Type X Collagen Gene Expression in Mouse Chondrocytes Immortalized by a Temperature-Sensitive Simian Virus 40 Large Tumor Antigen

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Abstract. Mouse endochondral chondrocytes were immortalized with a temperature-sensitive simian virus 40 large tumor antigen. Several clonal isolates as well as pools of immortalized cells were characterized. In monolayer cultures at the temperature permissive for the activity of the large tumor antigen (32°C), the cells grew continuously with a doubling time of ~2 d, whereas they stopped growing at nonpermissive temperatures (37°C–39°C). The cells from all pools and from most clones expressed the genes for several markers of hypertrophic chondrocytes, such as type X collagen, matrix Gla protein, and osteopontin, but had lost expression of type II collagen mRNA and failed to be stained by alcian blue which detects cartilage-specific proteoglycans. The cells also contained mRNAs for type I collagen and bone Gla protein, consistent with acquisition of osteoblastic-like properties. Higher levels of mRNAs for type X collagen, bone Gla protein, and osteopontin were found at nonpermissive temperatures, suggesting that the expression of these genes was upregulated upon growth arrest, as is the case in vivo during chondrocyte hypertrophy. Cells also retained their ability to respond to retinoic acid, as indicated by retinoic acid dose-dependent and time-dependent increases in type X collagen mRNA levels. These cell lines, the first to express characteristic features of hypertrophic chondrocytes, should be very useful to study the regulation of the type X collagen gene and other genes activated during the last stages of chondrocyte differentiation.

CHONDROCYTES are highly specialized cells that are derived from mesenchymal cells during embryonic development to form the various cartilages of vertebrates. Chondrocytes are characterized by the production of cartilage-specific extracellular macromolecules including the collagen types II, IX, and XI, and the proteoglycan aggrecan (16). In permanent cartilages, the phenotype of chondrocytes is stably maintained, whereas in endochondral cartilages, chondrocytes undergo a further differentiation process (16, 34). During this process, chondrocytes mature into hypertrophic cells around which the cartilage matrix becomes calcified, and subsequently undergo apoptosis, making way for invading osteoblasts. Hypertrophic chondrocytes still contain type II collagen and aggrecan RNAs, although at reduced levels (21, 38), they are characterized by the synthesis of type X collagen, which is uniquely expressed by these cells (10, 21, 39), and by the expression of markers typically associated with osteoblasts. Both hypertrophic chondrocytes and osteoblasts have the ability to induce mineralization of the extracellular matrix and to produce alkaline phosphatase (ALP)[1] (1), osteopontin (OP) (11, 29, 45), osteonectin (21), matrix Gla protein (MGP) (2, 12, 17), and the Ch21 protein (9). Bone Gla protein (BGP, also called osteocalcin), another osteoblast marker (35), is present at low levels in the calcified cartilage matrix surrounding hypertrophic chondrocytes in vivo (4, 28, 29). Studies with chondrocytes in culture have shown that after the cells became hypertrophic they produced type I collagen and BGP (3, 8, 24, 37, 41, 42), which led to the suggestion that these cells could acquire osteoblastic properties.

To better understand the molecular mechanisms that control the successive steps of chondrocyte differentiation, cell lines corresponding to the various chondrocytic phenotypes would be extremely useful. So far, however, no cell lines have been isolated which express the characteristics of hypertrophic cells, specifically the gene for type X collagen.

In the present study, we characterize a new cell line obtained by immortalization of primary mouse rib chondro-

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1. *Abbreviations used in this paper*: ALP, alkaline phosphatase; BGP, bone Gla protein; *COL2A1*, gene coding for the type II collagen α1 chain (identical nomenclature for other collagen genes); MCT, cell line of mouse chondrocytes immortalized by thermosensitive simian virus 40 large tumor antigen; MGP, matrix Gla protein; OP, osteopontin; RA, retinoic acid; SV40, simian virus 40; large T, large tumor antigen.

