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Author(s)	TSUJI, YOSHIRO; TAKESHITA, HIDEYUKI; KUSUZAKI, KATSUYUKI; HIRASAWA, YASUSUKE; UEDA, KAZUSHIGE; ASHIHARA, TSUKASA
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Cell Proliferation and Differentiation of Cultured Chondrocytes Isolated from Growth Plate Cartilage of Rat Rib

Yoshiro Tsuji[1], Hideyuki Takeshita[1], Katsuyuki Kusuzaki[1], Yasusuke Hirasawa[1], Kazushige Ueda[2], and Tsukasa Ashihara[2]

Department of Orthopaedic Surgery[1] and Pathology[2], Kyoto Prefectural University of Medicine, Kyoto 602

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Abstract

The present study was undertaken to investigate the relationship among cell morphology, proliferation, and maturation of chondrocytes in primary cultures. Chondrocytes were isolated from the growth cartilages of the rat ribs and cultured for 6 days. In situ DNA cytofluorometry using an inverted epi-illumination cytofluorometer (Nikon P1-I) and ^{3}H-thymidine autoradiography were carried out for the correlated analysis of cell morphology and proliferation. Cytoskeletal staining with fluorescent phalloidin and ^{35}S-sulphate autoradiography were also performed. In addition, in situ hybridization to c-myc mRNA was carried out using DNA probe.

According to the results obtained, the cultured chondrocytes were composed of mixed populations of large, polygonal cells and of small, round cells. The round cells showed a significantly higher ^{35}S uptake than the polygonal cells. The cytoskeletal staining clearly revealed stress fibers in the cytoplasm of the polygonal cells, whereas only a fine filamentous structure was shown in the cytoplasm of the round cells. In situ DNA cytofluorometry clearly demonstrated that cell proliferative activity was high in the polygonal cells and low in the round cells. In addition, ^{3}H-thymidine autoradiography with cumulative labeling method revealed that the polygonal cells were changing into the small, round cells. C-myc mRNA signals were detected in the cytoplasm of over a half of the round cells, whereas no evidence of c-myc expression were found in the polygonal cells.

From these results, it appears that as the shape of the cultured chondrocytes shifts from polygonal to round, the cell proliferative activity decreases in association with cell differentiation. It was also suggested that c-myc mRNA is amplified in the well differentiated round chondrocytes, and not in the proliferative polygonal cells.

Introduction

The chondrocytes in the growth plate cartilages display cell proliferation and cell differentiation during the endochondral ossification process[12]. Some authors also reported that there is a close relationship between cell shape and differentiation in cultured chondrocytes[5,14,26,41]. We have previously described two morphologically different types of chondrocytes in cultures obtained from the

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Present Address: Department of Orthopaedic Surgery, Kyoto Prefectural University of Medicine, 465 Kajiicho, Hirokoji-agaru, Kawaramachi-dori, Kamigyo-ku, Kyoto 602, Japan.

growth cartilages of the rat ribs: polygonal cells and round ones[16,38]. At the early stage, cultured chondrocytes consisting of polygonal cells and round ones had a moderate proliferative activity. On the contrary, at the confluent stage, cells consisting of only round cells showed no proliferative activity. The results obtained from immunocytochemical analysis showed that round cells produced S-100 protein, which is the marker for chondrocytic maturation[20]. In this study, we undertook to clarify the characteristic of both polygonal cells and round ones, and to investigate the relationship among cell morphology, proliferation, and maturation of chondrocytes in primary cultures. In situ DNA cytofluorometry[37,38] and ^{3}H-thymidine autoradiography were performed to correlate analysis of cell morphology and cell proliferation. Cytoskeletal staining with FITC conjugated phalloidin were per formed to analyze the relationship between cell shape and cytoskelton, and ^{35}S-sulphate autoradiography were also performed to analyze cell differentiation.

Overexpression of c-myc gene has been found in carcinomas[2,17,23,24], sarcomas[3,7,9,15,18,22,27,32,35], embryonic tissues and fetal tissues[29,34,39,40]. Ueda reported an expression of c-myc in the cartilage tissue of a fetus[40]. In this study, we undertook to detect the presence of c-myc mRNA in cultured chondrocytes using in situ hybridization[15,39].

Materials and Methods

(1) Cells and cell culture

The growth cartilages were carefully and aseptically resected from the ribs of 4-week-old male Wistar rats, weighing 80 g[36]. The cartilage was minced with scissors in Dulbecco's phosphate buffer saline (PBS). Chondrocytes were isolated from the cartilage fragments after matrix digestion with 0.2% type II collagenase (Worthington, USA) at 37°C for two and a half hours. The isolated chondrocytes were seeded onto 20 × 20 mm glass coverslips (Matsunami, Japan) in 35 mm plastic dishes (Corning, USA) to a density of 1×10^5 cells per dish. Cells were cultured in Ham F-12 medium (Gibco, NY) supplemented with 10% fetal calf serum (Gibco) and incubated at 37°C in humidified atmosphere at 5% CO_2. Medium was replaced after 3 days. The cultured chondrocytes were harvested from 4 to 6 days after seeding and used for the experiments.

