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Establishment of a Clonal Human Chondrosarcoma Cell Line with Cartilage Phenotypes[1]

Masaharu Takigawa,[2] Koji Tajima, Hai-Ou Pan, Motomi Enomoto, Akihiro Kinoshita, Fujio Suzuki, Yoshiro Takano,[3] and Yoichi Mori

Department of Biochemistry [M. T., H-O. P., M. E., A. K., F. S.] and the First Department of Anatomy [Y. T.], Osaka University Faculty of Dentistry, Suita, Osaka 565; Saiseikai Nakatsu Hospital, Osaka 530 [K. T.]; and Department of Cell Biology, Research Institute, Center for Adult Diseases, Osaka 537 [Y. M.], Japan

ABSTRACT

A clonal cell line with cartilage phenotypes and tumorigenicity during more than 3 years in culture was established from a human chondrosarcoma. In sparse cultures, the clonal line, named HCS-2/8, consisted of slightly elongated polygonal cells, which proliferated with a doubling time of 3.5 days. The cells became polygonal to spherical as they became confluent. After reaching confluence, the cells continued to proliferate slowly and formed nodules, which showed metachromasia when stained with toluidine blue. The nodules were three-dimensional in structure; cells were multilayered in the surface regions, overlying a thick layer of extracellular matrix, which showed metachromasia. Electron microscopically, the cells resembling chondrocytes *in vivo* were surrounded by an extracellular matrix consisting of thin collagen-like fibrils with numerous fine granules, presumably of proteoglycans. The cells actively synthesized proteoglycans as determined by [^{35}S]sulfate incorporation. The hydrodynamic size of major proteoglycan monomers synthesized by the cells was that of so-called cartilage-specific proteoglycans, as determined by glycerol gradient centrifugation. Immunostaining identified type II collagen but not type I collagen. Fluorography and immunoblotting of collagens separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis also demonstrated the synthesis of type II collagen but not type I collagen. Inoculation of HCS-2/8 cells into athymic mice resulted in the formation of chondrosarcomas that resembled the original tumor. Because of having these characters, HCS-2/8 cells should be useful not only in studies on the differentiated phenotypes of human chondrocytes but also in basic studies on the diagnosis, treatment, and etiology of human chondrosarcomas.

INTRODUCTION

Established differentiated cell lines, which have specific differentiated functions and can proliferate permanently in culture, should have many advantages for use in investigating various fundamental problems in cell biology. Chondrocytes isolated from normal cartilage form differentiated colonies that form cartilage-like tissue (1–3), show active syntheses of proteoglycans (4, 5) and type II collagen (6–8), which are important cartilage phenotypes, and responded well to hormones (4, 5, 9, 10), vitamins (4, 8, 11–13), and growth factors and other growth-promoting agents (14–16) in primary culture. But most of their differentiated phenotypes, such as a typical polygonal shape and the syntheses of proteoglycan and type II collagen, are lost during their serial passage in culture (6, 9).

Permanent cell lines are known to be established more easily from tumors than from normal tissues. However, malignant tumors arising from the skeletal system are rare, constituting less than 0.5% of all cancers (17) and chondrosarcomas constitute only about 20% of all malignant tumors of the skeletal system (18). Therefore, a permanent cell line of this rare tumor, especially of human origin, should be very useful for not only biological and biochemical studies on chondrocytes but also basic studies on this rare tumor. However, no permanent cell line of chondrosarcoma carrying cartilage phenotypes, such as the abilities to synthesize cartilage specific proteoglycans and type II collagen, has yet been reported.

As part of a program on study of the mechanism of expression of the differentiated phenotypes of human chondrocytes and the biology and biochemistry of human chondrosarcomas, in this work a new cell line carrying chondrocyte phenotypes was established from a human chondrosarcoma.

MATERIALS AND METHODS

Origin of the Human Chondrosarcoma (HCS[4]) Cell Line. The cell line HCS-2/8 was derived from a human chondrosarcoma of the proximal part of the humeral bone of a 72-year-old Japanese male who had received no surgical treatment or chemical or radiation therapy. On June 24, 1985, an operation was performed. Immediately after the operation, parts of the tumor specimen were fixed in formalin, embedded in paraffin, cut into thin sections, and stained with hematoxylin & eosin, periodic acid-Schiff, alcian blue, and toluidine blue (19). The tumor was identified as a well-differentiated type of chondrosarcoma. The remainder of the specimen was processed within 4 h for tissue culture.

Culture of HCS Cells. One piece of the tumor was cut into small fragments aseptically and treated with 0.25% trypsin in PBS for 15 min at 37°C. The resulting tumor cell suspension was passed through a nylon mesh, washed twice with Eagle's minimum essential medium (MEM: Nissui Pharmaceutical Co., Tokyo, Japan) containing 10% fetal bovine serum (FBS: GIBCO) and 60 μg/ml of kanamycin, and plated in 60-mm plastic Petri dishes (Falcon Plastic Corp., Oxnard, CA) at a density of 5×10^5 cells/5 ml of medium. Cultures were incubated at 37°C in a humidified atmosphere at 5% CO_2 in air. Cells were treated with 0.25% trypsin for several minutes, and subcultured at a dilution of 1:4 to 1:8 every 3-4 weeks. The medium was changed twice a week.

Cloning of HCS Cells. Cells from the 22nd passage were inoculated into six dishes at 200 cells/100-mm dish (Falcon) and fed twice a week with MEM supplemented with 20% FBS. After 2 months, more than 40 colonies were obtained and transferred to 24-well multiwell plates using stainless steel Penicylinders and grown in the same medium. When the cells reached confluence, they were expanded into 35-mm dishes. The cells were subcultured at a ratio of 1:4–8 when they became confluent. Clones with high activity for synthesis of proteoglycans, a chondrocyte phenotype, were selected biochemically by the following method and histochemically by toluidine blue staining (9).

