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ARTICLES

Establishment and Characterization of Chondrocyte Cell Lines From the Costal Cartilage of SV40 Large T Antigen Transgenic Mice

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Abstract Complete understanding of the physiology and pathology of the cartilage is essential to establish treatments for a variety of cartilage disorders and defects such as rheumatoid arthritis, congenital malformations, and tumors of cartilage. Although synthetic materials have been used in many cases, they possess inherent problems including wear of the materials and low mechanical strength. Autograft has been considered very effective to overcome these problems. However, the limitation of the transplant volume is a major problem in autograft to be overcome. The costal cartilage is the most serious candidate for donor site transplantation, since it is the largest permanent hyaline cartilage in the body. To investigate the possibility using the costal cartilage as a transplant source, we have established and characterized three mouse chondrocyte cell lines (MCC-2, MCC-5, and MCC-35) derived from the costal cartilage of 8-week-old male SV40 large T-antigen transgenic mice. At confluence, all the cell lines formed nodules that could be positively stained with alcian blue (pH 2.5). The size of nodules gradually increased during culturing time. After 2 and 6 weeks of culture, RT-PCR analysis demonstrated that all three cell lines expressed mRNA from the cartilage-specific genes for type II collagen, type XI collagen, aggrecan, and link protein. Furthermore, type X collagen expression was detected in MCC-5 and MCC-35 but not in MCC-2. Any phenotypic changes were not observed over 31 cell divisions. Immunocytochemistry showed further that MCC-2, MCC-5, and MCC-35 produced cartilage-specific proteins type II collagen and type XI collagen, while in addition MCC-5 and MCC-35 produced type X collagen. Treatment with 1α, 25-dihydroxyvitamin D_3 inhibited cell proliferation and differentiation of the three cell lines in a dose-dependent manner. These phenotypic characteristics have been found consistent with chondrocyte cell lines established from cartilage tissues other than costal cartilage. In conclusion, costal cartilage shows phenotypic similarities to other cartilages, i.e., articular cartilage and embryonic limbs, suggesting that costal cartilage may be very useful as the donor transplantation site for the treatment of cartilage disorders. Furthermore, the cell lines established in this study are also beneficial in basic research of cartilage physiology and pathology. J. Cell. Biochem. 81:571–582, 2001. © 2001 Wiley-Liss, Inc.

Key words: costal cartilage; chondrocyte cell line; gene expression; matrix formation; simian virus 40 large T-antigen; 1α, 25-dihydroxyvitamin D_3

Endochondral and intramembranous ossification are two major processes in the skeletogenesis. Long bones in limbs, vertebral bones, and costal bones are formed via endochondral ossification. This process is characterized by a series of events including the condensation of mesenchymal cells, their differentiation into chondrocytes [Grigoriadis et al., 1988; Johnstone et al., 1998], maturation into hypertrophic chondrocytes, mineralization of the extracellular matrix, and finally the replacement of the hypertrophic cartilage with bone tissue [Searls et al., 1972; Linsenmayer et al., 1973; Pechak et al., 1986; Long and Linsenmayer, 1995; Binette et al., 1998]. Thus, most of the cartilage tissues formed in the development process are eventually replaced with bone

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tissue. The induction of bone formation by demineralized bone matrix and bone morphogenetic proteins also involves a cascade of endochondral ossification [Reddi et al., 1995; Asahina et al., 1996; Sailor et al., 1996; Hoshi et al., 1997; Leboy et al., 1997; Erlacher et al., 1998; Haaijman et al., 1999; Sato et al., 1999].

In contrast, in permanent cartilages such as the articular cartilage on the joint surface and costal cartilage, chondrocytes do not undergo a phenotypic conversion to hypertrophic cartilage, and neither is being replaced with bone tissue throughout its life under physiological conditions. The conversion of phenotype from proliferating cartilage to hypertrophic cartilage is characterized by an increase of cell volume, a marked elevation of alkaline phosphatase (ALPase) activity, the onset of expression of type X collagen [Kirsch and von der Mark, 1991; Nerlich et al., 1992; Girkontaite et al., 1996] and the reduction of type II collagen expression [Lefebvre et al., 1995]. To date, the mechanisms by which the permanent cartilage ceases phenotypic maturation prior to hypertrophic conversion, as well as maintaining the highly differentiated phenotype for prolonged periods, are yet unclear, since only a few chondrocyte cell lines to study the physiology of chondrocytes and a few reports about the comparison between transient and permanent cartilage have been available.

To establish a treatment for cartilage disorders, e.g., rheumatoid arthritis, osteoarthritis, and cartilage defects, cell biological understanding of the differences between permanent and transient cartilage is essential. Hyaline cartilage of the articular and costal cartilage is the most important of the permanent cartilages. However, it is unknown whether all hyaline cartilages in different positions have the same phenotype. If costal cartilage has the same phenotype as the hyaline cartilage of the articular cartilage, it will be readily available to use as a source for transplantations to treat a variety of cartilage disorders such as rheumatoid arthritis and osteoarthritis of the joints.

Transgenic mice harboring a temperature-sensitive simian virus 40 large T antigen gene have been proven to be an excellent source to establish a variety of cell lines, i.e., bone marrow stromal cells, articular cartilage cells and hepatocyte cells [Yanai et al., 1991; Takazawa et al., 1999; Negishi et al., 2000]. The activity of SV40 large T antigen, which binds to p53 and Rb thereby suppressing their function, is restricted at the so-called non-permissive temperature (39°C), while it is retained at the permissive temperature (33°C). Using such mice, we have tried to establish a chondrocyte cell line from permanent cartilage.

In the present study, we report the establishment and characterization of three chondrocyte cell lines from costal cartilage of SV40 large T-antigen transgenic mice.

