Virchows Arch (1998) 432:529–534

ORIGINAL ARTICLE

Tokuhiro Chano · Hidetoshi Okabe
Yukikazu Saeki · Michihito Ishizawa
Keiji Matsumoto · Sinsuke Hukuda

Characterization of a newly established human chondrosarcoma cell line, CS-OKB

Received: 17 June 1997 / Accepted: 1 December 1997

Abstract A clonal cell line, CS-OKB, was derived from a human chondrosarcoma and characterized by cytogenetic study, immunocytochemical staining, and reverse transcriptase polymerase chain reaction (RT-PCR). Chromosomal abnormalities characteristic of malignant cartilaginous neoplasms were identified. CS-OKB cells were intensely stained with anti-type II collagen and anti-keratan sulphate antibodies. RT-PCR indicated that CS-OKB transcribes cartilage-specific genes such as type II, X procollagen, and aggrecan. This human chondrosarcoma cell line is stable and expresses well-differentiated chondrocyte-specific genes. It synthesizes well-differentiated chondrocyte-specific molecules in uncoated plastic dishes. CS-OKB may be useful for studies of human chondrocytes and in characterizing human chondrosarcomas.

Key words Human chondrosarcoma · Cell line · Type II collagen · RT-PCR · Immunocytochemistry

Introduction

Chondrocytes are cartilage-specific cells that secrete a series of cartilage-specific molecules including type II collagen [13] and proteoglycans [11]. Previously, primary cultures of normal chondrocytes from rabbit or chick have been used for cartilage research [2, 14, 18], but most of the differentiation characteristics of these cultures, in particular the typical polygonal shape and the production of type II collagen and proteoglycans, are lost after several passages in culture [2]. Furthermore, without contact with extracellular matrix, these chondrocytes show limited proliferation potential [14].

Generally, the establishment of a cell line from a tumour is not difficult; however, chondrosarcoma is very rare, onstituting about 0.05% of all malignant tumours [5]. Establishment of a cell line from this tumour, especially from humans, would be very useful for both biological and biochemical studies. Only two permanent cell lines of human chondrosarcomas, HCS-2/8 and 105KC, carrying cartilage phenotypes, have been reported [3, 17]. HCS-2/8 cells can synthesize cartilage-specific type II collagen [17], while 105KC cells can produce keratan sulphate-containing proteoglycans, but need to be alternated between monolayer and agarose suspension cultures [3].

In this paper, we describe a new cell line, CS-OKB, derived from a human chondrosarcoma. Using immunocytochemical staining and the reverse transcriptase–polymerase chain reaction (RT-PCR) we have shown that this cell line secretes type II collagen.

Materials and methods

CS-OKB was derived from a human chondrosarcoma. A 79-year-old man with parosteal chondrosarcoma of the left humerus of the shoulder was treated by disarticulation on 19 October 1989 [12]. When the patient was 85, the tumour metastasized to the left biceps femoris muscle. This lesion was resected on 26 October 1995. Immediately after the operation, parts of the tumour specimen were fixed in buffered-formalin, embedded in paraffin, cut into thin sections, and stained with haematoxylin-eosin, alcian blue, and toluidine blue. The metastatic tumour was identified as chondrosarcoma, grade 2, with a well-differentiated chondroid matrix (Fig. 1). The remainder of the specimen was used to create tissue culture.

Tumour tissue was minced aseptically and treated with 0.25% trypsin in PBS for 15 min at 37°C. The resulting tumour cell suspension was passed through a nylon mesh and washed twice with RPMI-1640 containing 10% fetal bovine serum (FBS: Gibco BRL, Life Technologies, Tokyo, Japan), penicillin (100 U/ml), and streptomycin (100 μg/ml). The resultant mixture was plated in 60-mm plastic petri dishes (Falcon, Becton Dickinson Labware, New Jersey, USA) at 10^5 cells/ml of medium. Cultures were incubated

T. Chano (✉) · M. Ishizawa · K. Matsumoto · S. Hukuda
Department of Orthopaedic Surgery,
Shiga University of Medical Science, Seta,
Ohtsu, Shiga 520-21-92, Japan
Tel.: +81-77-548-2252, Fax: +81-77-548-2254