Culture of Other Cells. Normal chondrocytes were isolated from growth cartilage of the ribs of young New Zealand rabbits weighing 200–500 g by treatment with EDTA, trypsin, and collagenase as de-

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[2] To whom requests for reprints should be addressed.

[3] Present address: The Second Department of Oral Anatomy, School of Dentistry, Hokkaido University, Sapporo, Hokkaido 060, Japan.

[4] The abbreviations used are: HCS, the generic name for cells obtained in this study from a human chondrosarcoma; HCS-2/8, a clonal human chondrosarcoma cell line established in this study; PBS, phosphate buffered saline; MEM, minimum essential medium; FBS, fetal bovine serum; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; NRK, normal rat kidney.

scribed (4, 5). Mouse skin fibroblasts were obtained from newborn ICR/Crj mice (Charles River Japan) by treatment with EDTA and trypsin as described (20). Normal rat kidney (NRK) cells and Swiss 3T3 fibroblasts were from Dainippon Pharmaceutical, Suita, Japan. These cells were cultured in MEM containing 10% FBS.

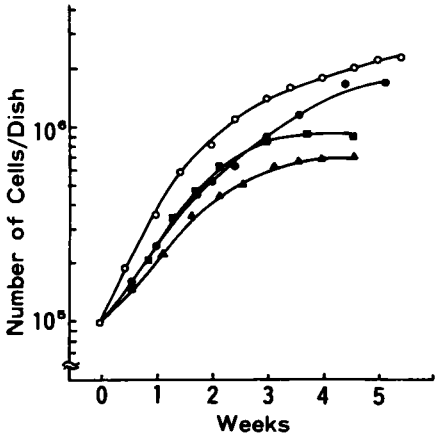

Fig. 1. Growth curves of uncloned HCS cells and the clonal line HCS-2/8. Uncloned HCS cells at passages 13 (●), 17 (■), and 23 (▲), and HCS-2/8 cells at passage 8 after cloning (passage 30 from the start of culture) (○) were plated at a density of 1×10^5 cells/35-mm dish and grown in MEM containing 20% FBS. Cell numbers were determined on the indicated days. Points are averages for values in duplicate dishes, which differed less than 10%.

Light and Electron Microscopy. The ultrastructural features of HCS-2/8 at passage 9 were assessed by electron microscopy. Cells grown to confluence in 35-mm dishes were rinsed in normal saline and fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3) at 4°C for 2 h. The cells were then postfixed in 1% osmium tetroxide at 4°C for 2 h, dehydrated by passage through a graded ethanol series, and embedded in Epon 812 (Taab, Berkshire). Semithin sections (2 μm) cut perpendicularly to the bottom of the culture dish were stained with 0.1% toluidine blue in 0.1 M sodium borate for light microscopic observation of the general morphology of cultured cell layers. Ultrathin sections were cut with a diamond knife, stained successively with aqueous uranyl acetate and lead citrate, and examined in a JEM 2000EX electron microscope.

Determination of Rates of Proteoglycan Synthesis. Proteoglycan synthesis was monitored by determining the incorporation of [^{35}S]sulfate into materials precipitated with cetylpyridinium chloride. Details of the method were described in previous papers (4, 5).

Determination of Relative Hydrodynamic Sizes of Proteoglycan Monomers. The hydrodynamic sizes of newly synthesized radiolabeled proteoglycans were investigated by density gradient centrifugation under dissociative conditions as described previously (11, 16), except that the gradients were centrifuged for 26 h at 27,500 rpm at 4°C in a SW28 rotor in a Beckman L5-50 ultracentrifuge.

Immunocytochemical Analysis of Collagen. Cultured cells were washed with PBS, fixed in 99% ethanol plus 1% acetic acid at 4°C for 1 h, and processed for indirect immunofluorescence staining. The cells were incubated in a moist chamber for 40 min at 37°C with rabbit anti-