MATERIALS AND METHODS

Cell Isolation and Culture

Costal cartilages were obtained from 8-week-old male transgenic mice harboring the temperature-sensitive simian virus 40 large T antigen regulated by the SV40 promoter. After removing the soft adhering tissue, costal cartilages were minced into small pieces, plated on 100 mm petri dishes, and cultured in α-MEM containing 10% fetal bovine serum (FBS), 50 μg/ml L-ascorbic acid phosphate magnesium salt n-hydrate (WAKO Pure Chemical Industries, Osaka, Japan), 1 × Glutamax® (GIBCO BRL, Grand Island, NY), 100 units/ml penicillin and 100 μg/ml streptomycin. After 1 week, the cells that had migrated from the cartilage pieces were washed with phosphate-buffered saline (PBS, pH 7.4) and dispersed with 0.05% trypsin–0.53 mM EDTA in the same buffer and subcultured. Thereafter, the cells were maintained in a monolayer culture in the same medium and subcultured when reaching 80–90% confluency. All the cultures were maintained at 33°C in a humidified atmosphere of 5% CO_2 in air with the medium being changed twice a week.

Cloning of Chondrocytes

The cells of the fifth passage were seeded at a cell density of 1×10^3 cells/dish in 150 mm culture dishes. Eighty-three discrete colonies were cloned using cloning cylinders. Three of the 83 clones showed high growth activity being maintained over 31 cell divisions. The following investigations were performed using these three clones (MCC-2, MCC-5, and MCC-35).

Cell Proliferation Assay

The three clones were seeded at a cell density of 5×10^3 cells/cm^2 in 24-well culture plates and

cultured in the same medium at both 33°C and 39°C. After 1, 3, 5, 7, and 10 days of culture, the cells were fixed with 1% glutaraldehyde in PBS for 15 min and cell proliferation kinetics was analyzed by the crystal violet staining method [Fedarko, 1995; Gronthos and Simmons, 1995]. Briefly, cells were stained for 30 min with a solution of 0.02% crystal violet and rinsed twice with distilled water. Crystal violet bound to the cell surface layer was extracted overnight at 4°C in 1 ml 70% ethanol. Absorbance was measured spectrophotometrically at 570 nm.

Alcian Blue Staining

The cells were seeded at a cell density of 5×10^3 cells/cm^2 in 12-well culture plates, cultured for 14, 28, and 42 days, and fixed with 10% neutral-buffered formalin for 10 min at room temperature. After washing with PBS twice, the cells were incubated in 3% acetic acid for 10 min and stained with 1% alcian blue (pH 2.5) in 3% acetic acid for 30 min at room temperature and rinsed with distilled water. Alcian blue bound to the cells was extracted at room temperature with 500 µl dimethylsulfoxide (DMSO) and subsequently the absorbance was measured spectrophotometrically at 650 nm.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) for Osteogenic and Chondrogenic Markers

The cells were seeded at a cell density of 5×10^3 cells/cm^2 in 6-well culture plates and incubated at 33°C. After 14 and 42 days of culture, total RNA was extracted using TRIzol® reagent (GIBCO BRL). First strand cDNA synthesis from 1 µg total RNA was performed using SUPERSCRIPT™ Pre-amplification System (GIBCO BRL, MD) after DNase I treatment. The PCR amplification was carried out under the following conditions: 94°C for 30 s, 55°C for 30 s and 72°C for 1 min for 23 cycles (G3PDH), 30 cycles (type II collagen, type X collagen, type XI collagen, ALPase, and osteopontin), and 94°C for 30 s, 64°C for 30 s, and 72°C for 1 min for 40 cycles (aggrecan and link protein) followed by a final extension at 72°C for 7 min. Primer sequences for PCR amplification are shown in Table I. PCR products were analyzed using ethidium bromide staining of a 1.8% agarose gel.

Immunocytochemistry

The cells were cultured in Lab Tek Chamber Slides (Nalge Nunc, Naperville, IL) for 7 days at 33°C. The cells were fixed for 10 min in 10%

TABLE I. Oligonucleotide Primers Used in RT-PCR

α1 (II) procollagen (GenBank Accession No. M65161)
- sense: 5′ (31276)-CACACTGGTAAGTGGGGCAAGACCG-3′(31300)
- antisense: 5′ (31448)-GGATTGTGTTTCAGGGTTCGGG-3′ (31424)
- amplified fragment length: 173 bp

α1 (X) procollagen (GenBank Accession No. X65121)
- sense: 5′ (1111)-GACAAAAAGGTGATCCTGGAGTG-3′ (1133)
- antisense: 5′ (1570)-TTAGAGAGAATGACAGTAAAAGC-3′ (1548)
- amplified fragment length: 460 bp

α1 (XI) procollagen (GenBank Accession No. X91014)
- sense: 5′ (90)-GGAAAGATGGGCTACCAGGACA-3′ (111)
- antisense: 5′ (501)-GGACGTCCTGGCAAACCAATTG-3′ (480)
- amplified fragment length: 412 bp

Alkaline phosphatase (GenBank Accession No. J02980)
- sense: 5′ (1741)-TACCGACCCTGTTCTGAGGG-3′ (1760)
- antisense: 5′ (2231)-ACCCTGGGTAGACAGCCAA-3′ (2213)
- amplified fragment length: 491 bp

Osteopontin (GenBank Accession No. J04806)
- sense: 5′ (455)-ACACTTTCACTCCAATCGTC-3′ (475)
- antisense: 5′ (694)-TGCCCTTTCCGTTGTTGTCC-3′ (675)
- amplified fragment length: 240 bp

Aggrecan (GenBank Accession No. L07049)
- sense: 5′ (5731)-CGGTACCCTACAGACACTTCAAGA-3′ (5756)
- antisense: 5′ (5928)-GTGACCCTGGAACTTGGTCCACCC-3′ (5905)
- amplified fragment length: 198 bp

Link protein (GenBank Accession No. AF098460)
- sense: 5′ (1081)-CAAGGTCTTCTCTCACCGAG-3′ (1100)
- antisense: 5′ (1750)-GAGTTTGGTGGGGTGGATCA-3′ (1731)
- amplified fragment length: 670 bp