H. Okabe
Department of Clinical Laboratory Medicine,
Shiga University of Medical Science, Shiga, Japan

Y. Saeki
Department of Basic Science for Health and Nursing,
Shiga University of Medical Science, Shiga, Japan

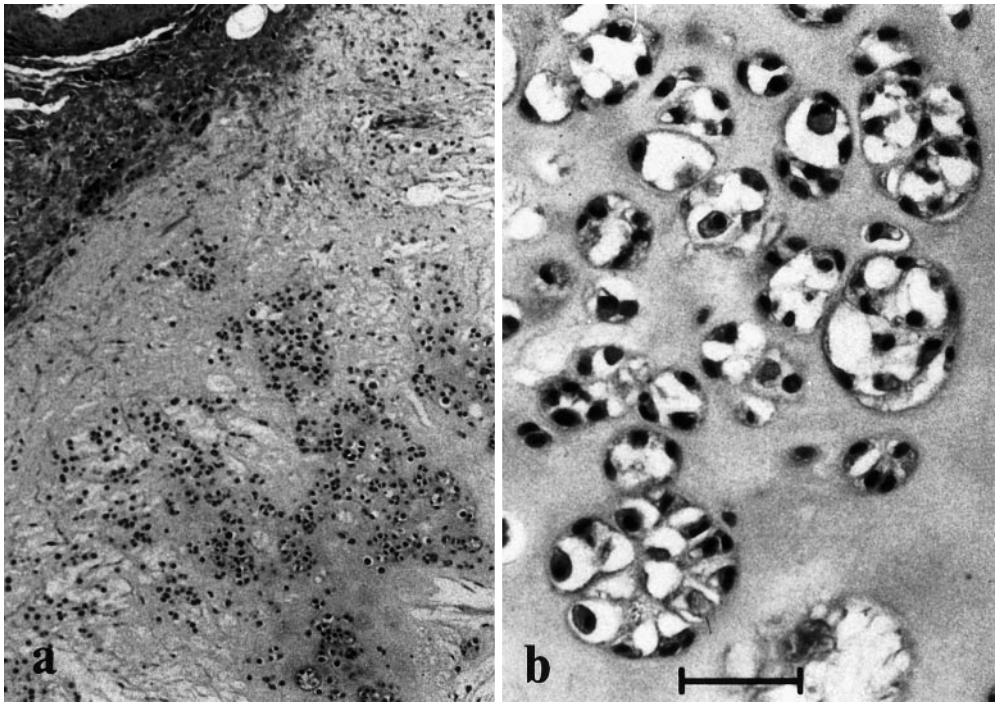

Fig. 1a, b Section of the lesion from which CS-OKB cells were extracted. It was a moderately cellular, cartilaginous tumour. The matrix was composed mainly of hyaline cartilage with myxomotous change. Chondrocyte nuclei were sometimes malformed. This tumour was diagnosed as chondrosarcoma, grade 2. H&E, **a** ×50, **b** ×400, *bar* 50 μm

Table 1 Primary antibodies used in the immunocytochemical analysis of CS-OKB

Antibodiy	Source	Dilution	Reactivity
Monoclonal antibodies			
Anti-human type II collagen	Fuji Yakuhin Kogyo, Takaoka, Japan	1:20	+
Anti-human type IV collagen	Progen Biotech., Heidelberg, Germany	1:20	–
Anti-keratan sulphate	ICN Pharma. Costa Mesa, USA.	1:100	+
Polyclonal antibodies			
Anti-rat type I collagen	LSL, Tokyo, Japan	1:500	+
Anti-rat type III collagen	Chemicon International, Temecula, USA	1:1,000	+
Anti-cow S-100 protein	DAKO, Copenhagen, Denmark	1:600	+