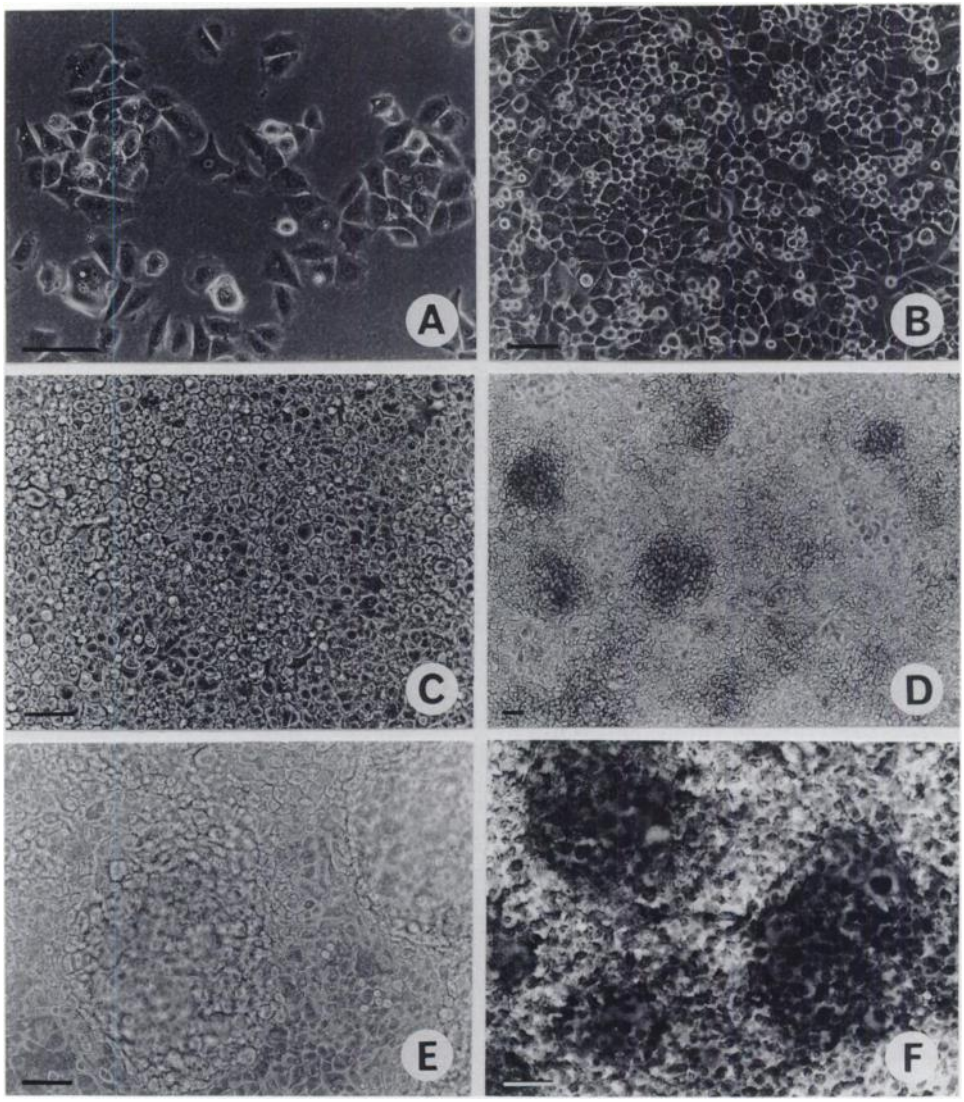

Fig. 2. Morphology of the clonal cell line isolated from a human chondrosarcoma. HCS-2/8 cells (passage 20) were plated at a density of 1×10^5 cells/35-mm dish and grown in MEM containing 20% FBS. Phase-contrast photomicrographs were taken on Day 5 (*A*), Day 10 (*B*), Day 14 (*C*), and Day 21 (*D* and *E*). Cultures on Day 21 were also stained with toluidine blue (*F*). *Bars*, 100 μm.

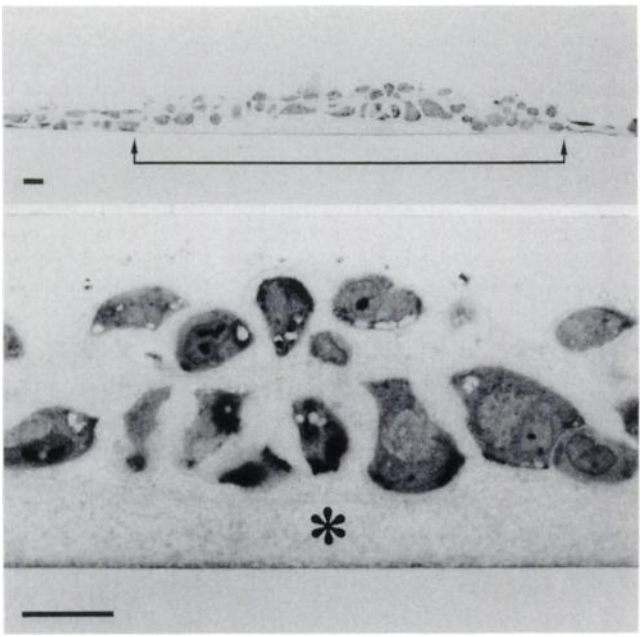

Fig. 3. Light micrographs of sections of nodules of HCS-2/8 cells. HCS-2/8 cells at passage 9 were cultured as described in the *legend* for Fig. 2 and nodules were fixed 7 weeks after inoculation. Specimens were stained with toluidine blue. *Top*, cells in the nodules (above the *bar with two arrows*) are mainly located in surface regions, overlying a thick layer of extracellular matrix. *Bottom*, higher magnification of the center portion of the nodule showing a coarse meshwork arrangement of metachromatic material in the extracellular matrix (*asterisk*). Dark staining in the cytoplasm are glycogen pools. *Bars*, 20 μm.

bovine type I collagen antibody (Advance Co., Tokyo) diluted 1:40 with PBS or with rabbit anti-bovine type II collagen antibody (Advance) diluted 1:150 with PBS. Then they were rinsed with PBS and incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Sigma) for 40 min at 37°C. After incubation, the specimens were mounted on cover slips in glycerin-PBS (9:1) and examined in a Nikon fluorescence microscope.

Biochemical Determination of Type of Collagen. When HCS-2/8 cells became confluent, they were cultured for 7 days in growth medium supplemented in 50 μg/ml ascorbic acid (Sigma) and then incubated for 24 h in Dulbecco's modified MEM (DMEM) (Nissui Pharmaceutical, Tokyo, Japan) with 2 μCi/ml of L-[2,3-^{3}H]proline (35.2 Ci/mmol; New England Nuclear), 50 μg/ml of ascorbic acid, and 0.5 mM β-aminopropionitrile (Sigma). Collagens were extracted by a published procedure (21) with some modifications. The medium was harvested, and then the cell layer was extracted with 1.0 M NaCl, 50 mM Tris-HCl (pH 7.4) for 24 h at 4°C. Next, the medium and the resultant extract and residue were mixed and dialyzed against 0.5 M acetic acid at 4°C overnight. The pepsin (100 μg/ml) was added and extraction was continued for 48 h at 4°C with dialysis. The material was then lyophilized. In some experiments, extracted collagens were precipitated with 1.2 M NaCl.