(2) In situ DNA cytofluorometry

In situ DNA cytofluorometry was carried out as previously described[37,38]. The chondrocytes cultured on coverslips were rinsed three times with PBS, and fixed with 4% paraformaldehyde (PFA) in PBS for 10 min. After that 4% PFA was removed, cells were treated with 0.5 mg/ml RNase (Sigma, USA) in PBS at 37°C for 1 hr to remove cellular RNA. Nuclear DNA content was determined after staining with 0.0025% propidium iodide[4] (PI, Sigma, USA) in sodium citrate buffer. Cell morphology was noted by phase contrast microscope, and the red fluorescence (672 ± 5 nm) due to PI-DNA binding was measured under green light excitation (532 ± 5 nm) by an inverted cytofluorometer, Nikon P1-I (Nikon, Japan). The relative fluorescent intensities were expressed by DNA content histograms plotted against cell frequency. Two hundreds cells were usually measured for each of the two populations.

(3) ^{3}H-thymidine autoradiography

Cells were incubated with 0.1 μCi/ml ^{3}H-thymidine for 0.5 hr, 12 hr, 24 hr, 36 hr, 48 hr, and 60 hr, as cumulative labeling study. In flash labeling study, cells were labeled with 0.1 μCi/ml ^{3}H-thymidine for 30 min at every other 12 hr during the same 60 hr of the cumulative labeling study. The cultured chondrocytes on the coverslips were rinsed 5 or 6 times with PBS and fixed with 4%

PFA. These coverslips were dipped into the nuclear emulsion, Sakura NR-M2 (Sakura Color, Japan), and exposed in a dark box at 4°C for 4 weeks, after drying of the emulsion. FD111 (Fuji Color, Japan) was used for the emulsion film development in the conditions as at 22°C for 6 to 8 min. After staining with hematoxylin and eosin, these coverslips were embedded on glass slides with balsam as facing cell attached surface to slide.

(4) ^{35}S-sulphate autoradiography

The cultured cells were labeled with 2.5 μCi/ml ^{35}S-sulphate for 30 min, then rinsed with PBS and fixed with 4% PFA. The procedures of the exposure and the development are as described in ^{3}H-thymidine autoradiography.

(5) Cytoskeletal staining with fluorescent phalloidin

The chondrocytes cultured on coverslips were fixed with 4% PFA, rinsed with PBS, treated with 0.1% Triton X-100 (Nakarai Tesc, Japan) in PBS for 2 min at room temperature, and incubated with 10 μg/ml FITC conjugated phalloidin (Sigma, USA) in PBS for 40 min at room temperature, as described by WULF et al.[42]. FITC emission from actin filaments was observed under fluorescent microscope.

(6) In situ hybridization to c-myc mRNA

In situ hybridization was performed as described by FUJITA[10] and UEDA et al.[39,40].

The chondrocytes on coverslips were fixed with 4% PFA for 20 min at room temperature, rinsed with PBS, and treated with 0.2 N HCl for 10 min. Proteins were removed with 1 μg/ml proteinase K (Merck, Germany) in 0.2 M Tris-HCl (pH 7.5)/2 mM $CaCl_2$ for 5 min, and the cells were fixed again with 4% PFA for 10 min. Subsequent to dehydration with ethanol, pre-hybridization with 50% formamide/2×SSC (standard sodium citrate, 1×SSC=0.15 N sodium chloride, 0.015 N sodium citrate) was carried out for 30 min. To detect c-myc mRNA, in situ hybridization with biotinylated DNA probes was carried out using c-myc DNA probe (1.5 Kb, second exon) and pSP64 DNA probe (Fig. 1), which were kindly gifted by YANO (Nippon Shinnyaku, Japan). Both DNA probes

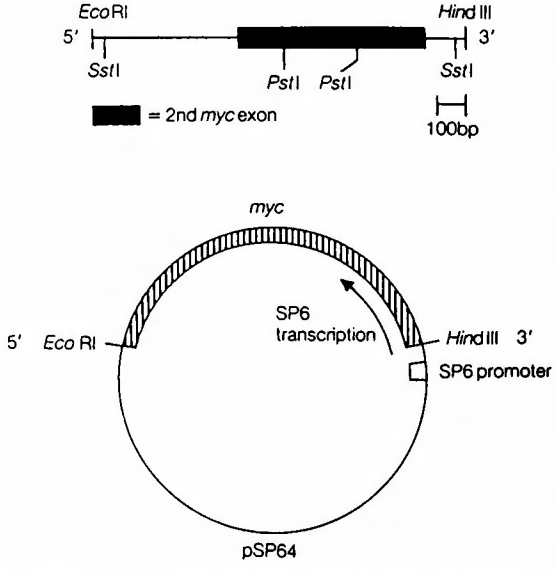

Fig. 1 DNA probe used for in situ hybridization to c-myc mRNA

were labeled with biotin by the nick translation method. The cells were incubated for 30 hr at 32°C with the biotinylated probes in hybridization solution. The final hybridization solution was as follows; 22.5% (v/v) formamide, 10% (v/v) dextran sulphate, 2×SSC, 1×Denhard's solution (0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 0.02% bovine serum albumin), 250 μg/ml salmon sperma DNA (Sigma), 20 mM Vanadyl Ribonucleoside Complex (BRL, USA), 150 μg/ml yeast tRNA (BRL), 20 mM phosphate buffer (pH 6.7) and 1 μg/ml biotinylated DNA probe. The cells were subsequently rinsed with 50% formamide/2×SSC, followed by 50% formamide/1×SSC and 5×SSC in order to remove non-specific binding. After reaction with streptavidin alkaline phosphatase conjugate (BRL), specific binding was visualized by reaction with NBT (nitro blue tetrazolium, BRL) and BCIP (5-bromo-4-chloro-3-indol phosphate, BRL).