Table 2 Primers used for RT-PCR amplification

Target cDNA	Primer sequence (5′ → 3′)	Fragment size (bp)
β-Actin [20]	S:5′-ATCTGGCACCACACCTTCTACAATGAGCTGCG-3′ A:5′-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3′	838
Type II procollagen [4]	S:5′-AACTGGCAAGCAAGGAGACA-3′ A:5′-AGTTTCAGGTCTCTGCAGGT-3′	621
Type X procollagen [4]	S:5′-AGCCAGGGTTGCCAGGACCA-3′ A:5′-TTTTCCCACTCCAGGAGGGC-3′	387
Aggrecan [4]	S:5′-ATGCCCAAGACTACCAGTGG-3′ A:5′-TCCTGGAAGCTCTTCTCAGT-3′	501/318
Type I procollagen [15]	S:5′-TGACGAGACCAAGAACTG-3′ A:5′-CCATCCAAACCACTGAAACC-3′	599
Type III procollagen [15]	S:5′-GCGGAGTAGCAGTAGGAG-3′ A:5′-GTCATTACCCCGAGCACC-3′	484
Type IV procollagen [8]	S:5′-CCTGCTTCATTGACCTCTACT-3′ A:5′-TTGGTTTTGGCAACACATAAT-3′	699

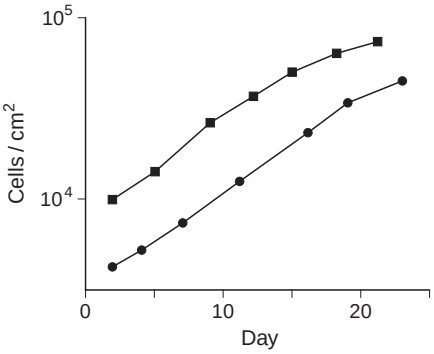

Fig. 2 Growth curves of the clonal line CS-OKB. The clonal CS-OKB cells at passage 17 were plated at 1×10^4 cells/cm^2 and grown in RPMI-1640–10% FBS (■). CS-OKB cells at passage 26 were plated and cultured as above (●). They had a doubling time of 5 days to a saturation of about 2×10^5 cells/cm^2

at 37°C in a humidified atmosphere at 5% CO in air. Cells were treated with 0.25% trypsin and subcultured at a dilution of 1:2 to 1:5 every 2–4 weeks. The medium was changed twice a week.

Cells from the 12th passage were inoculated into three dishes at approximately 200 cells/100-mm dish (Falcon) and allowed to form colonies. At this plating density colonies were widely spaced, facilitating picking of single colonies. When colonies were about 25–100 cells in size, single colonies were transferred to a 24-well plate by trypsinization within cloning cylinders (Belco Glass, Vineland, N.J., USA) and grown in identical medium. Upon confluency, cells were subcultured 1:2–5 and expanded into 35-mm dishes. Clones with strong immunocytochemical activity for type II collagen and S-100 protein, a chondrocyte phenotype, were selected and named CS-OKB.

Twentieth-passage CS-OKB cells were harvested and chromosome preparations were made in the usual manner. Chromosomes were banded by the G-banding method with trypsin and Giemsa [16], and analysed in the Mitsubishi Chemical BCL cytogenetic laboratory.

To identify the production of various matrix molecules, CS-OKB cells were analysed immunocytochemically (Table 1). CS-OKB cells of the 6th and 23rd passages were subcultured in Lab-Tek chamber slides (Nunc, Naperville, Ill., USA). Cultured cells were washed twice with PBS, fixed in 70% ethanol at 4°C for 3 min, and processed by immunocytochemical staining. For staining the slides were pretreated with 0.3% H_2O_2 and normal gout serum, and rinsed with Tris-buffered saline. The streptoavidine–biotin-peroxidase complex method was used for staining. All reagents were obtained from DAKO (Copenhagen, Denmark). Briefly, the slides were first incubated with each primary antibody at adequate dilution (Table 1) for 1 h at room temperature and stained with fresh diaminobenzidine. Following immunostaining, all sections were counterstained with methyl green, dehydrated, cleared with xylene, and mounted. Normal mouse or rabbit serum were substituted for primary antibody as a negative control.

Total RNA were extracted from subconfluent CS-OKB cells at the 18th and 23rd passages using TRIZOL reagent (Gibco). Isolated RNA samples were treated with TRIZOL to remove trace amounts of DNA. Complementary DNA was synthesized from 500 ng of total RNA in a 20-µl reaction mixture containing 1 × reverse transcriptase buffer, 5 mM $MgCl_2$, 4 mM dNTP mixture, 20 U of RNase inhibitor, 5 U of AMV reverse transcriptase, and 2.5 µM of random primers [nonadeoxyribonucleotide mixture; $pd(N)_9$]. All reagents were obtained from Takara Biomedicals (Osaka, Japan). The reaction proceeded at 30°C for 10 min, 42°C for 20 min, and 99°C for 5 min.