SDS-PAGE was performed by the method of Laemmli (22) in 4–20% gradient gel with human type I collagen (Sigma), bovine type II collagen (Nitta Gelatin, Tokyo), and type XI collagen purified from rabbit costal cartilage by a published procedure (21) as standards. Radioactive protein bands were detected by fluorography (23) with Autofluor (National Diagnostic, Sommerville, NJ). Briefly, the gels were stained, fixed, and immersed in Autofluor for 2 h. Then they were dried on filter paper, placed on film (Kodak X-Omat XAR-5, Eastman Kodak) and exposed at −76°C.

Electrophoretic transfer of proteins to nitrocellulose and immunoblotting were carried out by published procedures (24) with the following modifications. The electrophoretic blots were soaked in 1% bovine serum albumin in PBS for 30 min at 37°C to saturate additional protein binding sites. The sheets were then rinsed in PBS and incubated with rabbit antiserum against bovine type II collagen (Advance, Tokyo) diluted 1:500 with 1% bovine serum albumin in PBS for 1 h at 37°C. They were then washed with 0.05% Tween 20 in PBS (about five changes during 30 min) and incubated with peroxidase-conjugated anti-rabbit IgG (Cappel, Malvern, PA) diluted 1:300 for 1 h at 37°C. After another wash, the sheets were incubated with 0.01% H_2O_2 in a mixture of PBS and a methanolic solution of 4-chloronaphthol (3 mg/ml) (v/v, 5:1). The reaction was stopped by adding 4 N sulfuric acid.

Protein Determination. Protein was determined by the method of Lowry *et al.* (25) with bovine serum albumin as a standard.

Tumorigenicity in Athymic Mice. The tumorigenicity of HCS-2/8 cells was determined by s.c. injection of 5×10^6 cells in 0.3 ml of PBS into the backs of 6–7-week-old BALB/c:*nu/nu* nude mice (Curea Laboratory Animal Center, Osaka, Japan). The tumors were harvested when they became about 1.0–1.5 cm in diameter, usually 2 to 4 months after inoculation of cells. Each tumor was fixed and processed for examination by light microscopy.

RESULTS

Establishment of Cell Lines. The uncloned HCS cell line at early passages consisted of various types of cells including rounded polygonal cells with a refractile matrix resembling rabbit costal chondrocytes in primary culture, and fibroblast-like cells. The uncloned cells proliferated very slowly: their doubling time in the logarithmic phase was more than 1 week. During serial subcultivations, the population of polygonal cells gradually increased. At passage 13, their doubling time became

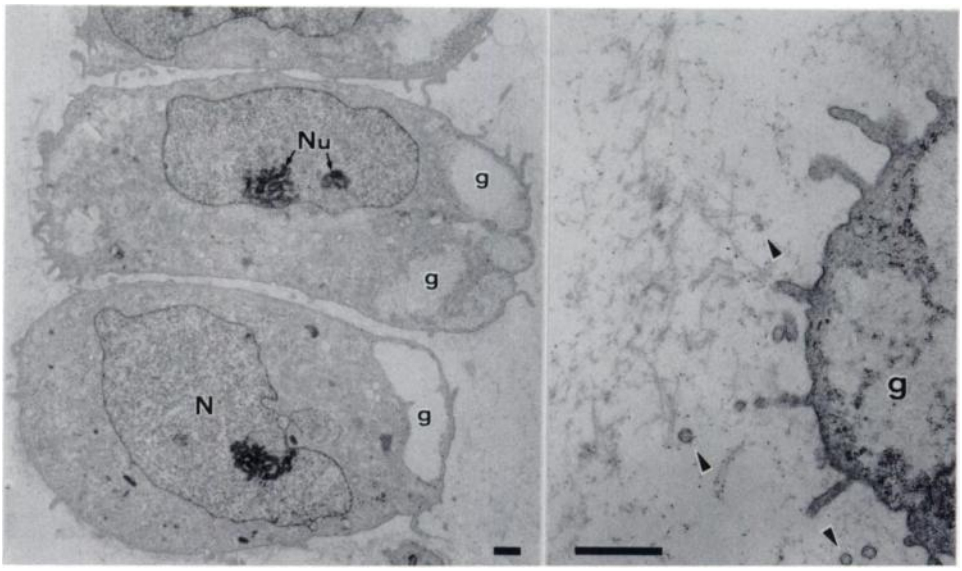

Fig. 4. Electron micrographs of clonal HCS cells. *Left*, typical HCS-2/8 cells located in the nodule. *N*, nucleus; *Nu*, nucleolus; *g*, glycogen (Some glycogen was washed away during histological processing). *Right*, a clonal cell at higher magnification, showing numerous microprojections. Note the presence of fine granules associated with fibrillar structures in the extracellular matrix. *Arrowheads*, transverse sections of microprojections; *g*, glycogen; *bars*, 1 μm.

Table 1 *Proteoglycan synthesis by clonal HCS cells and cells derived from other tissues*

HCS-2/8 cells were inoculated at a density of 2×10^5 cells/35-mm dish at the indicated passages. Other cells were inoculated at a density of 1×10^5 cells/35-mm dish. When the cells became confluent, they were labeled for 3 h in a mixture of Gey's solution and Hanks' solution (9:1, v/v) containing 1 μCi of [^{35}S]sulfate.

Type of cells	Passage no.[a]	[^{35}S]Sulfate incorporation (dpm/μg protein)
HCS-2/8	8 (30)[b]	108.8 ± 3.7[c]
	13 (35)	134.8 ± 16.1
	22 (42)	109.4 ± 31.6
Rabbit costal chondrocytes	0	159.0 ± 3.5
	1	64.5 ± 9.0
	4	21.5 ± 2.5
	9	7.5 ± 0.3
Mouse costal chondrocytes	0	48.4 ± 6.9
	1	33.0 ± 11.3
Mouse skin fibroblasts	0	7.8 ± 1.2
NRK	-[d]	4.5 ± 2.2
Swiss 3T3	-[d]	11.5 ± 1.4

[a] Passage numbers of clonal lines described in this table and the legends for all figures indicate passage numbers after cloning.
[b] Numbers in parentheses include passage numbers from before cloning.
[c] Values are means ± SD for four dishes.
[d] >30 passages.