Results

(1) Morphological characteristics of the cultured chondrocytes under phase contrast microscope

Chondrocytes cultures 4–6 days old showed two morphologically different cell populations consisting of large-polygonal cells, and small-round ones. The polygonal cells have a larger nucleus, and they contact each other closely. The colonies of the round cells were scattered through a background of the polygonal cells (Fig. 2).

(2) Cell kinetic analysis of the cultured chondrocytes by in situ DNA cytofluorometry

The photomicrograph in Fig. 3A displays the shape of the cultured chondrocytes under phase contrast microscope, while Fig. 3B shows the same cells as visualized by in situ DNA cytofluorometry. The fluorescence intensities of each cell are displayed as arbitrary unit on the figure. Seven cells (A, B, D, F, G, H and I) had fluorescence intensities ranging from 91 to 100,

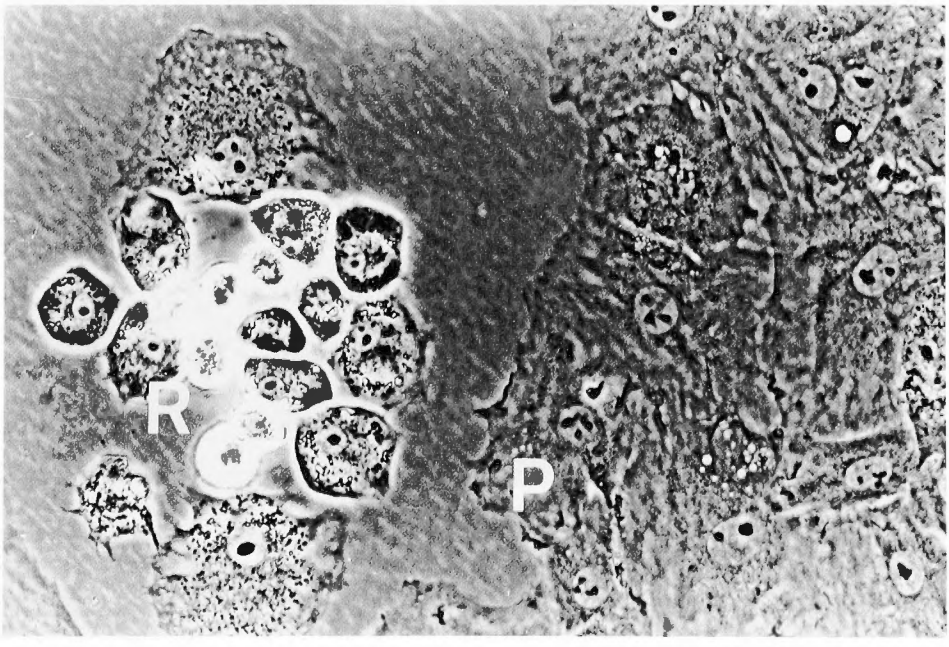

Fig. 2 Phase contrast photomicrograph of the chondrocytes on the 5th day of culture. The chondrocytes were composed of mixed populations of the large, polygonal (P) cells and the small, round (R) cells.

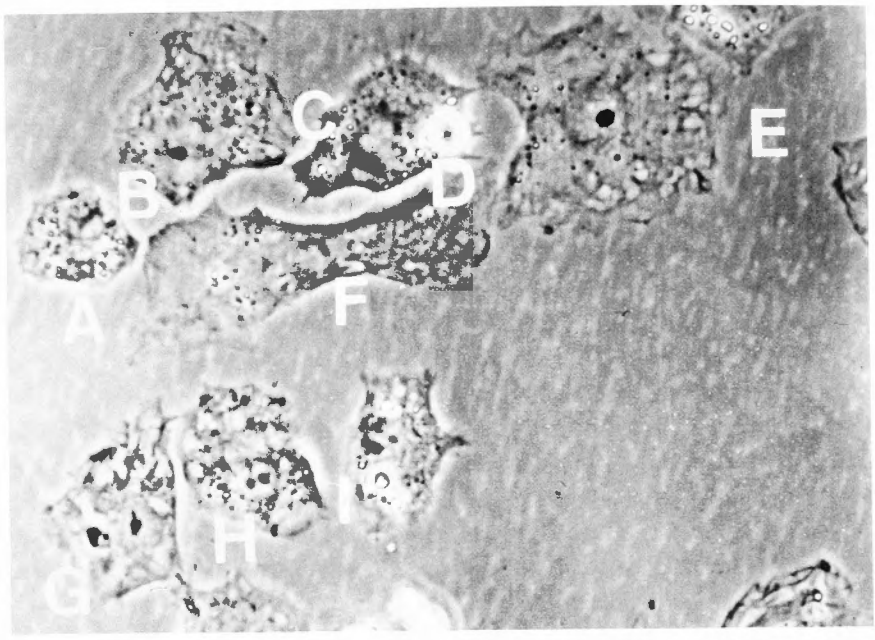

Fig. 3 (A)