Amplification reactions specific for the following cDNAs were performed: types I, II, III, IV, X collagen α-chain, aggrecan, and β-actin. PCR primer sequences are given in Table 2. Aliquots (1%) of the total cDNA were amplified in a 20-µl reaction mixture con-

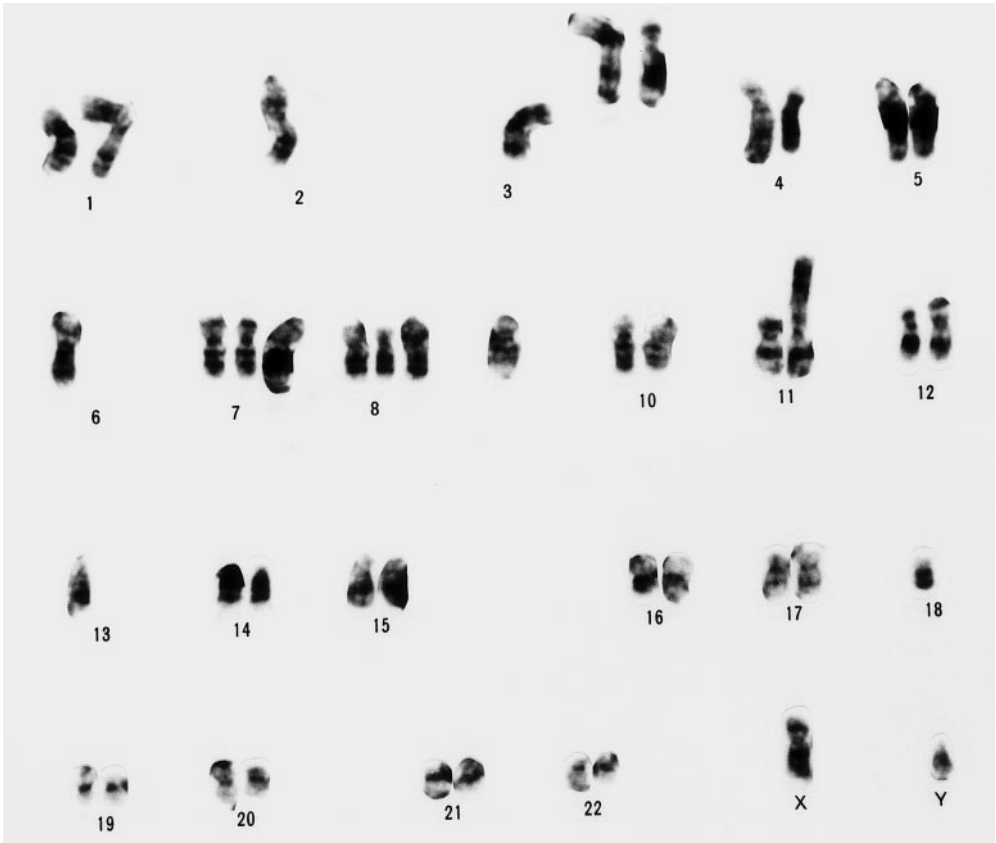

Fig. 3 G-banded karyotype of CS-OKB showing the chromosome number of 43, and XY, del(1)(p1)(p11 p22or31), –2, –3, –6, +7, –9, del(9)(p21 p24), dev(11)t(2;11)(q11;p15), –13, –18, +2mar[3]/44, idem, +8

