were counted by a scintillation spectrometer (Fig. 5). Radioactivities of the first three fractions increased sharply, and those of the proceeding fractions declined rapidly. Only a minimum amount of radioactivity could be detected for those fractions with cell volumes larger than 1,500 fl.

To confirm that the fractions containing elutriated cells of larger size were really composed of hypertrophic chondrocytes, cells released from thin layers of growth plate cartilage adjacent to the osteochondral junction were labeled with tritiated proline. After pooling these cells with unlabeled chondrocytes released from resting zone cartilage sections, the cell mixture was separated by elutriation. Radioactivities of the various elutriated fractions were recorded. As shown in Figure 6, elutriated chondrocyte fractions with cells smaller than 1,600 fl showed very low radioactivities. The radioactivities increased sharply as the cell volume increased until a peak was reached at the 2,700 fl fraction. A slight decrease then followed.

Chondrocytes in the pooled elutriation fractions were plated in 24-well culture plates and settled for 9 h before conducting the assays. Prolonged culture of the elutriated chondrocytes had also been performed. It was found that all the chondrocytes could maintain spherical

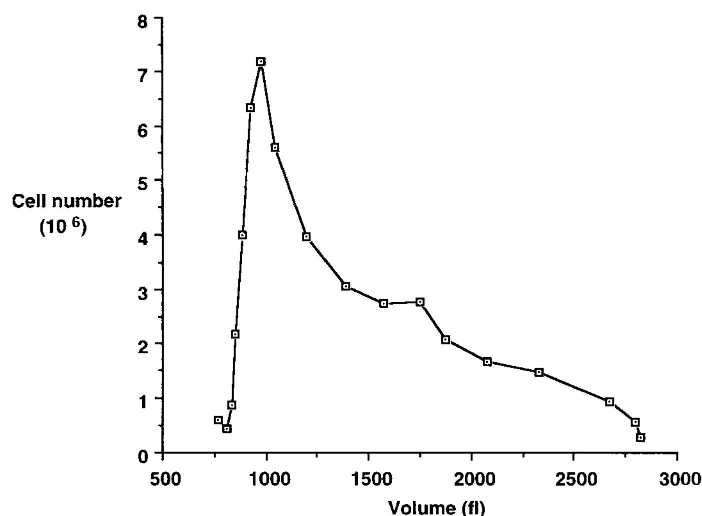


Fig. 3. Size distribution of the elutriated growth plate chondrocytes. Cellular volumes were determined by Coulter Counter-Channelyzer C256 system as described in Materials and Methods.

shapes like those of freshly isolated cells for the first 24 h. The cells then became flattened. When the medium was replaced with fresh medium every 2 days, the cultures became confluent after 7 days. The confluenced resting chondrocytes were granular, thick, and polygonal in shape, while the hypertrophic cells were thin and irregular (data not shown).

Figure 7 shows the alkaline phosphatase activities of cultured chondrocytes in various elutriated fractions. Alkaline phosphatase activities were maintained at a minimum detectable level in the fractions of cells smaller than 1,600 fl. The enzyme activities increased as the cell size increased. A plateau was observed in fractions of cells with a volume larger than 2,000 fl.

Figure 8 shows the thymidine incorporation rates of cultured chondrocytes in various elutriated fractions. The rate of thymidine uptake was very low in the chondrocyte subpopulation with a volume smaller than 1,518 fl. It increased drastically from about 75–275 fmol/10^6 cell/h for the fractions larger than 1,518 fl, with a peak at the 2,100 fl fraction. Then the rate declined to 25 fmol/10^6 cell/h again.

Figure 9 shows the sulfate incorporation of cultured chondrocytes in various elutriated fractions. For cells with volume less than 1,400 fl, the sulfate incorporation rate was around 1 nmol/10^6 cells/h. The rate then rose as the cell size increased and reached a peak of 5 nmol/10^6 cells/h for cells at 2,300 fl. A slight decrease then followed.