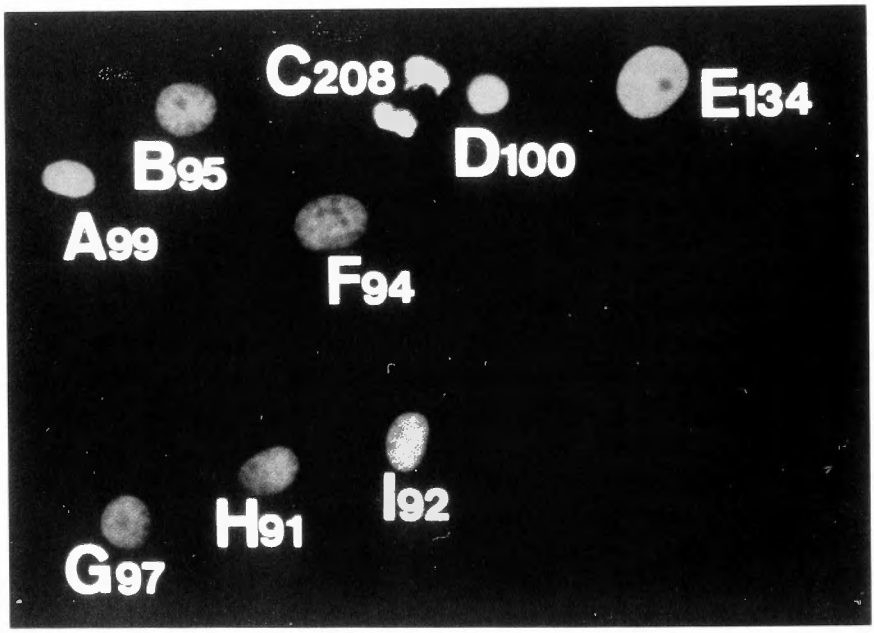

Fig. 3 (B)

Fig. 3 (A) Phase contrast photomicrograph of the cultured cells. (B) Fluorescent photomicrograph of the propidium iodide (PI)-stained cells which are same cells as shown in (A). The relative fluorescent intensities of individual cells are refered to the numerical values after identification of cell morphology.

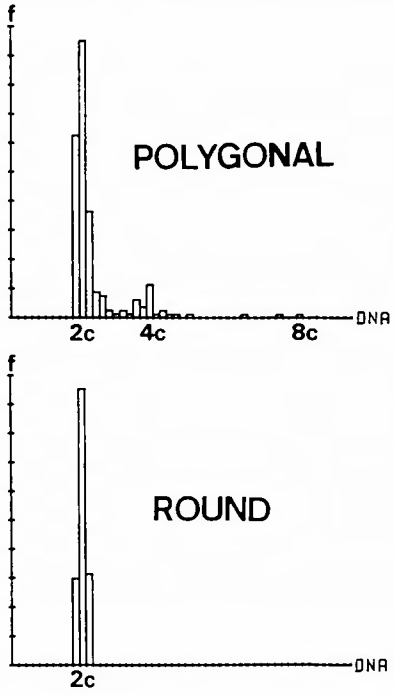

Fig. 4 Nuclear DNA content histograms of the cultured chondrocytes analyzed by in situ DNA cytofluorometry. The upper histogram is for the polygonal cells and the lower one for the round cells. In each graph, the ordinate shows the frequency of the measured cells and the abscissa shows DNA content values expressed in the relative fluorescence intensity.

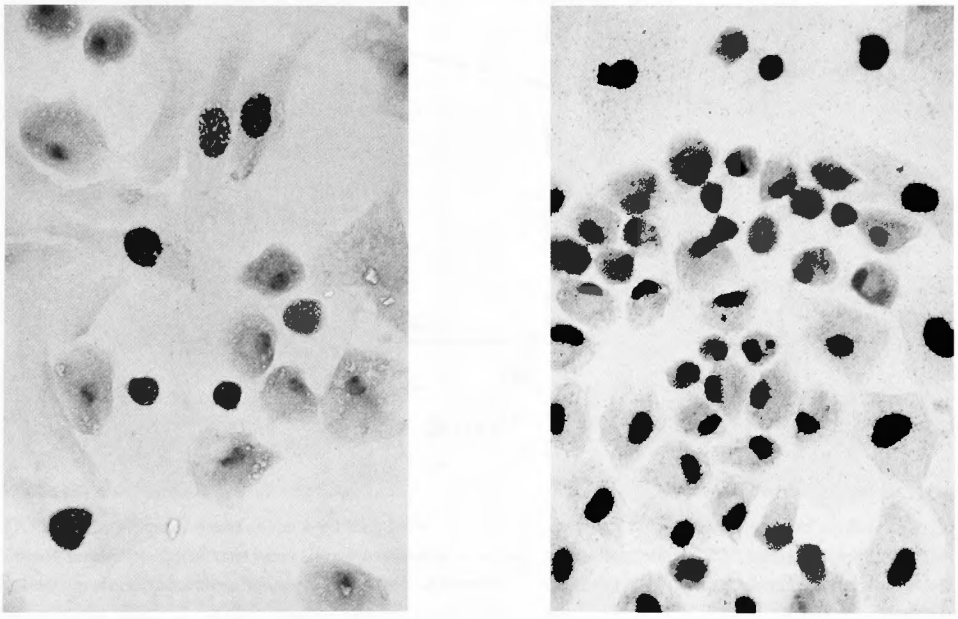

Fig.5 (A) Fig.5 (B)