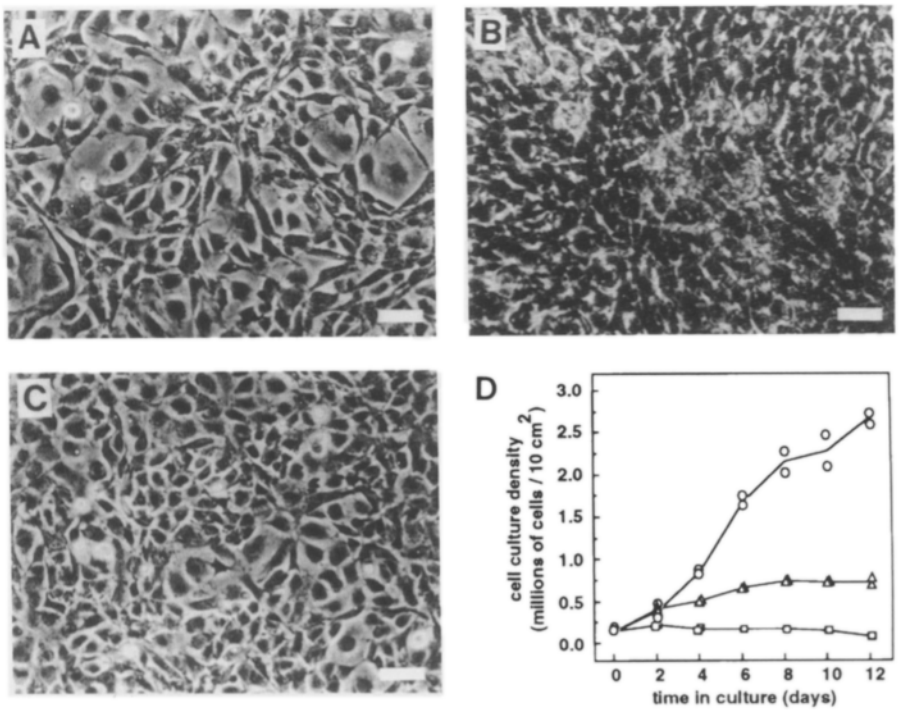

Figure 1. Morphology and growth kinetics of immortalized chondrocytes. (*A*) Nonconfluent culture of MCTs cells grown at 32°C for 3 d; (*B*) postconfluent MCTs cells grown for 8 d at 32°C; (*C*) MCTs cells plated near confluence and cultured for 3 d at 37°C; (*D*) MCTs cells were plated in low density monolayers and cultured at 32°C (*circles*), 37°C (*triangles*), or 39°C (*squares*) for 12 d. Cell densities were measured every other day by DNA assay in single or duplicate cultures and plotted as a function of time in culture. The 32°C cultures reached confluence at about day 5. Bars: (*A*, *B*, and *C*) 50 μm.

cytes with a thermolabile simian virus 40 (SV40) large tumor antigen (large T) (19). The cells are immortalized at the temperature permissive for large T (32–33°C) and are growth restricted at 37–39°C. They expressed the genes for several markers of hypertrophic chondrocytes, including type X collagen, OP, and MGP. They also expressed type I collagen and BGP, suggesting that they had acquired osteoblastic properties, and had lost expression of the main markers of differentiated chondrocytes, i.e., type II collagen and aggrecan. The cells also showed increased levels of mRNAs for type X collagen, OP, and BGP at nonpermissive temperatures, suggesting that the expression of these genes was upregulated upon growth arrest, as is the case in vivo. We believe that these cells will be useful to study the regulation of the type X collagen gene as well as other genes specifically expressed by endochondral chondrocytes during their differentiation into hypertrophic cells.

Materials and Methods

Culture Medium and Reagents

Standard culture medium consisted of DMEM (high glucose, without pyruvate, GIBCO BRL, Gaithersburg, MD) supplemented with penicillin (50 U/ml), streptomycin (50 μg/ml), L-glutamine (2 mM), and 10% FCS (heat inactivated for 30 min at 56°C) (GIBCO BRL). Where indicated, ascorbic acid was added at 50 μg/ml by dilution of a freshly prepared 100 × solution. To prevent a toxic effect of ascorbate on cells, 1 mM pyruvate and L-cysteine were included (43) in both ascorbic acid–supplemented and control cultures. β-Glycerophosphate was tested at 10 mM. All *trans*-retinoic acid (RA, Sigma Chem. Co., St. Louis, MO) was solubilized at 1 mg/ml in 95% ethanol and stored at −20°C in single use aliquots, and dilutions were added freshly at 1:1,000 in culture medium.

Immortalized Chondrocytes

Three independent preparations of primary chondrocytes cultured overnight under standard conditions (see below) were infected with the ZipSVtsa58 retrovirus for 24 h at 37°C (19). This was done by incubating the cells in conditioned medium of retrovirus-producing psi-2 cells to which 4 μg/ml polybrene had been added. Three days after infection, cells were split at a 1:10 ratio, propagated at 32°C in standard medium under 8% CO_2 and selected for resistance to G418 (300 μg/ml, geneticin, GIBCO BRL) for a total of 4 wk, constituting three pools of immortalized cells (pool 1, pool 2, pool 3). Several clones of cells were picked among the 3 pools after 10–15 d of selection and transferred to individual dishes. Clones and pools were further amplified for 1–4 mo and stored in liquid nitrogen.