G3PDH (GenBank Accession No. M32599)
- sense: 5′ (566)-ACCACAGTCCATGCCATCAC-3′ (585)
- antisense: 5′ (1017)-TCCACCACCCTGTTGCTGTA-3′ (998)
- amplified fragment length: 452 bp

neutral-buffered formalin and endogenous peroxidase was blocked by treating with 3% H_2O_2 in methanol for 30 min at room temperature. Membranes of the cells were made permeable by treating with 1% Triton X-100 (Boehringer Mannheim GmbH, Germany) in PBS containing 1% bovine serum albumin (BSA, Sigma, St. Louis, MO) for 10 min at room temperature. After washing with PBS twice for 5 min, fixed cells were incubated with mouse anti-SV40 T antigen, rabbit anti-mouse type II collagen (CHEMICON, Temecula, CA), type X collagen, type XI collagen (COSMO-BIO, LSL, Tokyo, Japan) antibodies diluted at 1:500 in PBS containing 1% BSA at 4°C overnight. After washing with PBS twice for 5 min, localization of SV40 T antigen, type II collagen, type X collagen, and type XI collagen was visualized by a labeled-avidin-biotin method using peroxidase (HISTOMOUSE™-SP KIT Zymed® Laboratories, South San Francisco, CA) and 3,3′-diaminobenzidine tetrahydrochloride (DAB) substrate (NICHIREI, Tokyo, Japan). Negative controls were performed with normal mouse serum, rabbit non-immune IgG or normal rabbit serum. Fixed cells were counterstained with hematoxylin and mounted.

Assay for ALPase Activity

ALPase activity was determined by the method of Lowry et al. [1954] with *p*-nitrophenyl phosphate as the substrate. Briefly, the cells were plated at a cell density of 5×10^3 cells/cm^2 in α-MEM containing 10% FBS and cultured for 14, 28, and 42 days. After washing with PBS, the cells were suspended in 1 ml 10 mM Tris–HCl buffer (pH 8.5), sonicated and centrifuged at $3000 \times g$ for 15 min at 4°C. The reaction mixture total volume of 240 μl contained 8.0 mM *p*-nitrophenyl phosphate, 0.5 M 2-amino-2-metyl-1-propanol (pH 10.0), 0.5 mM $MgCl_2$, and 40 μl supernatant. The reaction mixture was incubated at 37°C for 30 min and the absorbance was measured spectrophotometrically at 410 nm. One unit of enzyme activity was defined as the activity hydrolyzing 1 nmol of *p*-nitrophenyl phosphate per min at 37°C. Protein content was determined by the method of Bradford et al. [1976]. All measurements were performed in triplicate.

Effect of 1α, 25-Dihydroxyvitamin D_3

The effect of 1α, 25-dihydroxyvitamin D_3 (1α, 25-$(OH)_2D_3$) (Sigma) on the proliferation and differentiation of the cell lines has been investigated. Three clones (MCC-2, -5, and -35) were plated at a cell density of 5×10^3 cells/cm^2 in 24-well culture plates and cultured in α-MEM supplemented with 0.5% FBS at 33°C. To investigate the effect on cell proliferation, the medium was changed to a fresh medium i.e., α-MEM supplemented with 0.5% FBS containing various concentrations (0, 10^{-9} M, 10^{-8} M and 10^{-7} M) of 1α, 25-$(OH)_2D_3$ the following day. At different time intervals of culture, the cells were fixed with 1% glutaraldehyde in PBS for 15 min and cell proliferation kinetics was analyzed by the crystal violet staining method. Briefly, cells were stained for 30 min with a solution of 0.02% crystal violet and rinsed twice with distilled water. Crystal violet bound to cell surface layers was extracted overnight at 4°C in 300 μl 70% ethanol. Absorbance was measured spectrophotometrically at 570 nm. In order to investigate the effect of 1α, 25-$(OH)_2D_3$ on cell differentiation, the cells were cultured in α-MEM containing 0.5% FBS until reaching confluency. Subsequently, the medium was changed to α-MEM containing 0.5% FBS and various concentrations of 1α, 25-$(OH)_2D_3$ during 1 week. The cells were fixed with 10% neutral-buffered formalin for 10 min and stained with alcian blue (pH 2.5) for 30 min at room temperature and rinsed with distilled water. Alcian blue bound to cell cultures was extracted with 300 μl DMSO and the absorbance measured spectrophotometrically at 650 nm. At this point, total RNA was extracted using TRIzol® reagent and gene expression of type II collagen was also analyzed by RT-PCR.

Statistical Analysis

All results have been expressed as the mean ± SEM. Comparison between groups has been assessed by a paired Student's *t*-test. All experiments were repeated at least twice and gave equivalent outcomes.

RESULTS

Morphology and Growth of Three Chondrocyte Cell Lines

Of 83 clones isolated from the costal cartilage of SV40 large T antigen transgenic mice, 3 clones, designated MCC-2, MCC-5, and MCC-35 showed very high growth activity. When cultured at 33°C, these clones showed a spindle-shaped fibroblastic morphology in the growth