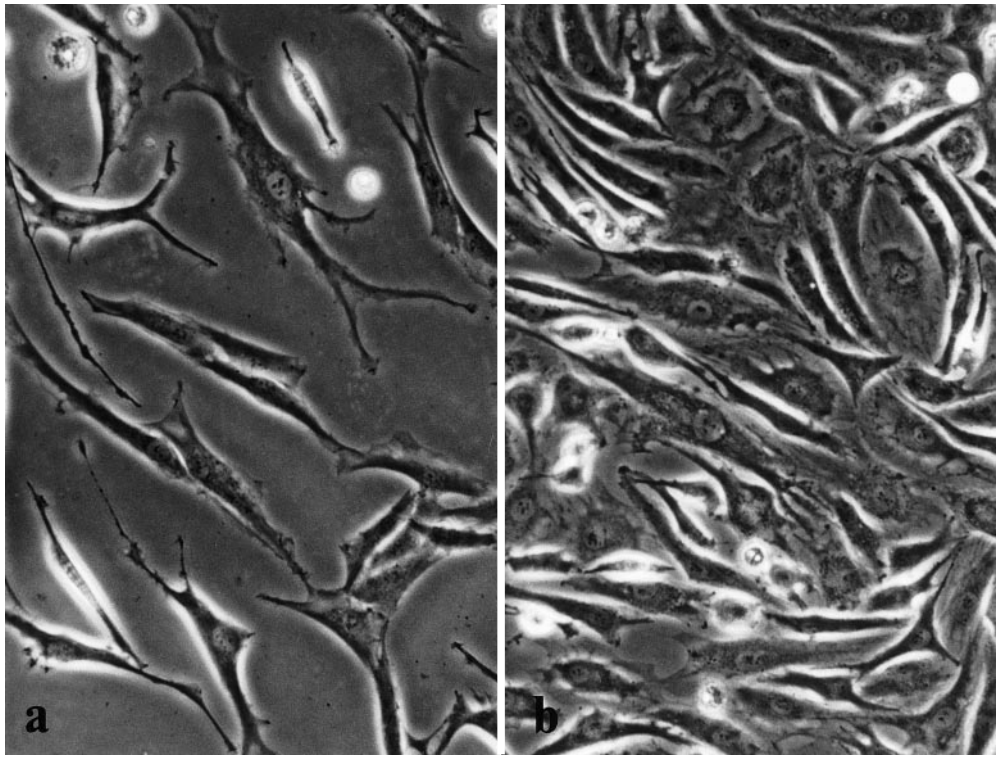

Fig. 4 **a** In sparse cultures, CS-OKB cells were elongated with polygonal and spherical shapes. ×420 **b** They showed polygonal and spherical at a confluent density. ×420

taining 500 nM of both sense and antisense primers, 1 × PCR buffer, 4 mM dNTP mixture, 2.5 mM $MgCl_2$, and 0.5 U of Taq polymerase. Amplifications were performed in a GeneAmp 9600 thermal cycler (Perkin-Elmer).

For the amplification of types II, III, and X collagen, aggrecan, and β-actin, Ampli-Taq Gold (Perkin-Elmer, Norwalk, Conn., USA) was used, and the initial denaturation and polymerase-activation step was at 95°C for 10 min. We used the following PCR characteristics: 40 cycles of (94°C, 30 s; 62°C, 1 min) with a final 10 min extension step at 72°. In the case of β-actin, only 34 cycles of PCR were applied.

For the amplification of type I collagen, Ampli-Taq (Perkin-Elmer) was used instead of Ampli-Taq gold, with 35 cycles of (94°C, 30 s; 55°C, 1 min; 72°C, 1 min) after an initial denaturation step at 95°C for 2 min.

The amplified DNA fragments were separated by electrophoresis in 1.5% agarose gels, and visualized by ethidium bromide staining.

Results

CS-OKB cells at passage 17 plated at 1×10^4 cells/cm^2 and cultured in RPMI-1640 containing 10% FBS had a doubling time of 5 days and reached confluency between days 14 and 20. The cells then continued to proliferate slowly to saturation at about 2×10^5 cells/cm^2 (Fig. 2). After passage 26, CS-OKB still proliferated with a doubling time of 5 days. CS-OKB cells had a chromosome number ranging from 43 to 44, and the following karyotype: 43, XY, del(1) (p1) (p11 p22or31), –2, –3, –6, +7, –9, del(9) (p21 p24), dev(11)t(2;11) (q11;p15), –13, –18, +2mar/44, idem, +8 (Fig. 3).

In sparse cultures, CS-OKB cells attached to the substratum and elongated with a polygonal shape (Fig. 4a). As they became confluent, cells exhibited polygonal and spherical shapes, remained attached to the substratum, and proliferated without contact inhibition (Fig. 4b). However, when CS-OKB cells were cultured in a type I-collagen-coated dish, they exhibited a spindle shape during subconfluent stage, and a spherical shape at confluency.