Other Cells

Rib chondrocytes and skin fibroblasts were prepared from transgenic and nontransgenic 1–3-d-old mice (B6D2Fl) as described previously (23). Transgenic mice carried a *COL2Al-lacZ* construct made of 3 kb of 5′ flanking sequences upstream of the transcription start site of the type II collagen α1 chain gene (*COL2Al*), exon 1 to exon 4, with exon 4 fused in frame to the *Escherichia coli* β-galactosidase gene (*lacZ*). Briefly, chondrocytes were isolated from rib cages by digestion of cartilages with bacterial collagenase after complete elimination of contaminating soft tissues by preliminary digestions with pronase and bacterial collagenase. Primary chondrocytes were plated in subconfluent monolayers (10^5 cells/cm^2).

Rat osteosarcoma cells (ROS 17/2.8) were kindly provided by Dr W. T. Butler (Department of Biological Chemistry, University of Texas Health Science Center, Houston, TX).

Chondrocytes, fibroblasts, and ROS cells were cultured in standard medium at 37°C under 8% CO_2.

DNA Assay

DNA was assayed with the DAPI (Sigma Chem. Co.) fluorometric method (6) and cell extracts were prepared as described previously (23).

RNA Preparation and Northern Blot Analysis

Total RNAs were purified from guanidinium isothiocyanate cell homogenates by CsCl density gradient ultracentrifugation. RNAs (~10 μg/sample) were heat denatured and electrophoresed in formaldehyde-agarose gel. 28S and 18S rRNAs were stained with ethidium bromide. RNAs were transferred to either Hybond-N^+ (Amersham Corp., Arlington Heights, IL) or Zeta-Probe GT (Bio-Rad Labs., Hercules, CA) blotting membranes in 10× SSC (1× SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.0) and fixed by UV cross-linking. Membranes were hybridized overnight at 65°C with

1–2 ng of labeled probe/ml in 0.3 M sodium phosphate buffer, pH 7.2, 7% SDS and 100 μg/ml sonicated and heat-denatured herring sperm DNA, and washed for 30-60 min at room temperature in 2× SSC/0.1% SDS, and then for 15–45 min at 65°C in 0.2× SSC/0.1% SDS. Autoradiography was performed at −70°C using intensifying screens. Membranes were hybridized consecutively with different DNA probes after stripping in 0.1× SSC/0.5% SDS at 95°C.

DNA Probes

RNAs for types I and II collagen α1 chain genes (respectively, *COL1A1* and *COL2A1*) were probed with cDNA inserts from pMCollal-1 and pMCol2al-1 (30). The *COL10A1* probe was a 1.2-kb Bam HI fragment from mouse *COL10* exon 3 (10); the ALP probe, a 2.5-kb Eco RI fragment from rat cDNA (31); the OP probe, a 1.3-kb Eco RI fragment from rat cDNA (45); the BGP probe, a 0.3-kb Eco RI–Xba I fragment of mouse cDNA (7); the MGP probe, a 0.3-kb Eco RI–Xba I fragment of mouse cDNA (26); and the GAPDH probe, a fragment of the human glyceraldehyde 3-phosphate dehydrogenase gene (Ambion, Austin, TX).

Probes were prepared by restriction enzyme digestion, purified by electrophoresis in low melting agarose gel, and labeled with [^{32}P]dCTP (Dupont New England Nuclear, Boston, MA) to a specific activity of at least 5 × 10^8 cpm/μg by random priming using the Multiprime DNA-labeling system (Amersham Corp.).

Results

Selection of Immortalized Cells, Growth Properties, and Morphological Aspects

Three pools and several clones of immortalized cells were isolated after infection of mouse rib primary chondrocytes with the ZipSVtsa58 retrovirus (see Materials and Methods). Soon after infection, some clones appeared differentiated and formed cartilage nodules (i.e., cells were round, enveloped in an abundant extracellular matrix, and piled up), others appeared dedifferentiated (i.e., cells were flattened and elongated or polygonal).

After 1–4 mo of amplification at 32°C, cells in both pools and clones appeared dedifferentiated and showed dependence on active large T antigen for growth. This is illustrated in Fig. 1 for one clone called MCTs (for mouse chondrocytes immortalized by SV40 temperature-sensitive large T antigen). At 32°C, MCTs cells grew continuously, even after they reached confluence (Fig. 1 *D*). Cells were polygonal or elongated (Fig. 1 *A*) and formed dense multilayer cultures (Fig. 1 *B*). After transfer to 37–39°C, cells stopped growing within a few days (Fig. 1 *D*). Cells plated at confluence formed regular monolayers of mostly polygonal cells (Fig. 1 *C*). Dead cells appeared within one week (not shown).