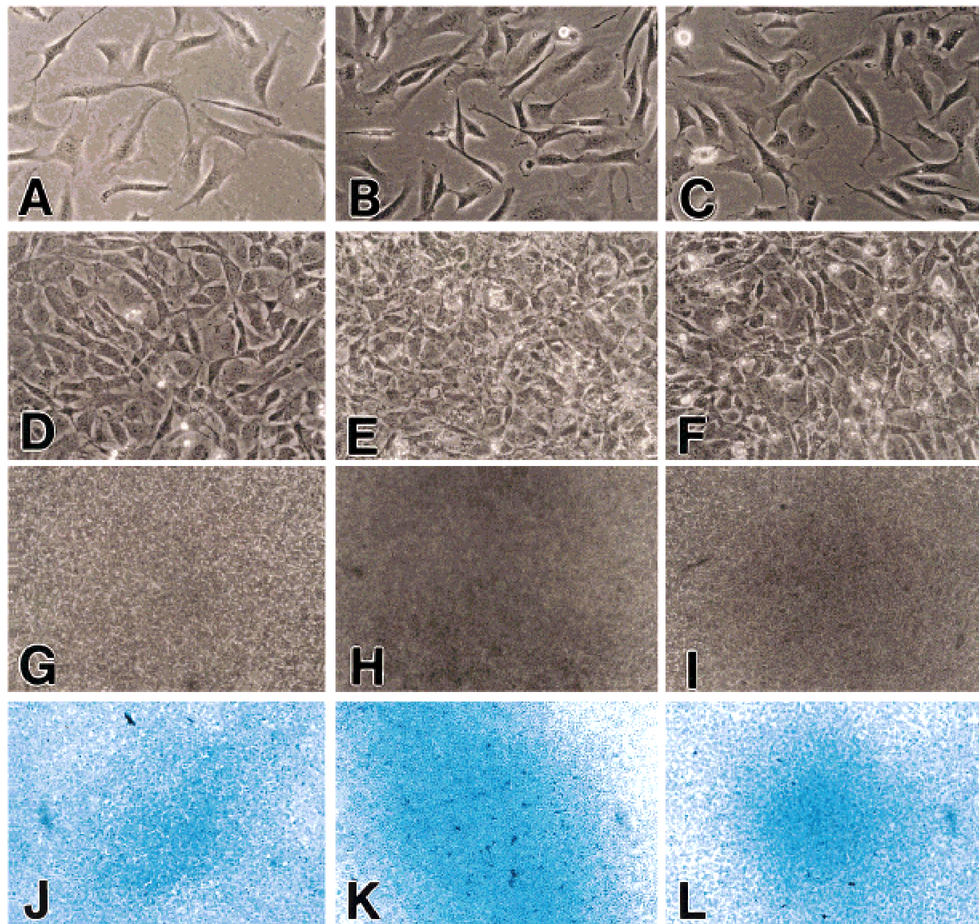

Fig. 1. Phase-contrast micrographs of MCC-2 (A, D, G, J), MCC-5 (B, E, H, K), and MCC-35 (C, F, I, L). The cells have been seeded at a cell density of 5×10^3 cells/cm^2 and grown at 33°C for 3 days (**A–C**), 7 days (**D–F**), and 42 days (**G–I**). At Day 42 of culture, cartilage nodule formation through positive staining with alcian blue (pH 2.5) has been observed (**J–L**). Original magnification: (A–F) × 88; (G–I) × 36; (J–L) × 19.

phase (Fig. 1A–C). In contrast, the cell lines exhibited a relative polygonal shape at confluence (Fig. 1D–F). When inoculated at a cell density of 5×10^3 cells/cm^2, all three clones reached confluency in 7 days (Fig. 2A). After reaching confluency, the cells formed nodules at some areas of the plate. The central region of these nodules looked dark under a phase-contrast microscope (Fig. 1G–I) and was positively stained with alcian blue (Fig. 1J–L). However, at 39°C, the growth rate of these clones was very low and none could reach confluence. Immunocytochemistry for SV40 T antigen revealed that SV40 T antigen was expressed in MCC-5 (Fig. 2B). MCC-2 and MCC-35 also expressed SV40 T antigen (data not shown). When these three clones were seeded at a density of 1×10^6 cells/ml in 0.33% noble agar and cultured for 42 days, all the cell lines could form cell aggregates (data not shown). All three clones could maintain the growth rate and phenotypical characteristics over 31 cell divisions.

Extracellular Matrix Formation

After 14 days of cell culture, these three cell lines were observed to start forming extracellular matrices stained positively with alcian blue (pH 2.5). Only a few alcian blue-stained clusters were observed at Day 14 of culture, after which both the number and area of alcian blue-positive nodules gradually increased with culturing time (Fig. 3).

Gene Expression of Bone- and Cartilage-Specific Markers

All three cell lines expressed mRNAs of a chondrocyte phenotype, and expression was