Fig. 5 Photomicrographs of the ^{3}H-thymidine autoradiography with 30 min labeling (A) and 60 hr labeling (B). (A) Most of the labeled cells were the polygonal cells. (B) The round cells also showed ^{3}H-thymidine uptake.

while the other cells (C and E) had high fluorescence intensities. Because C cell is apparently in mitotic (M) phase of cell cycle, it has twice fluorescence intensity of cells in G_0–G_1 phase, such as A, B, D, F, G, H, and I cells. E cell is in DNA synthetic (S) phase. Fig. 4 demonstrates DNA content

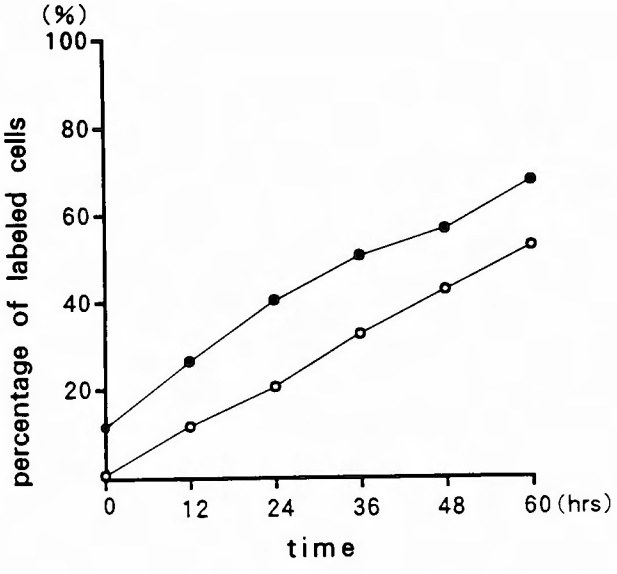

Fig. 6 (A)

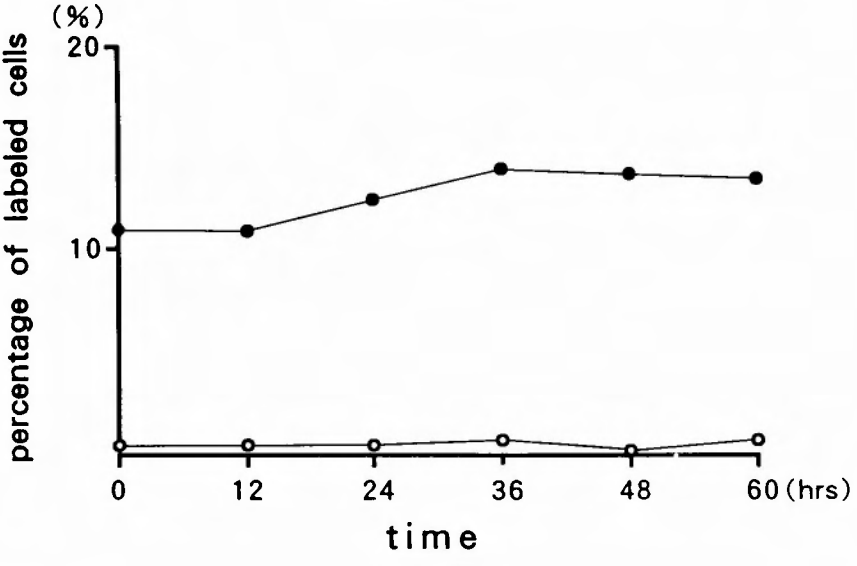

Fig. 6 (B)

Fig. 6 Relationships between time period and percentage of labeled cells by a cumulative labeling method (A) and by a flash labeling method (B). The open circles represent the round cells, and the closed circles are for the polygonal cells. (A) The labeling index gradually increased in both the polygonal cells and the round cells, but the increase for the latter cells was delayed than the increase for the former cells by 11.5–19.2 hours, estimated graphically. (B) Throughout the culture course, the labeling index was extremely low in the round cells and kept high in the polygonal cells.

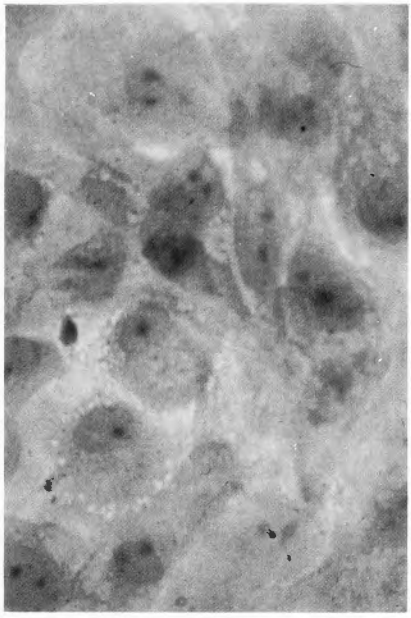

Fig.7 (A)

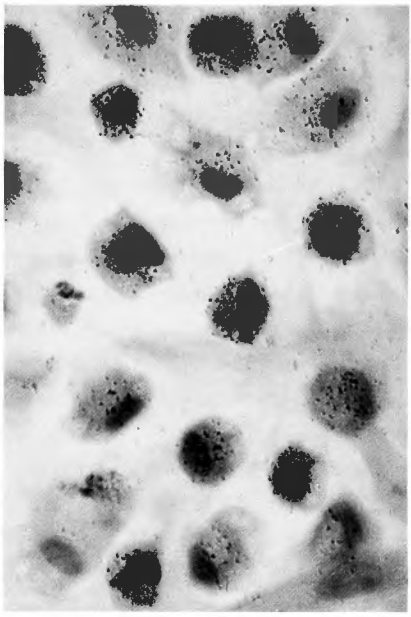

Fig.7 (B)

Fig. 7 Photomicrographs obtained by the ^{35}S-sulphate autoradiography. (A) The polygonal cells showed very low ^{35}S uptake, though (B) the round cells demonstrated a high uptake.