Alcian blue staining (40), which detects accumulation of cartilage-specific proteoglycans around differentiated chondrocytes (23), was negative for immortalized cells tested at 32, 37, and 39°C, after variable periods in culture, both at low and high cell densities, and whether ascorbic acid was present or not (not shown). Pool 1, pool 3, MCTs cells, and some other clones were obtained from transgenic mice harboring a *COL2-lacZ* construct (see Materials and Methods) whose specific expression in differentiated chondrocytes was detected in vivo (Metsäranta, M., S. Garofalo, P. Leppänen, C. Smith, K. Niederreither, B. de Crombrugghe, and E. Vuorio, manuscript submitted for publication) as well as in primary cultures of chondrocytes (23) by staining with X-gal, a chromogenic substrate for β-galactosidase. No positive X-gal staining of immortalized cells was observed (not shown).

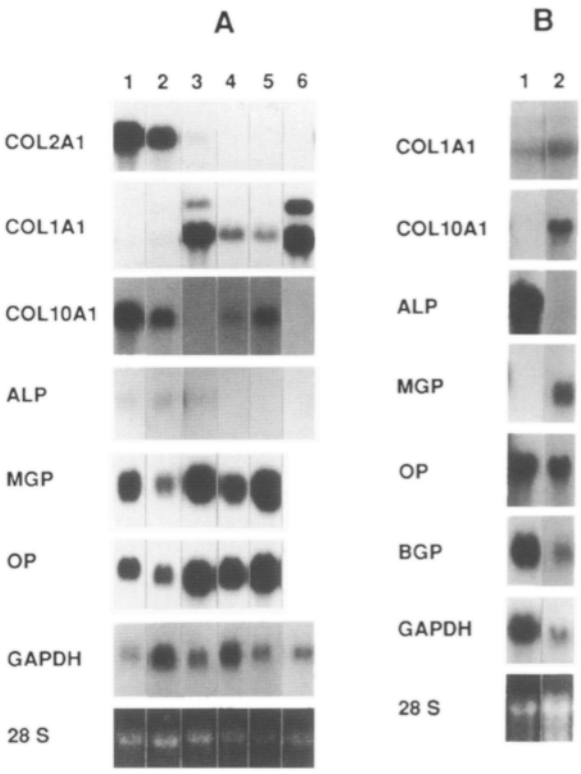

Figure 2. Comparison of RNAs expressed by immortalized cells, primary chondrocytes, and ROS cells. Northern blots were performed after fractionation of total RNAs isolated from the following cell preparations: (*A*, lane *1*) chondrocytes freshly isolated from cartilage (noncultured cells); (lanes *2* and *3*) primary and passage 3 chondrocytes, respectively, cultured until confluence (5 d); (lanes *4* and *5*) MCTs and pool 1 cells, respectively, cultured at 32°C to subconfluence (2 d), and then for 3 d at 37°C; (lane *6*) confluent skin fibroblasts at passage 2 (3 d); (*B*, lane *1*) confluent ROS cells; (lane *2*) MCTs cells cultured in standard medium at 32°C until subconfluence (3 d), and then for 3 d at 37°C. Membranes were hybridized with various probes and exposed to X-ray films for different time periods as follows: (*A*) *COL2A1*, 1 h; *COL1A1* and *COL10A1*, 20 h; *ALP*, 24 h; *OP*, 16 h; *MGP*, 6 h; (*B*) *COL1A1*, 2 h 30 min; *COL10A1*, 40 h; *ALP*, 24 h; *MGP*, 1 h; *OP*, 1 h; *BGP*, 8 d. Hybridizations with GAPDH probe and staining of 28S rRNA with ethidium bromide are shown as references for the amount of RNA loaded in each lane. Note that the level of GAPDH mRNA is lower in noncultured cells (lane *1*) than in cultured cells by comparison to the staining of 28S RNA.

Both morphological and histological data suggest that the immortalized cells no longer expressed a typical differentiated chondrocyte phenotype.

Comparison of the Phenotype Expressed by Immortalized Chondrocytes, Primary Chondrocytes, and Osteosarcoma Cells

At the time of their isolation (day 0) and after 5 d in primary culture (Fig. 2 *A*), mouse chondrocytes were fully differentiated: they produced considerable amounts of *COL2A1* mRNA and negligible amounts of *COL1A1* mRNA (23). These cells contained relatively high levels of mRNAs for type X collagen, ALP, OP, and MGP, indicating that a significant proportion of the cells were hypertrophic. After