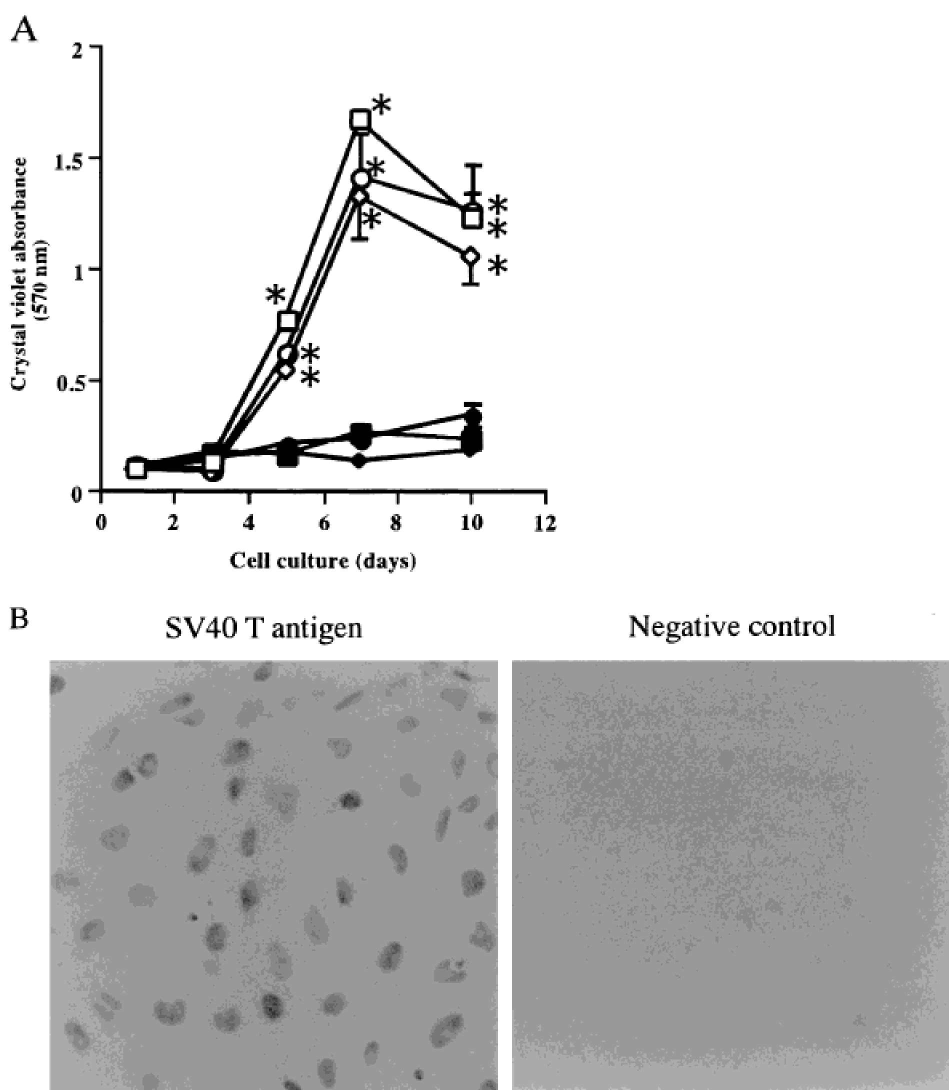

Fig. 2. (**A**) Growth curve of MCC-2 (□ and ■), MCC-5 (◇ and ◆) and MCC-35 (○ and ●). The cells have been seeded at a cell density of 5×10^3 cells/cm^2 and cultured in α-MEM supplemented with 10% FBS at 33°C (□, ◇ and ○) and 39°C (■, ◆ and ●) for 10 days. Data are shown as the mean ± SEM. (n = 8). $^*P < 0.001$ compared with cells cultured at 39°C. (**B**) Immunocytochemistry against SV40 T antigen of MCC-5. The cells have been seeded at a cell density of 5×10^3 cells/cm^2 and cultured at 33°C for 7 days. After fixation, the cells were analyzed immunocytochemically with anti-SV40 T antigen or normal mouse serum. Original magnification: × 223.

time-dependent (Fig. 4). MCC-2 expressed type II collagen constantly throughout the culture period. The mRNA levels of ALPase and osteopontin increased with time. Aggrecan mRNA was detected only at Day 14 of culture, and type XI collagen and link protein mRNA detected only at Day 42 of culture.

MCC-5 showed expression of mRNA for type II collagen at Day 14, could slightly increase with culturing time. In contrast, type X collagen mRNA could only be detected at Day 42 of culture. The mRNA levels of ALPase and osteopontin increased with time. Aggrecan mRNA was detected only at Day 14 of culture, and type XI collagen and link protein mRNA detected only at Day 42 of culture.

MCC-35 expressed mRNA for type II collagen and osteopontin at Day 14 of culture, with increasing expression levels with time. Type X collagen mRNA could only be detected at Day 42 of culture. ALPase mRNA expression was constant at days 14 and 42 of culture. Aggrecan mRNA could be detected throughout the culture period with levels decreasing with time. Type XI collagen and link protein mRNA could only be detected at Day 42 of cell culture.

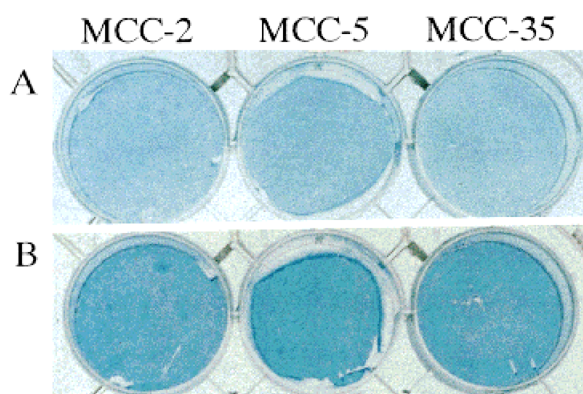

Fig. 3. Extracellular matrix formation by MCC-2, MCC-5, and MCC-35 being positively stained with alcian blue (pH 2.5). The cells have been seeded at a cell density of 5×10^3 cells/cm^2 and cultured in α-MEM supplemented with 10% FBS at 33°C. Alcian blue-positive extracellular matrix formation has been noted after Day 14 (**A**) and Day 42 (**B**) of cell culture.

Expression of Type II Collagen, Type X Collagen and Type XI Collagen

Immunocytochemistry of MCC-2, MCC-5, and MCC-35 revealed positive staining for type II collagen and type XI collagen (Fig. 5). In addition, MCC-5 and MCC-35 were stained positive for type X collagen.

ALPase Activity in MCC-2, MCC-5, and MCC-35

ALPase activity in MCC-2, MCC-5, and MCC-35 increased from Day 14 to 28 of culture and thereafter slightly decreased. In MCC-2, MCC-5, and MCC-35, enzyme activity at Day 28 of culture was respectively 4.3-, 9.1-, 3.7-fold higher than at Day 14 (Fig. 6).

Effect of 1α, 25-$(OH)_2D_3$ in MCC-2, MCC-5, and MCC-35

Treatment with 10^{-9} M, 10^{-8} M and 10^{-7} M of 1α, 25-$(OH)_2D_3$ inhibited the cell proliferation of MCC-2, MCC-5, and MCC-35 in a dose-dependent manner (Fig. 7). 1α, 25-$(OH)_2D_3$ also caused a significant inhibition of the subsequent cartilage-nodule formation in a dose-dependent manner (Fig. 8). RT-PCR demonstrated that the expression of type II collagen decreased in a dose-dependent manner upon treatment with 1α, 25-$(OH)_2D_3$ (Fig. 9). When these three cell lines were cultured in α-MEM containing 0.5% FBS and various concentrations of 1α, 25-$(OH)_2D_3$, type X collagen mRNA could not be detected in any cell line (data not shown).