DISCUSSION

Growth plate chondrocytes from chick have been extensively studied and characterized. These cells are basically classified into three subpopulations based on their histological and functional differences [O'Keefe et al., 1989]. The cartilage cells located farthest away from the osteochondral junction are the resting chondrocytes. These cells are small in size and homogeneous in appearance and represent the quiescent form of the growth plate chondrocytes. Next to the resting chondrocytes are the proliferative chondrocytes. These are the most active cells which are constantly involved in cell proliferation. The cells adjacent to the osteochondral junction and which gradually increase in size as they approach the osteochondral junction are hypertrophic chondrocytes. These distributions of chondrocytes were found both in chick growth plate [O'Keefe et al., 1989] and in porcine rib growth plate (Figs. 1a,b, 2a,b) in the present study.

Due to technical limitations in separating growth plate chondrocytes into their various differentiative subpopulations, many characteristics described in earlier studies by many researchers might have been the total or combined effect of a mixture of chondrocytes in different maturation stages rather than their true individual features. At the same time, red blood cell contamination often complicates the data interpretation. Much of the recent research sug-

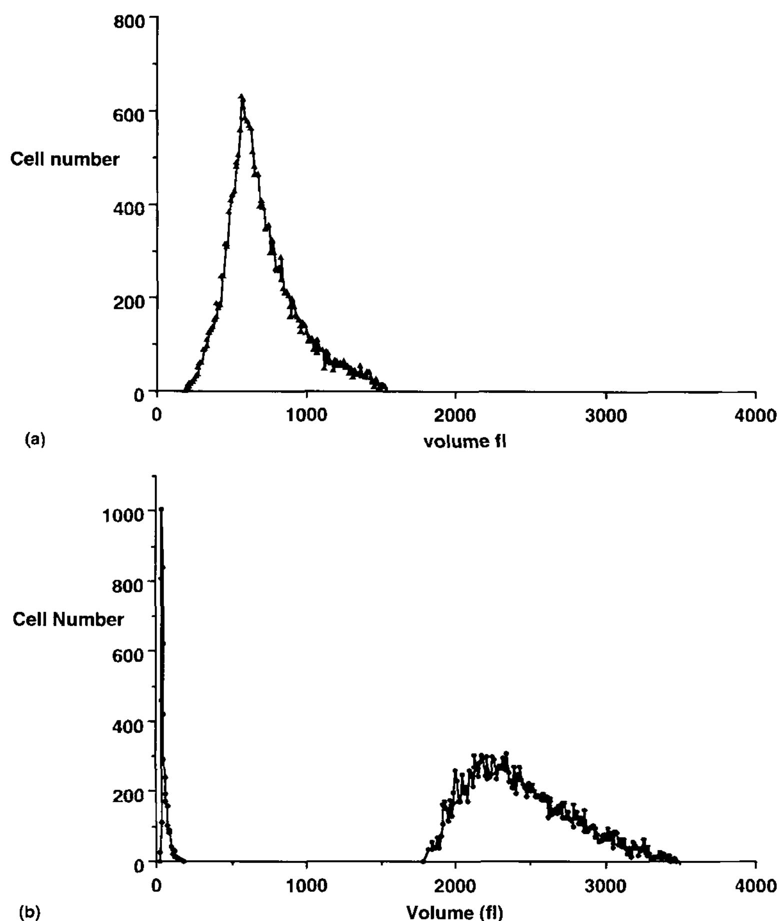


Fig. 4. Cell size distribution of freshly isolated chondrocytes. **a:** Chondrocytes from resting zone cartilage. The minimum, mean, and maximum cellular volumes of chondrocytes released were 212, 520, and 1518 fl, respectively. **b:** Chondrocytes released from the thin layer of growth plate cartilage adjacent to the osteochondral junction. The first population had maximum, mean, and minimum cell volumes of 15, 32, and 171 fl, respectively; the second population had minimum, mean, and maximum cell volumes of 1,583, 2,431, and 3,652 fl, respectively.

gested that cells in different differentiative stages have distinct physiological, biochemical, and morphological features [Rosier et al., 1989; O'Keefe et al., 1990; Gunter et al., 1990]. As a result, separation and identification of the different subpopulations become critical issues for studying growth plate chondrocytes as well as endochondral ossification.