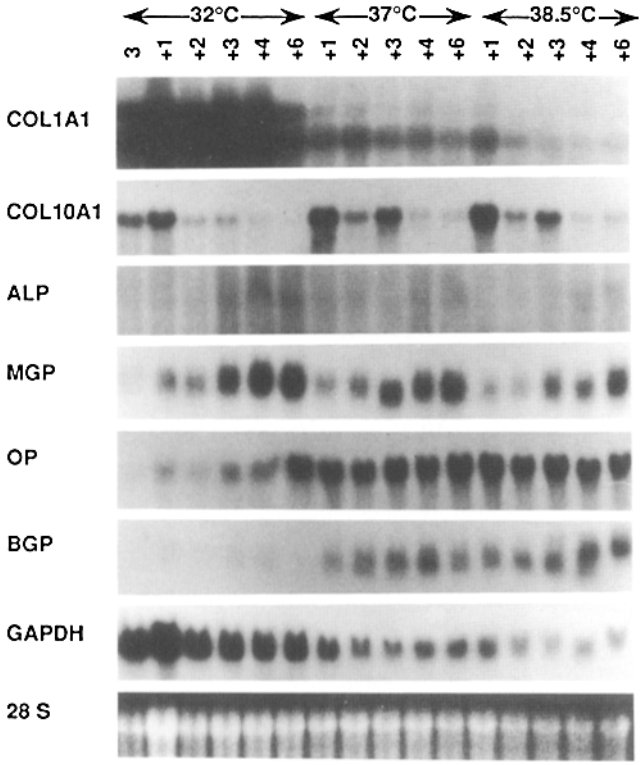

Figure 3. Comparison of RNAs of immortalized chondrocytes at permissive and nonpermissive temperatures. MCTs cells were grown until subconfluence (3 d) at 32°C in standard medium (3). The medium was then replaced and supplemented with ascorbic acid and β-glycerophosphate. Cells were further incubated at 32, 37, or 38.5°C for 1, 2, 3, 4, or 6 d (+1 to +6). Culture medium was renewed at days 2 and 4. Total RNAs were fractionated, blotted, and hybridized with various probes. Exposure was for different time periods as follows: *COL1A1*, 2 h 30 min; *COL10A1*, 40 h; *ALP*, 24 h; *MGP*, 1 h; *OP*, 1 h; *BGP*, 8 d. Hybridization with GAPDH probe and staining of 28S RNA, shown as references for the amount of RNA in each lane, indicate that lower amounts of RNA were loaded in the gel for samples from cultures at nonpermissive temperatures.

three passages in monolayer culture (Fig. 2 *A*), most chondrocytes had lost expression of the main markers of the chondrocyte differentiated phenotype as demonstrated by very low levels of *COL2A1* mRNA. These cells contained high levels of *COL1A1* mRNA but still maintained high levels of mRNAs for ALP, OP, and MGP, which is consistent with the notion that they had acquired osteoblast-like properties. No BGP mRNA was detected in either early or late passage primary chondrocyte cultures (not shown).

Type II collagen mRNA was undetectable in MCTs and pool 1 cells (Fig. 2 *A*), even after several days of exposure of the Northern blots. Both MCTs and pool 1 cells contained relatively high amounts of transcripts for type X collagen, MGP, OP, and type I collagen, but no significant amount of ALP mRNA (Fig. 2, *A* and *B*). BGP transcripts were found in MCTs cells, although at low levels by comparison to those present in rat osteosarcoma cells (Fig. 2 *B*). The other pools and clones of immortalized cells were all lacking type II collagen mRNA, 6 out of 7 clones contained type X collagen mRNA, and several also contained BGP mRNA (data not shown). No significant phenotypic changes were observed in MCTs cells maintained in culture for up to 4 mo (data not shown).

These results showed that the immortalized cells had retained several characteristics of hypertrophic chondrocytes, including expression of the genes for type X collagen, OP, and MGP, but had lost expression of type II collagen and ALP genes; they also displayed expression of type I collagen and BGP genes, consistent with the acquisition of osteoblast-like properties. They differed from late passage primary chondrocytes by expression of the *COL10* and BGP genes and by the absence of ALP transcripts.

Influence of Temperature and Culture Conditions on the Phenotype of Immortalized Chondrocytes

The phenotype of MCTs cells was further analyzed over a 6-d culture period at 32, 37, and 38.5°C (Fig. 3). Type X collagen transcripts were present at the end of the preculture at 32°C. One day later, higher amounts of *COL10* mRNA were