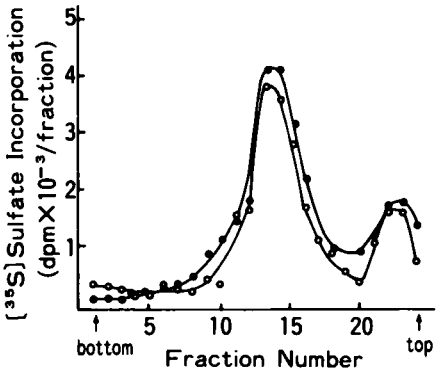

Fig. 5. Profile in a linear glycerol gradient under dissociative conditions of [^{35}S]sulfate-labeled proteoglycans synthesized by confluent cultures of HCS-2/8 cells. HCS-2/8 cells (○) (passage 10) were plated at a density of 2×10^5 cells/35-mm dish and cultured for 14 days. Rabbit costal chondrocytes (●) were plated at a density of 1×10^5 cells/35-mm dish and cultured for 10 days. The cells were labeled with 30 μCi/ml of [^{35}S]sulfate in a mixture of Gey's and Hanks' solutions (9:1). Proteoglycans extracted with 4 M guanidine-HCl were layered onto a linear gradient (17 ml) of glycerol (5–25%). Centrifugation was carried out under dissociative conditions.

about 6 days and their saturation density was 1.5×10^6 cells/35-mm dish (Fig. 1). When confluent cultures at passage 13 were stained with toluidine blue, the extracellular matrix of the polygonal cells stained metachromatically; the matrix showed purple color (data not shown). However, during further subcultivations, their doubling time became longer and the saturation density of cultures became less than 1×10^6 cells/35-mm dish. Moreover, the population of cells which appeared to be dedifferentiated increased. Therefore, to obtain cell lines that proliferated permanently and expressed the differentiated phenotype of chondrocytes, we cloned HCS cells at passage 22. More than 40 clones were isolated from six 100-mm dishes. One of these clones, named HCS-2/8, showed stable growth and high ability to synthesize proteoglycans during many serial passages. The present paper describes characters of the HCS-2/8 cell line.

Growth Properties. The clonal cell line HCS-2/8 at passage 8 after cloning (passage 30 from the start of culture) proliferated more rapidly than the uncloned cells. When HCS-2/8 cells were plated at a density of 1×10^5 cells/35-mm dish and cultured in MEM containing 20% FBS, they proliferated with a doubling time of 3.5 days, reaching confluence on Day 10. However, the cells then continued to proliferate slowly to a saturation density of about 2×10^6 cells/35-mm dish (Fig. 1). This growth pattern was similar when determined at passage 20 after cloning. The cells proliferated in almost the same way in Dulbecco's MEM containing 10% FBS as in MEM plus 20% FBS, but in Dulbecco's MEM containing 2% FBS their doubling time was twice that in Dulbecco's MEM containing 10% FBS.

Morphology and Histochemistry. In sparse cultures, HCS-2/8 cells became attached to the substratum and were a slightly elongated polygonal shape (Fig. 2*A*). As they became confluent, they became polygonal and then spherical, but still remained attached to the substratum (Fig. 2, *B* and *C*). On culture for more than 3 weeks, the cells formed nodules (Fig. 2, *D* and *E*). These nodules showed strong metachromasia when stained with toluidine blue (Fig. 2*F*); the extracellular matrix was stained strong pinkish purple. As shown in Fig. 3, the nodules were three-dimensional in structure. The cells in nodules were multilayered and were mostly ovoid, and they were surrounded by a considerable amount of extracellular matrix showing metachromasia when stained with toluidine blue. A thick layer of extracellular matrix with a coarse meshwork of fibrous components extended from the base of the cell layer to the bottom of the culture dish. Thickness of the cell layers in regions between nodules varied, but in most regions there were more than two layers of cells and these cells had similar morphological features to those in nodules.

Ultrastructural Observation. Clonal chondrosarcoma cells had an oval contour with numerous microprojections, rather like chondrocytes *in vivo* (Fig. 4). The nucleus was centrally located in the cytoplasm and contained a prominent nucleolus. Portions of the cytoplasm were occupied by variable-sized masses of glycogen granules (Fig. 4, *left*). The rough-surfaced endoplasmic reticulum was moderately developed and was mainly located in the periphery of the cytoplasm. Thin collagen-like fibrils were distributed throughout the extracellular matrix. At high magnification, numerous fine granules presumably of proteoglycans, were seen to be associated with matrix fibrils (Fig. 4, *right*).

Proteoglycan Synthesis. The incorporation of [^{35}S]sulfate into proteoglycans in HCS-2/8 cells at passage 8 was 70% of that of primary cultures of rabbit costal chondrocytes, 250% of that of primary cultures of mouse costal chondrocytes and 9 to 24 times that of cells derived from other tissues, such as NRK, Swiss 3T3, and mouse fibroblasts (Table 1). In contrast to rabbit costal chondrocytes, HCS-2/8 cells retained the ability to synthesize proteoglycans during many serial passages (Table 1).

Hydrodynamic Sizes of Proteoglycan Monomers. We have reported that rabbit chondrocytes in primary culture produced two populations of proteoglycans distinguishable by density gradient centrifugation under dissociative conditions (11, 16) (Fig. 5). We found that the faster-sedimenting proteoglycan was the major component in differentiated chondrocytes and must correspond to the cartilage-characteristic proteoglycan (26) and chondroblast-unique sulfated proteoglycan (27), while the slower-sedimenting proteoglycan was an ubiquitous species. Therefore, the faster sedimenting proteoglycan can be a good marker of cartilage phenotype, so we named the proteoglycan cartilage-specific proteoglycan. As shown in Fig. 5, the major component of proteoglycans synthesized by HCS-2/8 cells was the faster-sedimenting, cartilage-specific type.