In present study, we characterized the growth plate chondrocytes from porcine. With a yield of approximately 6×10^7 cells per gram of cartilage from each animal, the porcine rib growth plate provides an ideal source of chondrocytes for studying endochondral ossification. Moreover, in the porcine rib cartilage, a pure sample of resting chondrocytes can be obtained with relative ease from the resting zone cartilage since they are situated away from the growth plate (Fig. 2).

Countercurrent centrifugal elutriation (CCE) is a powerful cell separation technique which has been successfully applied by workers in cancer research for many years [Grading and Meistrich, 1980; Lord and Keng, 1984]. In 1989, this technique was been applied for the first time on avian growth plate cells by Rosier and his coworkers [O'Keefe, 1989]. In the present study, an optimized version of their method was adopted for investigation on a mammal model. Results obtained from our investigation showed that porcine growth plate chondrocytes can be effectively separated by size differences using CCE (Table I; Fig. 3).

The cell size distribution of freshly isolated chondrocytes was also determined. The results showed that the maximum cell volume of the resting chondrocyte population is not larger than 1,518 fl (Fig. 4a), and the cell sizes of hypertro-

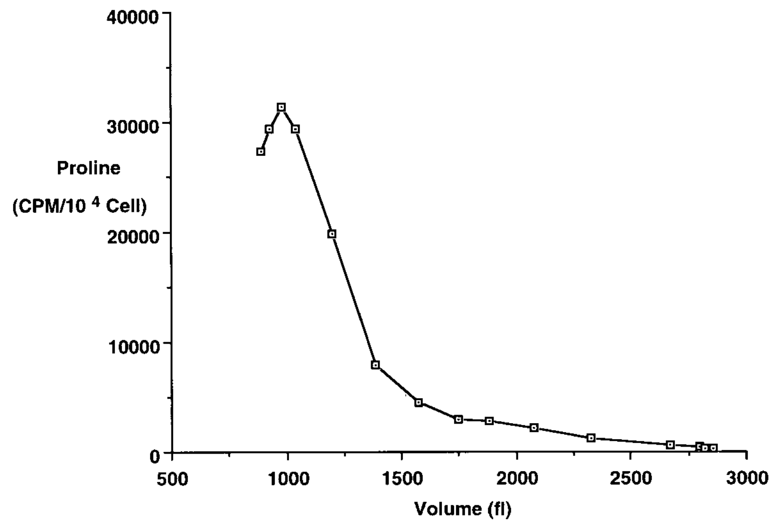


Fig. 5. Elutriation of labeled resting chondrocytes. Isolated chondrocytes from resting zone cartilage were labeled (as described in Materials and Methods) and then pooled with unlabeled chondrocytes from growth plate zone cartilage. The radioactivities of each elutriated fraction were recorded. Each data point represents the mean of four measurements. The C.V. was less than 1%.

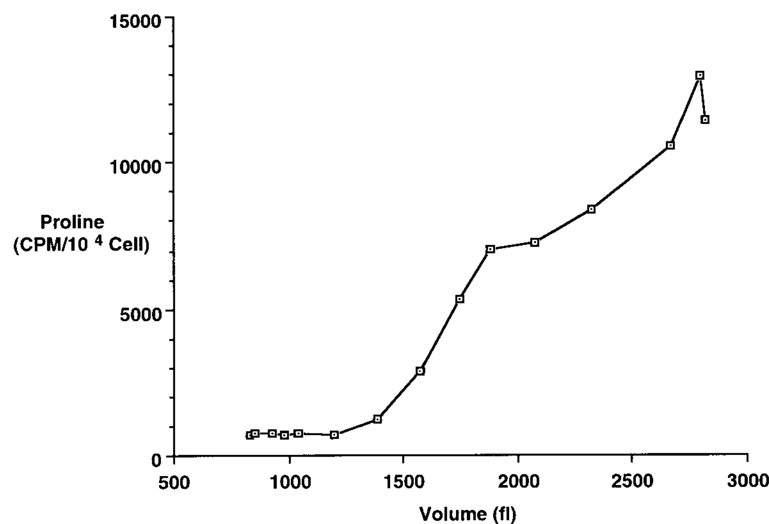


Fig. 6. Elutriation of labeled hypertropic chondrocytes. Isolated chondrocytes from thin layer growth plate cartilage adjacent to the osteochondral junction were labeled (as described in Materials and Methods) and then pooled with unlabeled chondrocytes from thick growth plate cartilage. The radioactivities of each elutriated fraction were recorded. Each data point represents the mean of four measurements. The C.V. was less than 1%.