CS-OKB cells stained strongly with antibodies against cartilage-specific substances, such as type II collagen (Fig. 5a), S-100 protein, and keratan sulphate (Fig. 5b). Cells also stained with anti-type I and III collagen antibodies, but not with anti-type IV collagen antibody. Immunoreactivity against type II collagen antibody was found to be proportional to cell density irrespective of the presence of a collagen substratum.

Agarose gels of the RT-PCR products from β-actin, types II and X collagen, aggrecan, and types III, IV and I collagen are shown in Fig. 6. Transcripts of cartilage-specific genes, for example types II, X collagen and aggrecan, were identified in these gels. However, genes for types I, III and IV collagens, which are not specific to cartilage tissue, were also found to be transcribed. Steady-state levels of these mRNAs were similar in CS-OKB cells at passage 18 and 23.

Discussion

Chondrocytes are difficult to culture in vitro, because of their limited proliferation potential, and usually cease dividing after several generations [9, 14]. They also tend to dedifferentiate phenotypically into fibroblastic cells [2, 18]. Establishment of a stable chondrocyte cell line would be of great value.

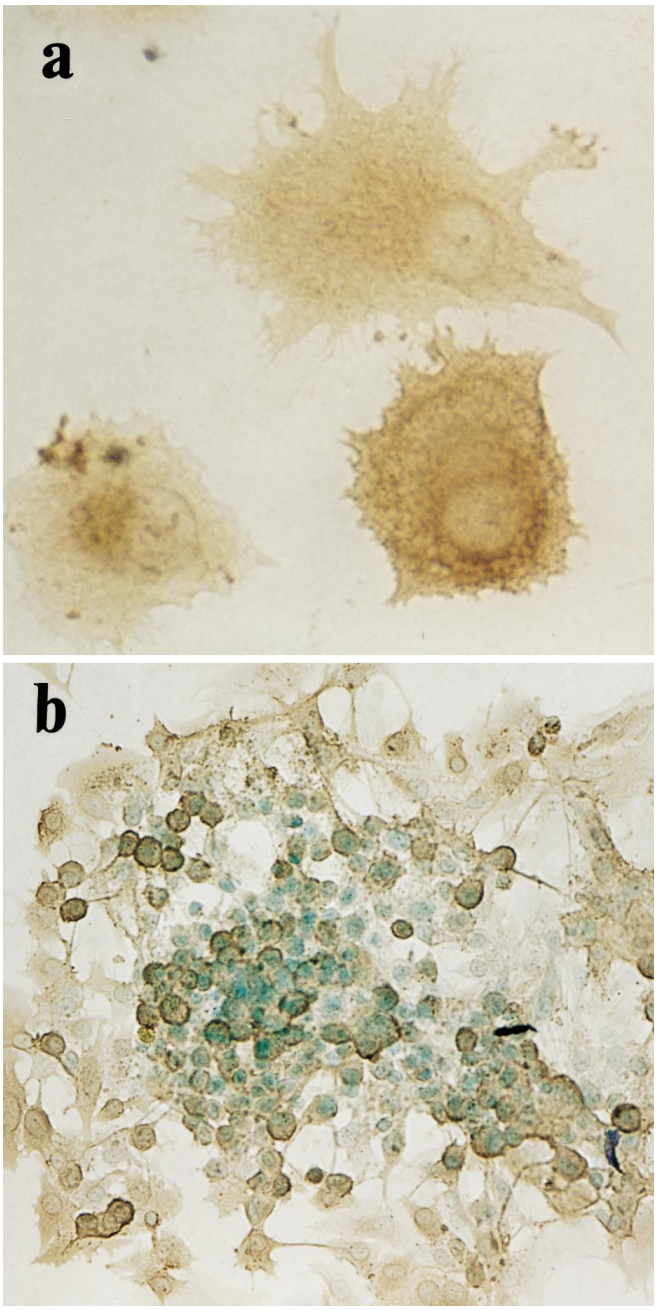

Fig. 5 **a** CS-OKB cells were immmunocytochemically stained with anti-type II collagen antibodies. ×1,000 **b** Keratan sulphate was also identified. ×230

Permanent cell lines are easier to establish from tumours than from normal tissues. However, chondrosarcoma is rare, but fortunately, we obtained a chondrosarcoma and were able to culture it in vitro. We cloned the cells after 12 passages and obtained 10 colonies, 1 of which we named CS-OKB. CS-OKB cells were stable and had a doubling time of 5 days. We continue to subculture these cells at 1:2–5, with a plating efficiency of about 70%. Presently, CS-OKB cells have been cultured for more than 100 generations (80 cell generations after cloning), indicating that the cell line is permanent. CS-OKB grows on non-coated plastic dishes without the contact inhibition. The tumorigenicity of CS-OKB in nude mice has not been determined; however, original tumour pieces were transplanted into nude mice and did not grow.