Production of Type II Collagen. As shown in Fig. 6, HCS-2/8 cells stained by the indirect immunofluorescence technique with anti-type II collagen, but not with anti-type I collagen.

Analysis of [^{3}H]proline-labeled collagens of HCS-2/8 cells by SDS-PAGE and fluorography revealed a major band corresponding to the α1 band of type I and II collagens and two minor bands of higher molecular weight (Fig. 7*A*, *Lane 1*). No

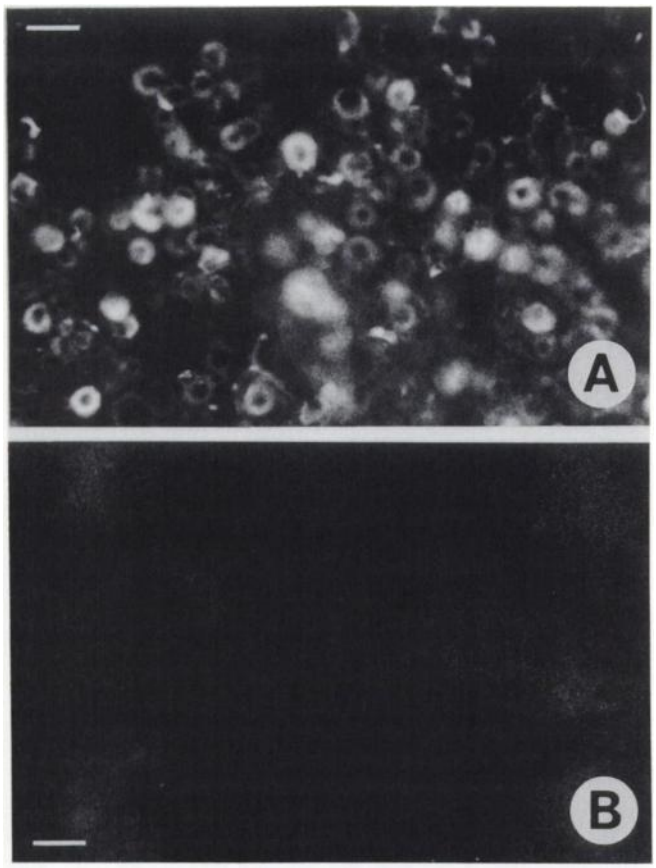

Fig. 6. Immunofluorescence staining for type II and type I collagens of HCS-2/8 cells. Inocula of 4×10^4 HCS-2/8 cells (passage 10) in 0.1 ml of medium were plated in stainless steel cylinders (length, 9 mm; inside diameter, 6 mm) placed in the center of 35-mm dishes. The cylinders were removed after the cells had become attached to the bottom of the dishes (24 h later) and the cells were fed with 2 ml of medium containing 50 μg/ml of ascorbic acid and cultured in the medium. They were fixed on Day 8 and stained with anti-type II collagen (*A*) or anti-type I collagen (*B*), as described in the text. *Bars*, 20 μm.

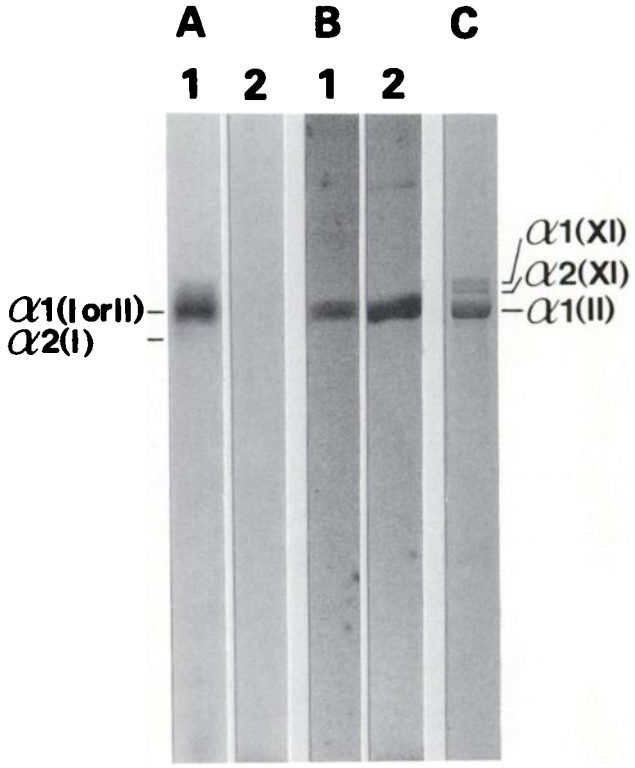

Fig. 7. Fluorography and immunoblotting of collagens separated by SDS-PAGE. HCS-2/8 cells (passage 20) were plated at a density of 1×10^6 cells/100-mm dish, grown in MEM containing 20% FBS for 2 weeks and then cultured in medium containing 50 μg/ml of ascorbic acid for 7 days. The cells were then labeled with 2 μCi/ml of [^{3}H]proline for 24 h and collagens were extracted by pepsin digestion as described in the text. In *A*, fluorograph of extracted collagens separated by SDS-PAGE; *Lane 1*, a major band corresponding to the α1 chain of type I and II collagens is seen, but no band of the α2 chain of type I collagen is detectable; *Lane 2*, treatment with collagenase resulted in disappearance of the proline-labeled collagen bands. In *B*, an identical sample (*Lane 1*) and type II collagen as a standard (*Lane 2*) were subjected to SDS-PAGE, transferred to nitrocellulose and immunoblotted with antibody against type II collagen. The major band stained with anti-type II collagen. In *C*, 1.2 M NaCl precipitate of extracted collagens was subjected to SDS-PAGE and stained with Coomassie blue. *α1 (I or II)*, *α1 (II)*, *α2*, *α1 (XI)*, and *α2 (XI)* indicate the positions of the α1 chain of type I or type II collagen, the α1 chain of type II collagen, the α2 chain of type I collagen, the α1 chain of type XI collagen and α2 chain of type XI collagen located by Coomassie blue staining.