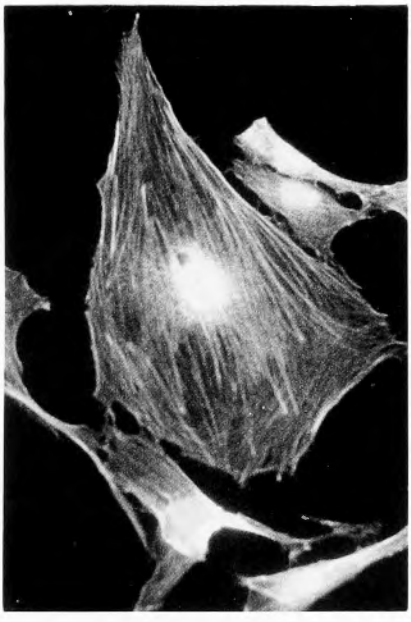

Fig.8 (A)

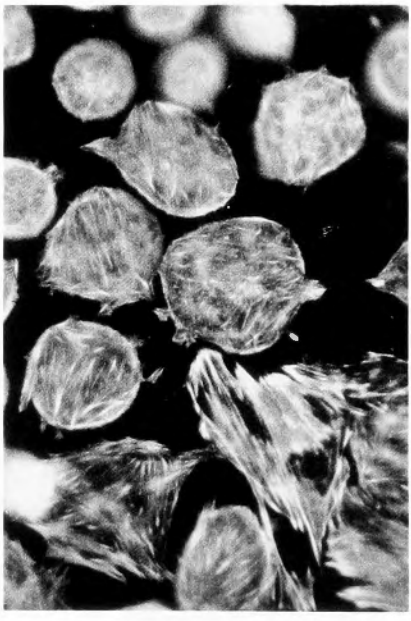

Fig.8 (B)

Fig. 8 Fluorescent photomicrographs of the cultured chondrocytes stained with FITC-phalloidin. (A) Stress fibers were clearly displayed in the cytoplasm of the polygonal cells. (B) A fine filamentous structure was shown in the round cells.

histograms obtained from in situ DNA cytofluorometric analysis. After the cells were identified as polygonal or round by phase contrast microscope, the respective fluorescence intensities were immediately measured by cytofluorometer.

The upper graph in Fig. 4 is DNA content histogram of polygonal cells. The polygonal cells were composed of many diploid (2c) cells, a small number of tetraploid (4c) cells, and a few cells having with DNA content between 2c and 4c. In contrast, 100% of the round cells were 2c, as demonstrated in the lower graph in Fig. 4, suggesting that they had extremely lower proliferative activity, compared to the polygonal ones.

(3) Cell kinetic analysis of the cultured chondrocytes by ^{3}H-thymidine autoradiography

After the first 30 min of labeling, the percentage of labeled cells (labeling index: LI) of the polygonal chondrocytes was 11%, whereas that of the round cells was only 0.5% (Fig. 5A). The LI of both cell populations increased in a linear fashion with labeling time. After 60 hr of labeling, 50% of the round cells were labeled, whereas 70% of the polygonal cells were labeled (Fig. 5B). Although the LI of the polygonal cells was higher than that of the round ones, the rate of ^{3}H-thymidine accumulation was same in both cell groups (Fig. 6A). The flash labeling studies for 30 min at every other 12 hr revealed that the LI of both cell groups were mostly constant during 60 hr (Fig. 6B). This suggests that cell proliferative activity of the cultured chondrocytes was constant during this period.

(4) Analysis of matrix production activity of the cultured chondrocytes by ^{35}S-sulphate autoradiography

Matrix production was assessed by ^{35}S incorporation, and the round chondrocytes showed significantly higher uptake of ^{35}S than the polygonal ones (Fig. 7). This means that cartilage matrix