phic chondrocytes ranged from 1,583–3,652 fl (Fig. 4b). To confirm these findings, cells released from resting zone cartilage were labeled with tritiated proline and pooled with unlabeled chondrocytes released from the respective cartilage sections. When the cell mixture was then separated by elutriation, results showed that radioactivities of those fractions with cells larger than 1,500 fl were very low (Fig. 5). Similarly, when the mixture of labeled and unlabeled cells of thin layers of growth plate cartilage was subjected to CCE, only cells with sizes larger than 1600 fl exhibited radioactivities (Fig. 6).

The alkaline phosphatase (ALP) activities of the individual fractions of chondrocytes after elutriation were determined. ALP is an important maturational marker, and its activity increases in the hypertrophic zone of the growth

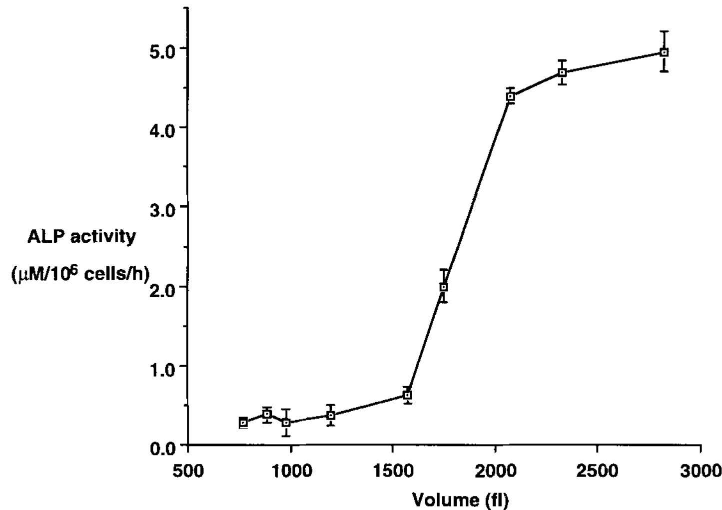


Fig. 7. Alkaline phosphatase (ALP) activities of cultured chondrocytes in various elutriated fractions. The enzyme activities were measured after 9 h of cultivation (as described in Materials and Methods). Data are expressed as mean ± S.D. (n = 4).

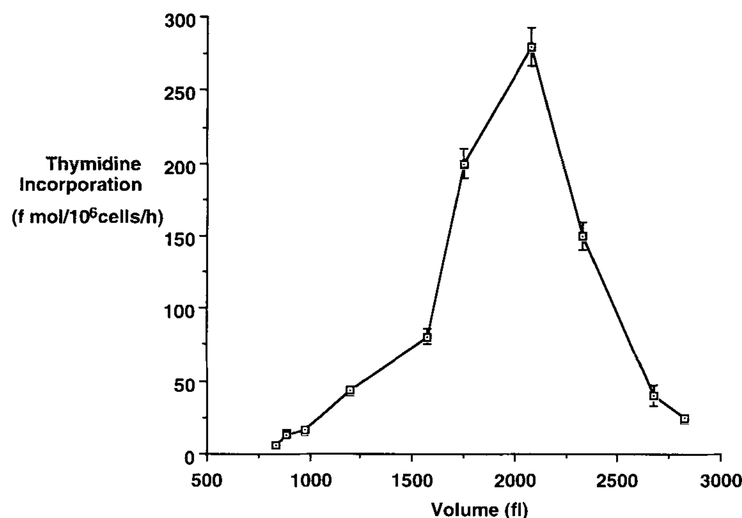


Fig. 8. Incorporation rates of thymidine of the cultured chondrocytes in various elutriated fractions. The incorporation rates of radiolabeled thymidine were determined after 9 h of cultivation (as described in Materials and Methods). Data are expressed as mean ± S.D. (n = 4).