α2 band of type I collagen was observed. Treatment with collagenase resulted in the disappearance of these bands, indicating that they were those of collagens (Fig. 7*A*, *Lane 2*). Immunoblotting analysis revealed that the major band was that of the α1 chain of type II collagen (Fig. 7*B*). When extracted collagens were partially purified and subjected to SDS-PAGE, the minor bands became clear to correspond to α1 and α2 chains, respectively, of type XI collagen (Fig. 7*C*).

Tumorigenicity. Inoculation of total numbers of 5×10^6 viable HCS-2/8 cells at passages 9, 16, and 21 into three nude mice resulted in development of palpable s.c. tumors, which grew to 5–10 mm in diameter within a month after cell inoculation and to 15–25 mm in diameter within 6 months. Grossly, the tumors were poorly vascularized and were solid at the periphery with central necrotic and cystic degeneration. Histologically, they were well-differentiated chondrosarcomas retaining the characters of the original tumor (Fig. 8, *A–D*). They showed metachromasia when stained with toluidine blue (Fig. 8, *E* and *F*) and stained with alcian blue (data not shown).

DISCUSSION

There are two main problems in establishment of tumor cell lines from chondrosarcomas: one is overgrowth of contaminating fibroblasts, and the another is change of chondrosarcoma cells to fibrosarcomatous cells. We subcultured uncloned cells at about 4-week intervals during early passages. Under these conditions, the cultures became confluent within at least 2 weeks, and tumor cells, which do not show contact inhibition, continued to proliferate while contaminating fibroblasts, which show contact inhibition, did not. Therefore, even if tumor cells proliferated more slowly than contaminating fibroblasts, their population increased during serial passages. However, when we tried to clone cells at early passages, the clonal cells did not proliferate well. Therefore, contaminating fibroblasts may have acted as a feeder layer during early passages.

The doubling time of the uncloned cells was over 1 week until passage 10, and 6 days at passage 13, and then became longer again (Fig. 1). The saturation density also decreased after 17 passages (Fig. 1). The clonal cell line HCS-2/8 proliferated faster than the uncloned cells: their doubling time was 3.5 days. Because the clonal cells were derived from the uncloned cultures, the population of cells from which the clonal cells originated should have increased in the uncloned cultures during subcultivation and consequently the doubling time and saturation density of the uncloned line should also have increased. The reason why the cells from which the HCS-2/8 cells originated could not proliferate well in the uncloned cultures is unknown. Dedifferentiated cells appeared in the uncloned line at about passage 17, so these cells may have produced a growth inhibitor of the cells which were cloned later.