DISCUSSION

In this study, three chondrocyte cell lines (MCC-2, MCC-5, and MCC-35) derived from the costal hyaline cartilage of SV40 large T antigen transgenic mice have been established and phenotypically characterized. All three cell lines were grown in low serum conditions at the permissive temperature of 33°C and maintained phenotypic characteristics over 31 cell divisions. However, the cell lines did not reach

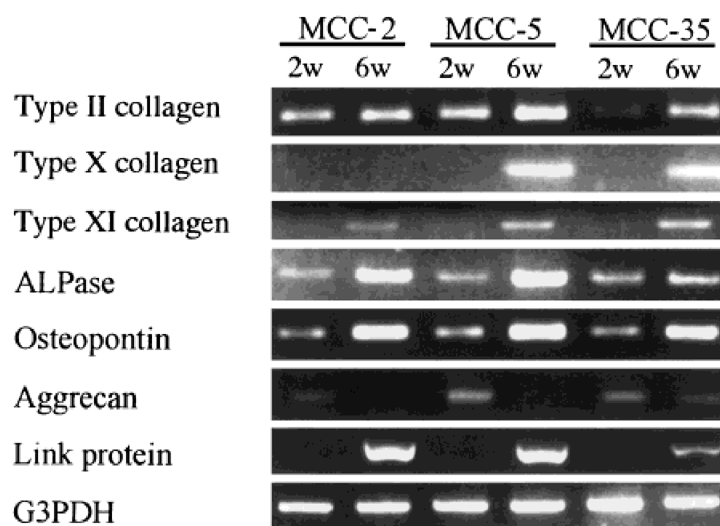

Fig. 4. Gene expression of bone- and cartilage-specific markers in MCC-2, MCC-5, and MCC-35. The cells have been seeded at a cell density of 5×10^3 cells/cm^2 and cultured in α-MEM supplemented with 10% FBS at 33°C for 2 and 6 weeks. Total RNA has been extracted and gene expressions were analyzed using semi-quantitative RT-PCR.

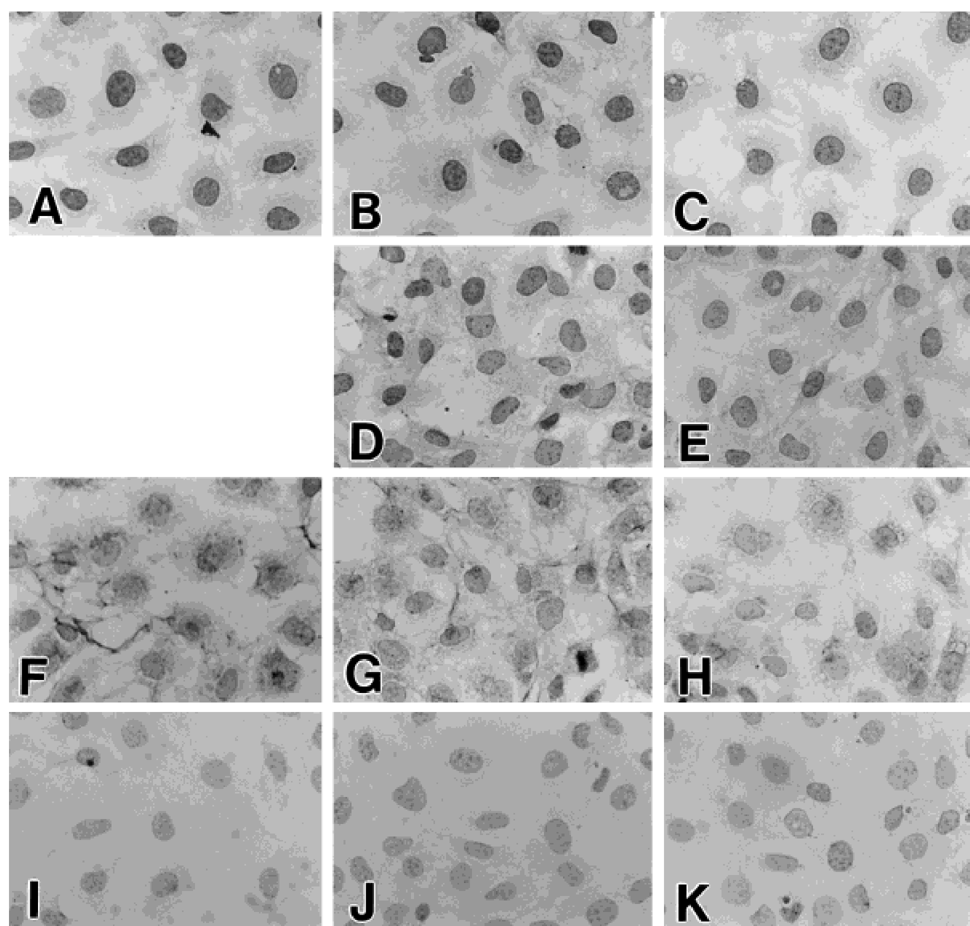

Fig. 5. Immunocytochemistry of MCC-2 (A, F), MCC-5 (B, D, G) and MCC-35 (C, E, H) using polyclonal antibodies raised against type II collagen (**A**–**C**), type X collagen (**D**, **E**) and type XI collagen (**F**–**H**). The cells have been seeded at a cell density of 5×10^3 cells/cm^2 and incubated at 33°C for 7 days. Negative controls have been carried out using non-immune IgG (**I**) and normal rabbit serum (**J**, **K**) instead of specific primary antibodies. Original magnification: × 223.

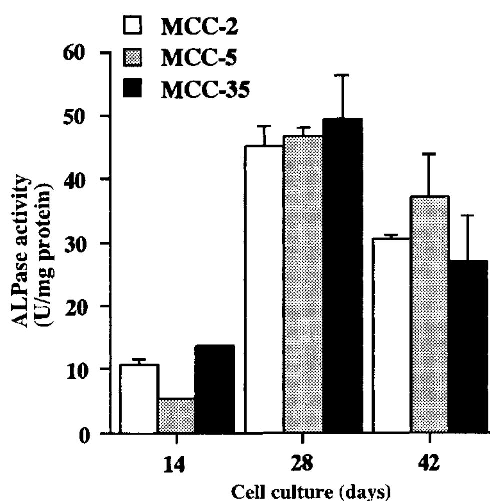

Fig. 6. ALPase activity of MCC-2, MCC-5, and MCC-35. The cells have been seeded at a cell density of 5×10^3 cells/cm^2 and cultured at 33°C for 14, 28 and 42 days in α-MEM supplemented with 10% FBS. Data are shown as the mean ± SEM. (n = 3).