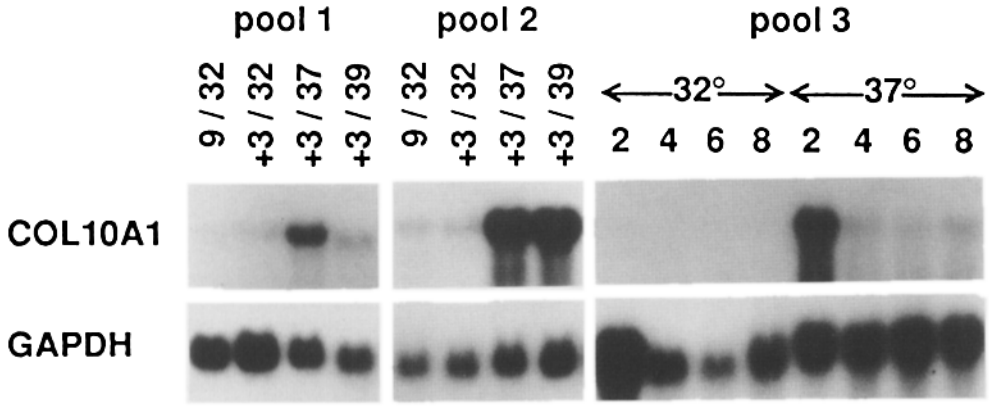

Figure 4. Northern blot analysis comparison of the steady-state levels of *COL10* mRNAs present in pools of immortalized cells at permissive and nonpermissive temperatures. Immortalized chondrocytes from pools 1 and 2 were cultured for 9 d at 32°C (9/32), and then for three additional days at 32, 37, or 39°C (+3/32, +3/37, +3/39). Pool 3 cells were cultured for 2, 4, 6, or 8 d at either 32°C or 37°C, as indicated. Total RNAs were fractionated, blotted, and hybridized with the *COL10* probe. Blots were exposed for 4 d for pools 1 and 2, and 10 d for pool 3. Hybridization with GAPDH probe is shown as reference for the amount of RNA loaded in each lane.

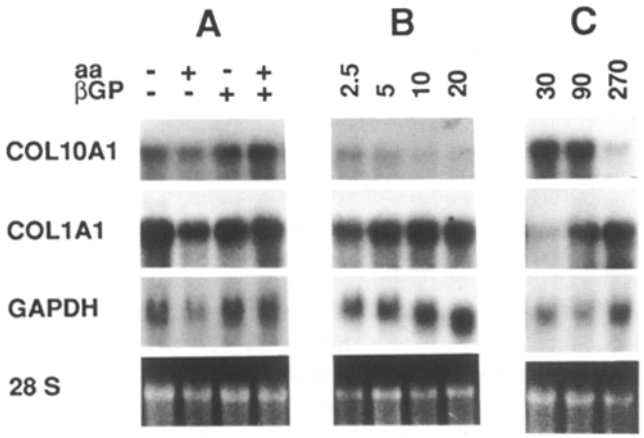

Figure 5. Influence of ascorbic acid, β-glycerophosphate, FCS, and cell culture density on the steady-state levels of *COL10* mRNAs of immortalized cells. In *A*, MCTs cells were cultured for 1 d at 37°C in standard medium, and then for 4 d at 37°C in the presence (+) or absence (−) of ascorbic acid (*aa*) and β-glycerophosphate (*βGP*). In *B*, MCTs cells were cultured for 1 d at 37°C in standard medium and then for 2 d at 37°C in medium supplemented with ascorbic acid, β-glycerophosphate, and as indicated, 2.5, 5, 10, or 20% FCS. In *C*, MCTs cells were plated at about 30, 90, and 270% confluence (30, 90, 270) and cultured for 4 d at 37°C in medium supplemented with ascorbic acid and β-glycerophosphate. All culture media were renewed every day. Total RNAs were fractionated, blotted, and hybridized with *COL10A1* and *COL1A1* probes, and blots exposed for 3 d. Hybridization with GAPDH probe and staining of 28S RNA are shown as references for the amount of RNA loaded in each lane.

recorded in the cells shifted to 37°C and 38.5°C than in the cells left at 32°C. During the following days, the levels of *COL10* mRNA were higher 1 d after medium renewal than after 2 d. Moreover, a progressive decrease in the steady-state levels of *COL10* mRNA was apparent with time in culture at all temperatures (compare days 1 with days 3, and days 2 with days 4 and 6). Whereas *COL1A1* mRNA was present at similar levels in all culture samples, no *COL2* mRNA was detected in any sample (not shown). Only traces of ALP mRNA were detectable. MGP mRNA levels were similar at all temperatures and increased with time in culture. OP mRNA levels were higher at nonpermissive than at permissive temperatures and did not vary significantly with time in culture. BGP mRNA levels were extremely low at 32°C and significantly higher at nonpermissive temperatures.

Increase in the steady-state levels of *COL10* mRNA also occurred in the three pools of immortalized cells upon shift from permissive to nonpermissive temperatures (Fig. 4). This effect was more pronounced in the pools than in the MCTs cells.

These data suggested that transfer of immortalized chondrocytes from permissive to nonpermissive temperatures resulted in upregulation of the expression of the genes for type X collagen, OP and BGP, which all are markers of nonproliferative hypertrophic chondrocytes.