plate [Kuhlman, 1965; Lewinson et al., 1982]. Although its role is not fully understood, ALP is essential for calcification of the matrix [Fallon et al., 1986]. Extensive investigations carried out by other workers suggest that this enzyme plays an important role in both bone formation [Farley and Baylink, 1986; Wlodarski and Reddi, 1986] and extracellular mineralization [Fauran-Clavel and Oustrin, 1986; Register et al., 1986]. However, the mechanism by which the enzyme promotes calcification in mineralizing tissues (i.e., bone and cartilage) is poorly understood. De Bernard et al. [1986] provided evidence that cartilage-derived ALP binds calcium ions with high affinity. Results from the present study (Fig. 7) showed that elutriated porcine chondrocytes exhibited ALP activities differentially; for example, hypertrophic chondrocytes have the highest level of activity. The increase of enzyme activity was about sevenfold in hypertrophic chondrocytes compared with resting chondrocytes. This difference could not be accounted for by the greater surface area of the plasma membrane in the hypertrophic chondrocytes. The

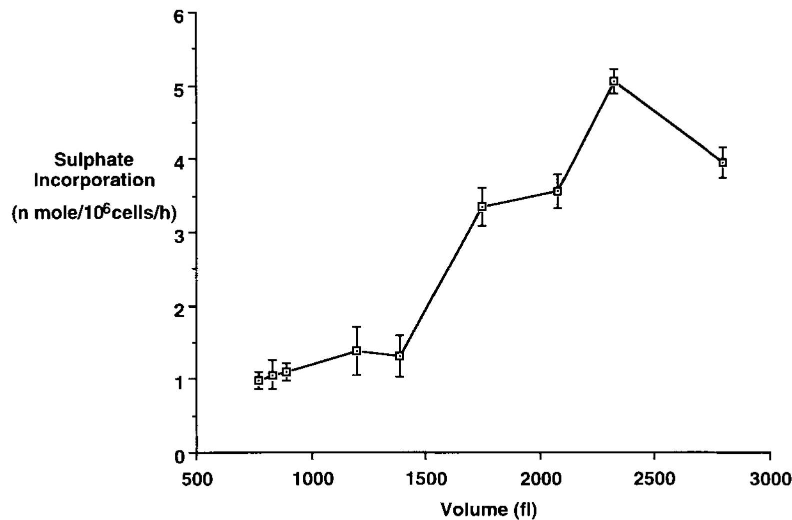


Fig. 9. Sulphate incorporation rates of cultured chondrocytes in various elutriated fractions. The incorporation rates of radiolabeled sulphate were determined after 9 h of cultivation (as described in Materials and Methods). Data are expressed as mean ± S.D. (n = 4).

reason is that the increase in volume of 4.5-fold corresponds only to an increase in cellular surface area of 2.2-fold. The difference in enzyme activities is therefore not due solely to the increase in total area of plasma membrane in the hypertrophic chondrocytes but may be due to a specific biochemical change associated with cellular hypertrophy. The content of this enzyme per unit area of membrane may increase as chondrocytes become mature in the growth plate. The present finding is consistent with earlier observations in bovine [Grant et al., 1985] and avian chondrocytes [O'Keefe et al., 1990].

Thymidine incorporation rate is the primary indicator of DNA synthesizing activity as well as cell proliferative potential [Puzas and Brand, 1986; Puzas and Felter, 1988]. Figure 8 shows that thymidine incorporation rates varied with the changes of mean cellular volumes of the elutriated chondrocytes. The smaller cells showed a low incorporation rate, indicating the minimum proliferative potential of the resting cells. The thymidine incorporation rate rose sharply starting from the fractions with cell size of 1,518 fl and subsequently declined quickly from the 2,100 fl fraction. From this result, we can conclude that those fractions of elutriated chondrocytes with mean cell volume just larger than the resting chondrocytes are proliferative chondrocytes which are active in DNA synthesis. Our findings are consistent to those of Rosier et al. [1989] who showed that avian proliferative chondrocytes are also active in DNA synthesis.