Chromosomes 6, 13, 8, 1 and 7 of CS-OKB showed abnormalities, with loss of chromosome 6 shown by cytogenetic study. Chromosome 6 abnormalities have been suggested to be associated with the majority of cartilaginous neoplasms [16]. The prominent involvement of 12q13-15, identified in CS-OKB, has also been identified as possibly significant for cartilaginous tumours [16]. The aberrations of chromosome 8, 1, and 7 are consistent with the previously reported cytogenetic studies of malignant neoplasms [16].

To characterize CS-OKB, we looked for chondrocyte-specific products such as type II collagen, using immunocytochemical staining, and the expression of chondrocyte-specific genes using RT-PCR. We found that CS-OKB secretes type II collagen and keratan sulphate in uncoated plastic dishes and transcribes cartilage-specific genes such as type II, type X procollagen, and aggrecan. In addition, CS-OKB cells synthesize S-100 protein, which has previously been detected in chondrocytes [19]. It is also reported that the expression of type X procollagen [10] and S-100 [19] protein is associated with the hypertrophic zone of cartilage. Therefore, CS-OKB cells are probably well-differentiated hypertrophic chondrocytes. Interestingly, CS-OKB cells produce type I and type III, which are normally expressed in a more immature mesenchymal cells. RT-PCR analysis of the expression of the aggrecan shows two nucleotide bands at 501 and 318 bp, indicating the alternative splicing of exon 18 [1]. The bigger band corresponds to the complement-regulatory protein domain, whilst the smaller band lacks this domain. It has been re-

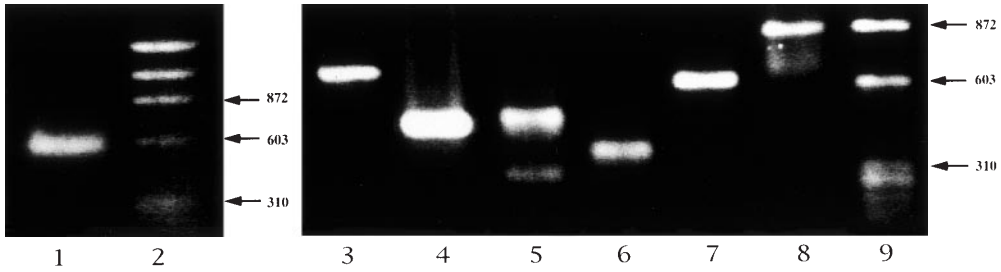

Fig. 6 RT-PCR analysis of mRNA isolated from CS-OKB cells at passage 23. PCRs were separately performed and the products were identified by ethidium bromide-stained agarose gel electrophoresis. Lanes correspond to types I (lane *1*), IV (lane *3*), III (lane *4*), X (lane *6*) and II (lane *7*) procollagen, and aggrecan (lane *5*). Lanes *2* and *9* are molecular-sized marker; ϕX174-HaeIII digest

ported that the small band is associated with dedifferentiation [4, 7]. This suggests that CS-OKB is slightly dedifferentiated in uncoated plastic dishes. However, further studies are necessary to confirm this result.

Type IV collagen in cartilaginous tissue is associated with cartilage canals in perichondral invaginations of blood vessels [6]. Transcription of this gene in CS-OKB may relate to vessel induction in malignant sarcomas. However, production of type IV collagen was not detected immunocytochemically.

The human chondrosarcoma cell line CS-OKB is a stable line that expresses well-differentiated chondrocyte-specific molecules (type II collagen, S-100 protein and keratan sulphate-containing proteoglycans) and cartilage-specific genes (type II, X procollagen and aggrecan genes) during prolonged culture. It will be useful for studies on the differentiated phenotypes of human chondrocytes and in characterizing human chondrosarcomas.