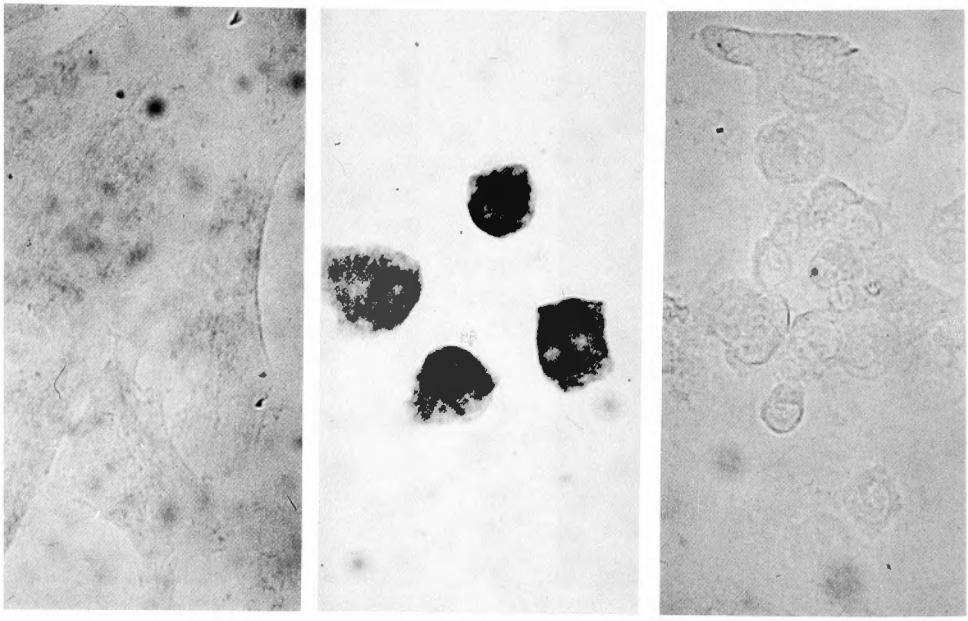

Fig.9 (A) (B) (C)

Fig. 9 Photomicrographs of in situ hybridization to c-myc mRNA (A, B) and to pSP 64 mRNA (C). The signals of in situ hybridization to c-myc mRNA were not observed in the cytoplasm of the polygonal cells (A), whereas signals were clearly detected in the cytoplasm of the round cells (B). No remarkable pSP 64 signals were observed in the round cells (C)

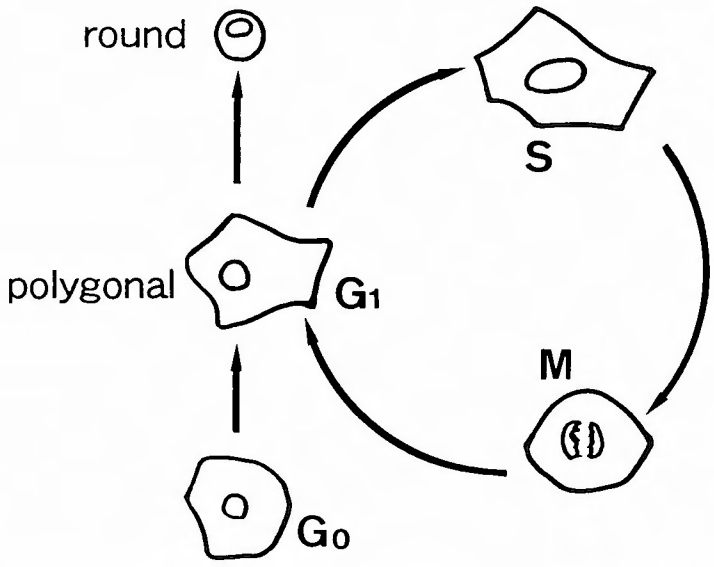

Fig. 10 The postulated model of the relationship between the polygonal chondrocytes and the round ones. The polygonal cells participate in the cell cycle. A part of them subsequently exit from it, and differentiate into the round cells.

production activity was higher in the round cells than in the polygonal cells.

(5) Cytoskeletal staining with FITC conjugated phalloidin

The polygonal cells showed stress fibers arranged longitudinally in the cytoplasm, whereas only fragmented fibers were seen in the cytoplasm of the round ones (Fig. 8).

(6) In situ hybridization

There were no detectable signals of c-myc in the polygonal cells (Fig. 9A). The signal was detected in 50% to 80% of the round ones, and it was mainly distributed in the cytoplasm (Fig. 9B). As control study, pSP64 probe did not show any signal in the round ones (Fig. 9C), confirming the specificity of the binding.

Discussion

The endochondral ossification process at the growth plate cartilage is characterized by chondrocytes proliferation and hypertrophic changes followed by degeneration. Hypertrophic change is considered to be maturative process of chondrocytes[12].

Cultured chondrocytes demonstrated two morphologically different populations represented by the polygonal cells and the round ones[16,38]. Becasue there are very few chances to be contaminated with other cells such as fibroblasts in this culture method[32], both cells are absolutely originated from chondrocytes. In situ DNA cytofluorometry[37,38] revealed that the polygonal cells have high proliferative activity, while the round ones are quiescent. Supporting that, the LI of the polygonal cells is significantly higher than that of the round ones in the ^{3}H-thymidine flash labeling study. However, cumulative labeling study suggested that the polygonal ones could transform to the round ones, because labeling indicies of both cells are continuously increasing associated with cumulative labeling time. This relationship between the polygonal chondrocytes and the round ones in vitro are

similar to that between proliferative chondrocytes and hypertrophic cells in vivo during endochondral ossification[19].