Even after cloning, the HCS cells proliferated slowly. This

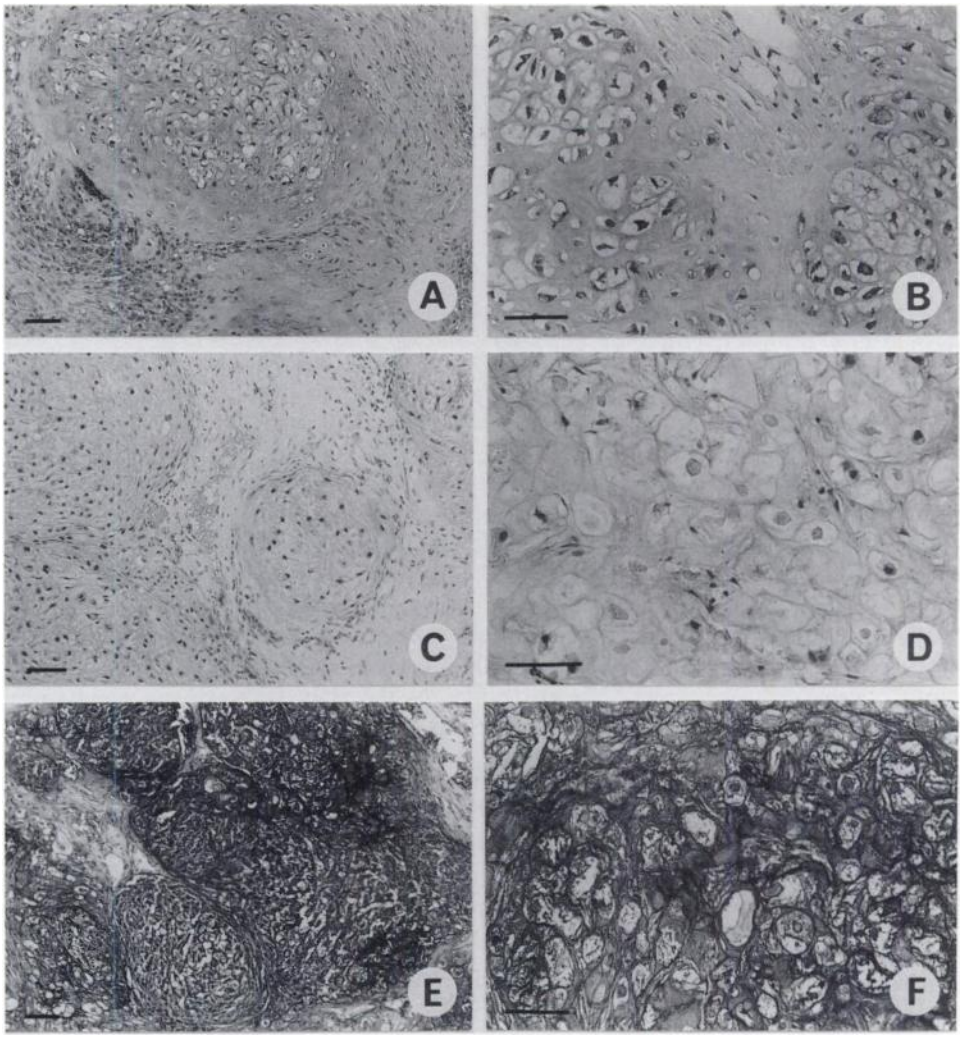

Fig. 8. *A* and *B*, the human chondrosarcoma from which HCS cells were derived; *C–F*, an HCS-2/8 cell (passage 16) tumor from a nude mouse. *A–D*, hematoxylin & eosin staining; *E* and *F*, toluidine blue staining. The tumor from a nude mouse was a proteoglycan-producing chondrosarcoma and retained the characters of the original tumor. *Bars*, 50 μm.

character may have reflected the characteristics of the original tumor, because chondrosarcomas are reported to be slow-growing tumors with a doubling time of 176 and 366 days, unlike other sarcomas such as osteosarcomas which have a doubling time of 32 days (18). Another possible reason for their slow growth is that the cells were well differentiated. If proliferation became more rapid, the cells might lose their cartilage phenotypes and become fibrosarcoma-like cells.

Two typical markers of chondrocytes are abilities to synthesize proteoglycans, especially cartilage-type proteoglycan (11, 16), and type II collagen (6–8), which is the principal collagen of cartilage (28, 29). HCS-2/8 cells have proliferated continuously and shown these characteristics during more than 43 passages in about 3 years from the start of culture (Figs. 2–8). Moreover, as seen in primary cultures and early subcultures of rodent (1, 2) and chick (3) chondrocytes, the cells accumulated large amounts of extracellular matrix that consisted of these macromolecules (Figs. 2–4). We have subcultured the cell line at a ratio of 1:4–8 and its plating efficiency of the cells is about 70%. The cells have now been cultured for about 130 cell generations from the start of culture (60 cell generations after cloning), indicating that the line has become a permanent cell line. There are no previous reports of maintenance of various cartilage phenotypes in cultured chondrocytes or chondrosarcoma cells for such a long time.

In addition to type II collagen, HCS-2/8 cells synthesized two minor collagen chains which appeared to be α1 and α2 chains of type XI collagen. Because type XI collagen is known to be present in cartilage (21, 29, 30) and to be synthesized by cultured chondrocytes (8), this finding also supports the evidence that HCS-2/8 cells maintain cartilage phenotypes very well.

Cells isolated from a Swarm rat chondrosarcoma, which have been maintained by *in vivo* transplantation, can express the cartilage phenotype in primary culture (31) but do not accumulate large amounts of extracellular matrix in culture (32), unlike rodent and chick chondrocytes (1–3) and HCS-2/8 cells (Fig. 3). Moreover, these cells are difficult to maintain in culture for a long time due to an alteration to a more fibroblastic phenotype (33). Recently, the ability of these cells to produce alcian blue-positive material was found to be maintained for 14 days when the cells were cultured in agarose gel, which has often been used to induce reexpression of the chondrocyte phenotype by dedifferentiating chondrocytes (33). However, it is unknown whether these cells can synthesize type II collagen or how long they can proliferate and express these phenotypes in culture. Miller *et al.* (34) tried to culture cells of a human chondrosarcoma, but their cells did not produce a metachromatic matrix even in primary culture. Thein and Lotan (35) reported chondrosarcoma cell lines, but these cell lines were fibroblast-like in morphology, and their expression of markers of chondrocytes was not examined.

It is unknown why our clonal cell line did not change to fibrosarcoma-like cells, unlike Swarm rat chondrosarcoma cells and the human chondrosarcoma cells reported by Thein and Lotan (35), but retained abilities to synthesize cartilage-specific

proteoglycans and type II collagen, which are two major phenotypes of cartilage. Cloning was undoubtedly helpful in establishing the cell line, because the growth rate of the uncloned cells decreased after 17 passages with the appearance of dedifferentiated cells. Although cloning may have selected specific cells that did not reflect the characters of the original tumor, HCS-2/8 cells formed chondrosarcomas that resembled the original tumor, suggesting that the characters of HCS-2/8 cells are those of the original tumor cells.

In the case of normal chondrocytes, no one has succeeded in establishing even a permanent cell line without cartilage phenotypes. For example, rabbit costal chondrocytes lose cartilage phenotypes after four passages and stop proliferating after 10 passages (9). On the other hand, Katoh and Takayama (36, 37) transformed hamster sternal cartilage cells *in vitro* by exposing them to 4-nitroquinoline 1-oxide and established clonal chondrogenic cell lines from the transformed cells. They maintained these cell lines for about 1 year and showed that they synthesized a metachromatic matrix. However, they did not determine whether the cells synthesized cartilage-specific proteoglycan and type II collagen, which are specific markers of chondrocytes or how long the cells could be maintained *in vitro* without losing their ability to produce metachromatic matrix. Moreover, these cells were transformed cells that did not form tumors, and could not be used for studies on chondrosarcomas.

The HCS-2/8 cell line established in this work should be useful for studies on the differentiated phenotypes of human chondrocytes because it is of human origin and can proliferate permanently and synthesize a matrix consisting of cartilage-specific proteoglycan and type II collagen. Moreover, it is tumorigenic and forms tumors in nude mice that are similar to the original tumor, and are of human origin, so it should be useful for studies on the diagnosis, treatment, and etiology of human chondrosarcomas.

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