In order to characterize the proteoglycan synthesis ability of the chondrocyte subpopulations isolated from CCE, sulfate incorporation assay was performed. Sulfate-containing glycosaminoglycans, such as chondroitin sulfate and keratan sulfate, are the predominant macromolecules in cartilage matrix proteoglycan. Thus, the incorporation rate of radioactive sulfate into cellular and extracellular macromolecules is a good indication of proteoglycan synthesis [O'Keefe et al., 1989]. Data obtained here show that the synthesis of proteoglycan was greatest in the larger chondrocytes (Fig. 9). In addition, results of these experiments indicate clearly that chondrocytes with mean volume less than 1,400 fl exhibited a low proteoglycan synthesizing rate, whereas those cells with increasing cell sizes exhibited a larger rate. It seems reasonable to speculate that cellular proteoglycan synthesis may be triggered by the onset of chondrocyte differentiation. Although the terminally differentiated hypertrophic chondrocytes showed a decline in proteoglycan rate (Fig. 9), they are still metabolically active even after they were isolated and plated in culture plates as primary monolayer cultures when compared with the resting cell subpopulation.

We found that porcine cultured chondrocytes maintained their initial phenotypic characteristics found in freshly separated cells. Previous investigations on chick cartilage also demonstrated the ability of cultured chondrocytes in maintaining their specific characteristics [Prins

et al., 1982]. Monolayer cultures, established after 16 h of cultivation in vitro, also demonstrated the chondrocyte phenotype both morphologically and biochemically, even after growth to confluence [O'Keefe et al., 1989, 1990]. Our finding also confirms the results of earlier investigations which showed that the chondrocytes from different regions of the growth plate maintain a specific cellular subphenotype [Skantze et al., 1985; Wier and Scott, 1986]. Such findings bear important implications, as they permit the study of subpopulations of chondrocytes in culture. By using a particular phenotypic subpopulation of chondrocytes, specific interventions or stimuli can be evaluated with special reference to their ability in promoting or inhibiting maturation. Thus, culturing of chondrocytes in vitro which have been separated by CCE may be a powerful tool for the investigation of hormones or growth factors which control endochondral ossification. Actually, we have found that porcine chondrocytes elutriated in our study could be maintained in culture medium as confluent cultures. Histomorphometric studies have demonstrated that individual hypertrophic cells are responsible for the production and maintenance of the increased areas of matrix [Hunziker et al., 1985]. This finding suggests an increase in metabolic activity by these cells and is supported by the observation of increased numbers of mitochondria and secretory organelles on a subcellular basis [Buckwalter et al., 1986]. In the present study, the rates of synthesis of DNA and proteoglycan in chondrocytes were measured (Figs. 8, 9). Proliferative and hypertrophic chondrocytes were found to be metabolically active cells and can synthesize large amount of proteoglycan. Therefore, the change in the larger size of these cells is not due to cellular swelling which represents the preceding of cell death but is due to the result of an active process.

In conclusion, by using porcine rib growth plate chondrocytes and applying the technique of CCE, effective isolation and identification of various subpopulations of the growth plate chondrocytes were achieved. CCE was found to be a reproducible and accurate separation technique which effectively eliminated the problem of red blood cell contamination during cell isolation in our study. Three distinct types of chondrocytes were identified and characterized in this study, each with its own morphological and biochemical characteristics. CCE was firstly used to separate growth plate chondrocytes in chick [O'Keefe et al., 1989, 1990; Rosier et al., 1989]. The present study represents an attempt to use this technique to separate mammalian growth plate chondrocytes. It is of interest to investigate whether this technique can also be used in other mammalian systems, especially in humans. We believe that data on the study of the biochemistry of chondrocytes can provide valuable information on the process of ossification, and further studies should give an insight into the etiology of various bone diseases, such as cartilaginous tumors, fracture callus formation, and heterotopic ossification.

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