The result obtained from ^{35}S-sulphate autoradiography demonstrated that the round cells have a higher rate of cartilage matrix production than the polygonal ones, suggesting that the round ones are more differentiated than the polygonal ones. Some authors have reported that round or globe shaped chondrocytes, cultured in agarose gels or cultured by cell suspension methods, have a higher rate of cartilage matrix production than flat chondrocytes in monolayer culture[5,12,14,26,41]. ROSEN et al.[33] showed that cytochalasin B[6], one of the inhibitors of actin synthesis, transforms undifferentiated mesenchymal cells in vitro to round shaped chondrocytes which produce cartilage matrix. Our FITC conjugated phalloidin staining studies reported here demonstrate that the round chondrocytes have no cytoplasmic stress fibers, differently from the polygonal ones, which showed that stress fibers clearly. All of the data described above lead us to postulate that morphologic transformation of the polygonal chondrocytes to the round ones in vitro is closely related to chondrocytic maturation. Results from studies on proliferation and differentiation of the cultured chondrocytes strongly suggest that the proliferative polygonal chondrocytes differentiate into the round chondrocytes (Fig. 10), similar to the chondrocytes in the proliferative zone of the growth plate which differentiate into the hypertrophic chondrocytes in vivo. In fact, the round cells did not incorporate ^{3}H-thymidine after the first 30 min of labeling, whereas the polygonal ones did. As shown in Fig. 5B, in the cumulative labeling study, most round cells showed labeled nuclei, supporting the hypothesis that they derived from polygonal precursors which retained ^{3}H-thymidine into the nucleus.

It is interesting to note that c-myc mRNA was detected in the round chondrocytes but not in polygonal chondrocytes using in situ hybridization. Overexpression of c-myc oncogene has previously been found in various carcinomas[2,17,23,24], sarcomas[3,7,9,15,18,22,27,31,32,35]. Expression of this gene has also been detected in many normal tissues[8,13,28,43], especially embryonic tissues and fetal tissues [29,34,39,40]. There have been no reports regarding c-myc expression in the cultured chondrocytes. ALEMA et al.[1] demonstrated that proliferative activity of chondroblasts increased after transfection of c-myc gene, supporting that it is closely related with cell proliferation[11]. On the other hand, NATH et al.[25] have reported that c-myc mRNA was elevated in the differentiating lens cells, and LACHMAN et al.[21] and PROCHOWNIK et al.[30] have suggested that c-myc is related to differentiation of murine erythroleukemia cells. On the basis of our results, which shows that c-myc is expressed in the well differentiated round chondrocytes and not in the less differentiated polygonal cells, we conclude that in cultured chondrocytes, c-myc expression appears to be related to differentiation rather than proliferation.

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和文抄録

培養軟骨細胞の分化機能発現と細胞増殖動態に関する実験的研究

京都府立医科大学　整形外科
辻　　吉郎，竹下　秀之，楠崎　克之，平澤　泰介

京都府立医科大学　第一病理
上田　和茂，芦原　　司

従来の培養軟骨細胞を用いた研究から，軟骨細胞の形態と分化機能発現の間には，関連性のあることが示されている．著者らは，成長軟骨細胞の培養系において細胞形態，機能が明らかに異なっている2種類の細胞が存在することを見いだした．本研究では，この培養系を用い，軟骨細胞の形態・細胞増殖動態・分化機能発現の3者の関連性を総合的に把握することを目的とした．このための方法論として，細胞形態別増殖動態解析には，in situ DNA 顕微蛍光測光法と ^{3}H−サイミジンオートラジオグラフィーを行い，分化機能の検索には ^{35}S オートラジオグラフィーを用いた．また，FITC−ファロイジン染色法により，軟骨細胞の形態と細胞骨格の関係についても調べた．更に，本研究では，悪性腫瘍以外に，胎生期の細胞や分化途上の細胞にも出現し，細胞の分化・増殖に深く関係があると考えられている c-myc 遺伝子の発現の有無を，in situ DNA-mRNA hybridization 法を用いて検索した．

実験には，ラット肋軟骨から分離・培養した成長軟骨細胞を用いた．培養開始4～6日目頃の成長軟骨細胞は，大型多角形の扁平な胞体を持ち，大きな核を有する細胞（以下，多角形細胞と略す）と，比較的小型で類円形ないし球状の胞体と小さな核を有する細胞（以下，円形細胞と略す）の2種類の細胞から構成されていた．in situ DNA 顕微蛍光測光法による細胞増殖動態解析の結果，多角形細胞は，活発な増殖性を示す2倍体細胞と少数の4倍体から構成されているのに対し，円形細胞は，ほとんど増殖活性を持たない2倍体細胞から構成されていることが判った．^{3}H−サイミジンの30分標識の結果から，多角形細胞の標準率は11%，円形細胞の標識率は0.5%であり，その標識率の経時的変化はほとんど認められなかった．^{3}H−サイミジンの持続標識実験の結果から，多角形細胞が円形細胞に形態的に変化することが示唆された．また，^{35}S オートラジオグラフィーより，多角形細胞は，軟骨基質の産生能が低く，他方，円形細胞では，基質産生が亢進していることがわかった．FITC−ファロイジン染色によるアクチンの細胞内分布パタンを，両細胞で比較したところ，多角形細胞ではストレスファイバーがよく発達しているのに対し，円形細胞には，分断された線維性構造のみが観察された．以上の結果をまとめると，培養軟骨細胞の形態・増殖・分化の3者の間には，たがいに密接な関連が有り，多角形細胞から円形細胞への形態変化に伴って，増殖活性が低下し，分化機能が発現されることが判明した．

次に，c-myc 遺伝子の発現の有無を in situ hybridization 法を用いて検索したところ，円形細胞の過半数に，c-myc mRNA のシグナルが検出された．このことから，軟骨細胞では，分化機能発現と関連して c-myc 遺伝子が発現される可能性が示唆された．