confluence at 39°C. These cell lines showed chondrocytic characteristics, including formation of cartilage nodules that could be stained with alcian blue, and mRNA expression for type II collagen, type XI collagen, ALPase, osteopontin, aggrecan, and link protein. After 6 weeks of cell culture, MCC-5 and MCC-35 also expressed mRNA of type X collagen, while MCC-2 expressed no detectable levels of mRNA for type X collagen. Immunocytochemistry revealed the production of type II collagen and type XI collagen in the three cell lines and type X collagen in both MCC-5 and MCC-35. When the three clones were seeded at a cell density of 1×10^6 cells/ml in 0.33% noble agar and cultured for 42 days, they all showed aggregate formation (data not shown). From these findings, these cell lines were considered to be chondrocytic cell lines. The difference in gene expression between MCC-2 and MCC-5/

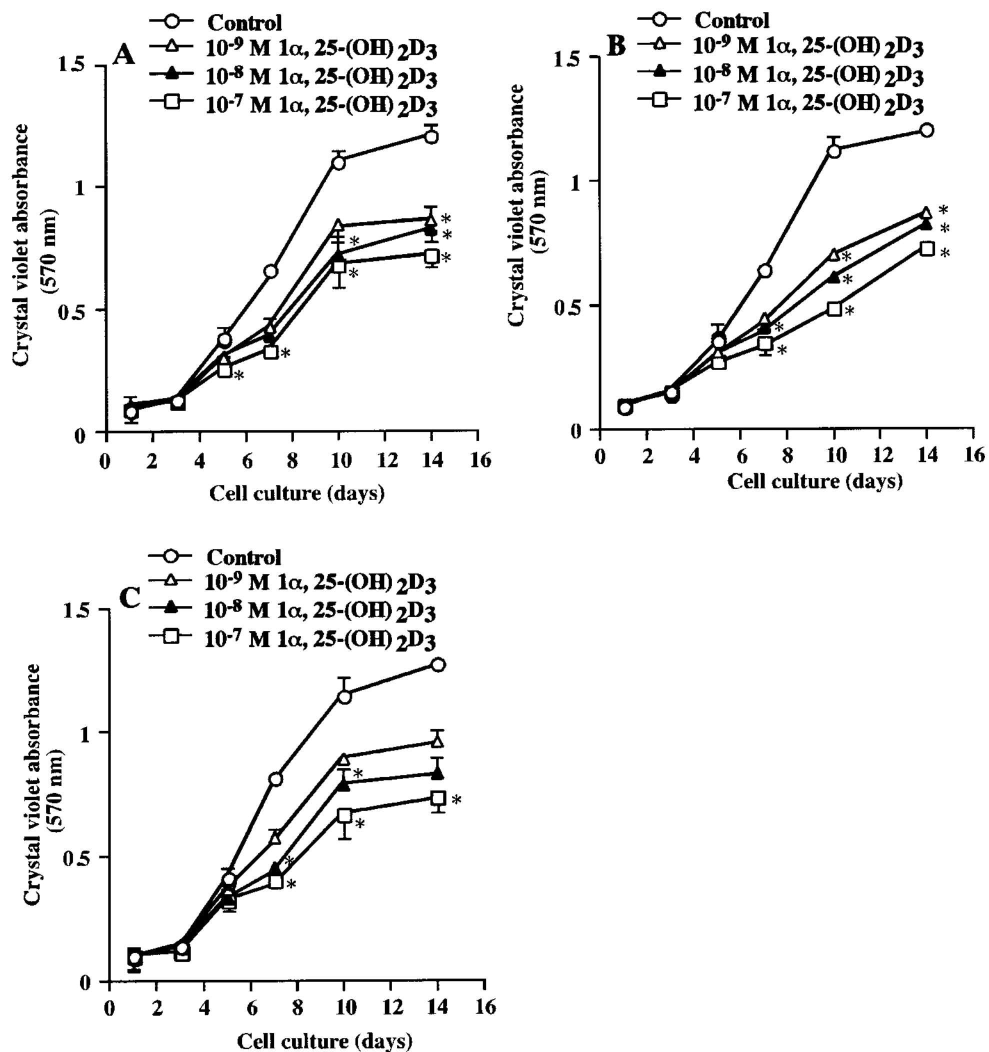

Fig. 7. Effect of 1α, 25-(OH)$_2$D$_3$ on the proliferation of MCC-2 (**A**), MCC-5 (**B**), and MCC-35 (**C**). The cells were seeded at a cell density of 5×10^3 cells/cm^2 and cultured at 33°C. The medium was changed to fresh medium with or without various concentrations (10^{-9} M, 10^{-8} M and 10^{-7} M) of 1α, 25-(OH)$_2$D$_3$ next day. Data are shown as the mean ± SEM. (n = 3). *$P < 0.001$ vs. control.

MCC-35 may result from the difference in the differentiation stage of each cell line and/or the difference in the anatomic zone from which each cell line was derived.

The effect of 1α, 25-(OH)$_2$D$_3$ on the cell proliferation and differentiation of MCC-2, MCC-5, and MCC-35 was analyzed by crystal violet staining, alcian blue staining, and RT-PCR. Treatment with 1α, 25-(OH)$_2$D$_3$ significantly inhibited the cell proliferation of the three cell lines in a dose-dependent manner. 1α, 25-(OH)$_2$D$_3$ caused inhibition of cartilage-nodule formation and accumulation of proteoglycan in MCC-2, MCC-5, and MCC-35. The expression of mRNA for cartilage-specific markers also was inhibited upon treatment with 1α, 25-(OH)$_2$D$_3$ in a dose-dependent manner. These findings suggest that 1α, 25-(OH)$_2$D$_3$ not only suppresses cell proliferation, but also cell differentiation of the three chondrocytic cell lines in a dose-dependent manner. These results are consistent with previous studies reported by others [Akiyama et al., 1996]. In addition, it has been reported that 1α, 25-(OH)$_2$D$_3$ causes enhanced differentiation of chondrocytes derived from the post-proliferaive zone of rat costochondral cartilage [Boyan et al., 1988; Schwartz et al., 1988, 1989]. Judging from these reports, the cell lines established in the present study are considered not to be from