Addition of either ascorbic acid, β-glycerophosphate, or a combination of both agents, for 1 (not shown) or 4 d at 37°C (Fig. 5 *A*) did not significantly affect *COL10* mRNA levels of MCTs cells. After 2 d at 37°C, high concentrations of FCS slightly decreased *COL10* mRNA levels but did not affect *COL1A1* mRNA levels (Fig. 5 *B*). When MCTs cells were plated at 30, 90, or 270% confluence and analyzed after 1 (not shown) or 4 d (Fig. 5 *C*) at 37°C, the levels of *COL10* mRNAs present in the cells were inversely proportional to the density of the cultures, whereas the levels of *COL1A1* (and GAPDH) mRNAs increased with cell density.

In summary, cell culture density, high concentrations of FCS, time in culture, and infrequent medium renewal were all associated with decreases in the levels of *COL10* mRNA.

Retinoic Acid Increases COL10 mRNA Levels in Immortalized Chondrocytes

RA stimulated the steady-state levels of *COL10* mRNAs produced by MCTs cells in both a dose-dependent (Fig. 6 *A*) and time-dependent manner (Fig. 6 *B*). A significant effect of RA

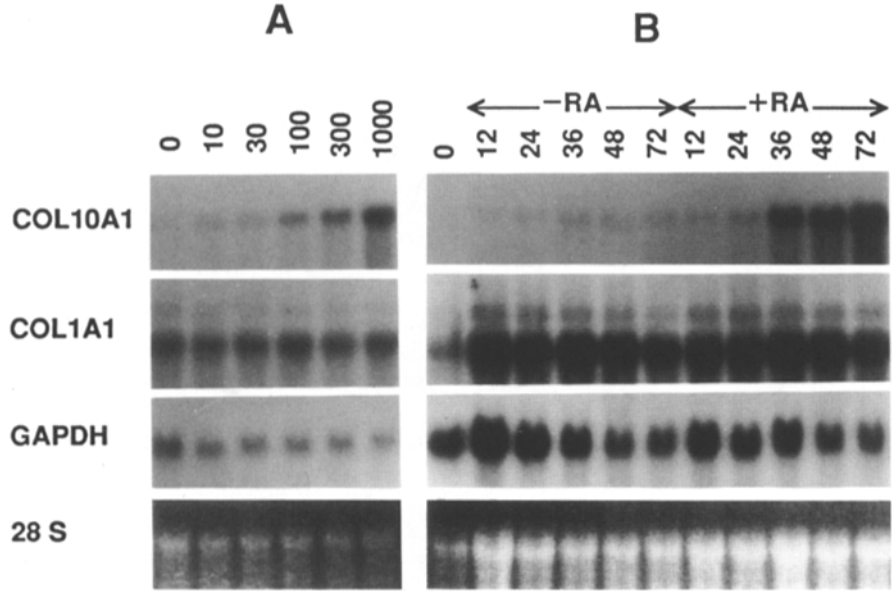

Figure 6. Increase of *COL10* mRNA steady-state levels in immortalized chondrocytes treated with retinoic acid. MCTs cells were plated at subconfluence in standard medium and cultured for 1 d at 37°C. The medium was then replaced and supplemented with ascorbic acid, β-glycerophosphate, and either retinoic acid or its solvent. In *A*, cells were treated for 54 h at 37°C with increasing concentrations of retinoic acid, from 0 to 1,000 nM as indicated. In *B*, retinoic acid was added at the concentration of 0 or 300 nM, and cultures were incubated for increasing time periods, from 0 to 72 h as indicated. In *A* and *B*, culture media were renewed after 24 and 48 h. Total RNAs were fractionated, blotted, and hybridized with the *COL10A1* and *COL1A1* probes. Blots were exposed for 4 d and 20 h, respectively. Hybridization with GAPDH probe is shown as reference for the amount of RNA loaded in each lane.

was observed at physiological doses of the vitamin (10–30 nM) and increased at higher concentrations. The response to RA appeared to be slow despite the high concentration of 300 nM used in the time kinetics, being barely detectable 12–24 h after addition of RA. However, it was pronounced after 36 h, and continued to increase at 48 and 72 h. *COL1A1* mRNA levels were not significantly affected by RA.