Acknowledgments We thank Sei Yoshida and Mari Asada, Pharmaceutical Research Laboratory III, Pharmaceutical Research Division, Takeda Chemical Industries, for their kind advice on RT-PCR.

References

1. Baldwin CT, Reginato AM, Prockop DJ (1989) A new epidermal growth factor-like domain in the human core protein for the large cartilage-specific proteoglycan. Evidence for alternative splicing of the domain. J Biol Chem 264:15747–15750
2. Benya PD, Shaffer JD (1982) Dedifferentiated chondrocytes reexpressed the differentiated collagen phenotype when cultured in agarose gels. Cell 30:215–224
3. Block JA, Inerot SE, Gitelis S, Kimura JH (1991) Synthesis of chondrocytic keratan sulfate-containing proteoglycans by human chondrosarcoma cells in long-term cell culture. J Bone Joint Surg [Am] 73:647–658
4. Bonaventure J, Kadhom N, Cohen-Solal L, Ng KH, Bourguignon J, Lasselin C, Freisinger P (1994) Reexpression of cartilage-specific genes by dedifferentiated human articular chondrocytes cultured in alginate beads. Exp Cell Res 212:97–104
5. Dorfman HD, Czerniak B (1995) Bone cancers. Cancer [Suppl] 75:203–210
6. Ganey TM, Ogden JA, Sasse J, Neame PJ, Hilbelink DR (1995) Basement membrane composition of cartilage canals during development and ossification of the epiphysis. Anat Rec 241: 425–437
7. Grover J, Roughley PJ (1993) Versican gene expression in human articular cartilage and comparison of mRNA splicing variation with aggrecan. Biochem J 291:361–367
8. Hernandez MR, Ye H, Roy S (1994) Collagen type IV gene expression in human optic nerve heads with primary open angle glaucoma. Exp Eye Res 59:41–52
9. Horton WE Jr, Cleveland J, Rapp U, Nemuth G, Bolander M, Doege K, Yamada Y, Hassell JR (1988) An established rat cell line expressing chondrocyte properties. Exp Cell Res 178: 457–468
10. Kimura JH, Hardingham TE, Hascall VC, Solursh M (1979) Biosynthesis of proteoglycans and their assembly into aggregates in cultures of chondrocytes from the Swarm rat chondrosarcoma. J Biol Chem 254:2600–2609
11. Kuettner KE (1992) Biochemistry of articular cartilage in health and disease. Clin Biochem 25:155–163
12. Matsumoto K, Hukuda S, Ishizawa M, Saruhashi Y, Okabe H, Asano Y (1993) Parosteal (juxtacortical) chondrosarcoma of the humerus associated with regional lymph node metastasis. A case report. Clin Orthop 290:168–173
13. Mayne R (1989) Cartilage collagens. What is their function, and are they involved in articular disease? Arthritis Rheum 32: 241–246
14. Mayne R, Vail MS, Mayne PM, Miller EJ (1976) Changes in type of collagen synthesized as clones of chick chondrocytes grow and eventually lose division capacity. Proc Natl Acad Sci 73:1674–1678
15. Rickard DJ, Kassem M, Hefferan TE, Sarkar G, Spelsberg TC, Riggs BL (1996) Isolation and Characterization of osteoblast precursor cells from human bone marrow. J Bone Mine Res 11:312–324
16. Sandberg AA, Bridge JA (1995) The cytogenetics of bone and soft tissue tumors. Springer, Berlin Heidelberg New York
17. Takigawa M, Tajima K, Pan HO, Enomoto M, Kinoshita A, Suzuki F, Takano Y, Mori Y (1989) Establishment of a clonal human chondrosarcoma cell line with cartilage phenotypes. Cancer Res 49:3996–4002
18. Von der Mark K, Gauss V, Von der Mark H, Muller P (1977) Relationship between cell shape and type of collagen synthesized as chondrocytes lose their cartilage phenotype in culture. Nature 267:531–532
19. Weiss AP, Dorfman HD (1986) S-100 protein in human cartilage lesions. J Bone Joint Surg [Am] 68:521–526
20. Obtained from CLONTECH Lab. Inc., Palo Alto, Calif. Catalog #5401-1