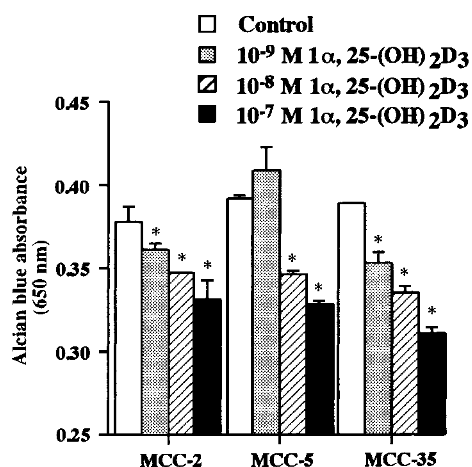

Fig. 8. Effect of 1α, 25-$(OH)_2D_3$ on the alcian blue-stainable proteoglycan content of MCC-2, MCC-5, and MCC-35. The cells have been exposed to various concentrations (10^{-9} M, 10^{-8} M, and 10^{-7} M) of 1α, 25-$(OH)_2D_3$ during 1 week at 33°C, and reaching confluency stained with alcian blue. Alcian blue bound to cultures has been extracted with 300 µl of DMSO and the absorbance subsequently measured spectrophotometrically at 650 nm. Data are shown as the mean ± SEM (n = 3). *$P < 0.05$ vs. control.

the growth plate region, at least not from the lower hypertrophic cartilage next to the costal bones.

A considerable number of primary cultures of isolated chondrocytes has already been reported [Binette et al., 1998]. Primary chondrocytes isolated from normal cartilage tissue showed expression of cartilage-specific genes such as type II collagen and type X collagen, and response to calcitropic hormones and cytokines, i.e., parathyroid hormone and 1α, 25-$(OH)_2D_3$ [Akiyama et al., 1996; Quatro et al., 1997]. However, it has been very difficult to generate a stable cell line maintaining the original phenotype of chondrocytes, since multiple cell divisions resulted in a rapid loss of the proliferation and differentiation capacity. This has been a critical limitation in the research of the physiology and pathology of chondrocytes and cartilage tissue. Recently, the establishment of some bone marrow stromal cell lines having the ability of differentiation to chondrocyte has been reported, i.e., TBR31-2 [Negishi et al., 2000], MMSC [Satomura et al., 2000], as well as five murine chondrocyte cell lines, ATDC5 [Atsumi et al., 1990], CFK2 [Bernier et al., 1990], WT2 [Wang et al., 1993], MC615 [Mallein-Gerin et al., 1995], and TC6 [Mataga et al., 1996]. CFK2, WT2, and ATDC5 were established from rat calvaria, mouse *c-fos*-induced cartilage tumor and mouse embryonic carcinoma, respectively. Each cell line showed chondrocytic phenotypes, i.e., expression of type II collagen and the response to calcitropic hormones and cytokines such as PTH [Shukunami et al., 1996, 1997, 1998] and 1α, 25-$(OH)_2D_3$. MC615, established from mouse limbs by a transfection of the SV40 large T-antigen gene, has been shown to possess the phenotypic characteristics of proliferative cartilage, i.e., the presence of type II collagen expression and the absence of type X collagen expression [Mallein-Gerin et al., 1993, 1995; Valcourt et al., 1999]. The phenotype of MCC-2 in the

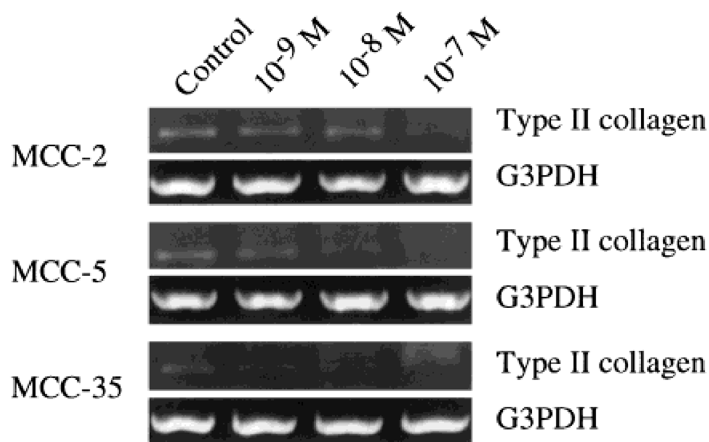

Fig. 9. Effect of 1α, 25-$(OH)_2D_3$ on gene expression of type II collagen in MCC-2, MCC-5, and MCC-35. The cells have been exposed to various concentrations (10^{-9} M, 10^{-8} M and 10^{-7} M) of 1α, 25-$(OH)_2D_3$ for 1 week at 33°C after reaching confluency. Total RNA has been extracted from these three clones and the expression of mRNA for type II collagen analyzed using RT-PCR.

present study has been considered being similar to that of MC615. TC6 has been established from the articular cartilage of SV40 large T-antigen transgenic mice, forming alcian blue-positive nodules and expressing the cartilage-specific proteins type II collagen, link protein, and aggrecan. Moreover, this cell line has been found to form cartilage tissue in vivo after implantation into cavity-shaped full-thickness defects created in the articular cartilage of mouse femora. Other reports described reconstruction of cartilage tissue by cultured chondrocytes derived from the articular cartilage [Takazawa et al., 1999] or the rib [Shimomura et al., 1975]. Recently, it was reported that rat costochondral resting zone chondrocytes would retain their chondrocytic phenotype even in mouse muscle and that PDGF-BB promoted the retention of the hyaline-like chondrogenic phenotype [Lohmann et al., 2000]. This report shows the possibility that the costal cartilage would be the preferred source of commited chondrocytes for cartilage repair.

Thus, costal cartilage is considered to be a more useful source to obtain chondrocytes due to an abundance of tissue volume and the ease of surgical procedure, causing less damage to the donor site. It is yet unclear whether chondrocytes are able to withstand local mechanical stresses in the articular cartilage. From this point of view, it is essential to understand the physiology of chondrocytes in costal cartilage, as well as the difference between costal and articular cartilage. The chondrocyte cell lines reported in this study are considered very useful in basic research on tissue engineering aiming to treat cartilage defects and disorders such as rheumatoid arthritis and osteoarthritis.

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