Discussion

We have immortalized mouse growth plate chondrocytes with a temperature-sensitive SV40 large T antigen and have characterized the phenotype of these cells. MCTs cells and other clones and pools of cells grew continuously at a temperature permissive for large T, as expected for transformed cells, whereas their growth was arrested within a few days after transfer to nonpermissive temperatures. In all clones and pools, a shift to the non-permissive temperatures strongly increased the levels of type X collagen, OP, and BGP mRNAs. Although we cannot exclude a direct effect to the inactivation of large T itself, our results suggest that the decrease in growth rate and growth arrest were responsible for the higher levels of *COL10*, OP, and BGP transcripts. We propose that at the nonpermissive temperatures, our immortalized cells were blocked from progressing through the cell cycle, which we suggest is needed to express higher levels of *COL10*, BGP, and OP RNA. In vivo, growth plate chondrocytes also start to express type X collagen only when they have terminated their proliferative phase and mature into hypertrophic cells (10, 25, 39). Furthermore, in vivo, the highest levels of osteopontin and BGP in cartilages are in the calcifying hypertrophic zone (29). For chondrocytes in culture, type X collagen expression was also correlated with growth arrest (14, 20). Moreover, constitutive overexpression of c-*myc* (18) and constitutive expression of v-*myc* (36) in chick chondrocytes maintained the cells in a proliferative state and blocked their maturation into hypertrophic cells. The levels of *COL10* mRNA present at 32°C varied from one cell clone or pool to another and were inversely related to the proliferative capacity of the cells (not shown). It thus appears likely that a major reason for the lack of expression of significant amounts of type X collagen mRNAs in previously reported chondrocyte cell lines (15, 18, 27, 36, 44) was the high rate of cell proliferation. The modifications in OP and BGP gene expression upon transfer of our transformed chondrocytes to nonpermissive temperatures resembled those described during the development of the hypertrophic phenotype in chick chondrocyte cultures (24). Indeed, in both cases, OP expression increased severalfold and BGP expression was induced even if BGP mRNA levels remained low by comparison to those detectable in osteoblastic cells.

At the time of infection with the retrovirus coding for large T, our primary chondrocytes expressed high levels of type II collagen and cartilage-specific proteoglycans (23), typical markers of differentiated chondrocytes. These initial populations of primary chondrocytes also contained mRNAs for type X collagen, MGP, OP, and ALP, indicating that an important proportion of the cells were hypertrophic. The immortalized cells lost expression of type II collagen and aggrecan but maintained expression of *COL10*, MGP, and OP; they also lost expression of ALP. In contrast to the initial primary chondrocytes, immortalized cells also contained type I collagen and BGP mRNAs. These two mRNAs were shown by others to be present in hypertrophic chondrocyte cultures after the cells had reached a further stage of differentiation corresponding to an osteoblast-like phenotype (3, 8, 24, 29, 37, 41, 42). Expression of the MGP and OP genes is also characteristic of the osteoblastic phenotype. Overall, our immortalized cells thus appeared to express a phenotype composed of markers of both hypertrophic chondrocytes and osteoblasts.

Hence, we believe that in our experiments the presence of SV40 large T during the selection process froze the cells in a specific stage of differentiation. However, to fully express markers like *COL10* that are characteristic of hypertrophic chondrocytes, these cells need to exit from the cell cycle.

Although positive effects of ascorbic acid (13, 22), β-glycerophosphate (13), and serum (5) on the production of type X collagen by chick chondrocytes were observed previously, none of these agents significantly increased the steady-state levels of *COL10* mRNAs present in MCTs cells. Ascorbic acid, β-glycerophosphate, and serum probably helped chick chondrocytes mature to hypertrophy whereas our immortalized cells were already at the hypertrophic stage.

Retinoic acid increased the levels of type X collagen transcripts in MCTs cells in a dose- and time-dependent manner, suggesting an RA-induced upregulation of the expression of the *COL10* gene. Similar observations were previously reported with primary chick chondrocytes (32, 33). As for chick chondrocytes, we observed that a significant response to RA occurred at physiological doses of the vitamin, but very high concentrations were necessary to obtain maximal stimulation. The slowness of the response to RA may indicate that it acted indirectly on *COL10* expression.

This study describes the first chondrocyte cell line that expresses the type X collagen gene and other hypertrophic chondrocyte markers. Similarly to the in vivo situation, a strong increase in expression of *COL10*, OP, and BGP genes was observed upon arrest of cell growth when the cells were shifted to nonpermissive temperatures. Upregulation of type X collagen gene expression also occurred upon stimulation of the cells by RA. Expression of type I collagen and BGP suggests that the cells could also have acquired certain properties of osteoblasts. We believe that these cell lines should greatly benefit investigations on hypertrophic chondrocyte differentiation and fate. Their advantage over primary hypertrophic chondrocytes is their immortality, the homogeneity of the cell populations, and the stability of their phenotype. In particular, they should be very useful for studies on the transcriptional mechanisms involved in the regulation of the type X collagen gene and of other genes which are activated during the ultimate stages of chondrocyte differentiation. Indeed, our cell lines should be suitable for DNA transfection experiments aimed at identifying *cis*-acting DNA elements which control expression of the *COL10A1* gene specifically in hypertrophic chondrocytes. These cell lines should also serve to identify hypertrophic chondrocyte-specific DNA-binding proteins and could be used to prepare cDNA libraries used for the isolation of clones encoding such cell-specific polypeptides.

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