Type X Collagen Synthesis during In Vitro Development of Chick Embryo Tibial Chondrocytes

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Abstract. In the developing chick embryo tibia type X collagen is synthesized by chondrocytes from regions of hypertrophy and not by chondrocytes from other regions (Capasso, O., G. Tajana, and R. Cancedda, 1984, *Mol. Cell. Biol.* 4:1163–1168; Schmid, T. M., and T. F. Linsenmayer, 1985, *Dev. Biol.* 107:375–381). To investigate further the relationship between differentiation of endochondral chondrocytes and type X collagen synthesis we have developed a novel culture system for chondrocytes from 29–31-stage chick embryo tibiae. At the beginning of the culture these chondrocytes are small and synthesize type II and not type X collagen, but when grown on agarose-coated dishes they further differentiate into hypertrophic chondrocytes that synthesize type X collagen. The synthesis of type X collagen has been monitored in cultured cells by analysis of labeled collagens and in vitro translation of mRNAs. When the freshly dissociated chondrocytes are plated in anchorage-permissive dishes, most of the cells attach and dedifferentiate, as revealed by their fibroblastic morphology. Dedifferentiated chondrocytes, after several passages, can still reexpress the differentiated phenotype and continue their development to hypertrophic, type X collagen–synthesizing chondrocytes. Hypertrophic chondrocytes, when plated in anchorage permissive dishes, attach, maintaining the differentiated phenotype, and continue the synthesis of type X collagen.

THE development and growth of long bones mainly occurs by endochondral bone formation. During this process, chondrocytes in the bone growth region pass sequentially through a proliferative, a hypertrophic, and a degenerative stage (44). Differentiating chondrocytes synthesize collagens and other extracellular matrix macromolecules which are deposited in the cartilage. For several years type II collagen has been recognized as the only cartilage-specific collagen (29). More recently the existence of other minor collagens has been demonstrated, including 1α, 2α, 3α chains (6), and type IX (33, 34, 45) and X collagens. In particular, type X collagen, first detected in cultures of chick embryo tibial chondrocytes (8, 9, 37, 38) and in cultures of chick embryo sternal chondrocytes grown within collagen gels (16, 17) and subsequently in other species (35), becomes a major collagen in the regions where cartilage is removed and replaced by bone tissue. Its synthesis by hypertrophic chondrocytes and its association with endochondral development have been shown by metabolic labeling of proteins made by the cells (10, 20) and by immunofluorescent staining of cartilage with specific monoclonal antibodies (40). From the same regions of the tibiae the type X collagen deposited in the cartilage in vivo has been purified in milligram quantities (9, 32). After performing cultures of chondrocytes from sterna of chick embryos at different stages of development, Gibson and Flint (14) reported synthesis of type X collagen by chondrocytes derived from the presumptive calcification region but not by chondrocytes derived from the permanent cartilaginous region.

In an attempt to establish a better correlation between differentiation of endochondral chondrocytes and synthesis of type X collagen, we developed a culture system in which tibial chondrocytes from 29–31-stage chick embryos (stage I chondrocytes) can continue their differentiation to endochondral hypertrophic chondrocytes synthesizing type X collagen (stage II chondrocytes) provided that their growth is anchorage independent. Under conditions of anchorage-dependent growth, most of the chondrocytes dedifferentiate, assume a fibroblastic morphology, and switch from the synthesis of type II to the synthesis of type I collagen; these cells express chondrogenic capability when retransferred to anchorage-independent growth conditions. In turn, stage II chondrocytes when plated on anchorage-permissive dishes maintain the differentiated phenotype and continue the synthesis of type X collagen.

Materials and Methods

Materials

Culture medium was Coon's modified F12 (3) lacking ascorbic acid. The medium was supplemented with 10% fetal calf serum (Seralab Ltd. [Sussex, England] or Flow Laboratories Ltd. [Irvine, Scotland]). Collagenase I and II

were obtained from Cooper Biomedical Inc. (Malvern, PA); agarose (type II), trypsin, pepsin, and ascorbic acid from Sigma Chemical Co. (St. Louis, MO, USA); and chicken serum from Flow Laboratories. Radiolabeled products were from Radiochemical Centre (Amersham, England) or from New England Nuclear GmbH, (Braunschweig, Federal Republic of Germany). All other reagents were analytical grade.

Cell Culture

Chondrocyte Suspension. Whole tibiae from stage 29–31 (6.5 d) stage chick embryos (19) were rinsed in Ca^{2+}, Mg^{2+}-free phosphate-buffered saline and digested with, per ml, 7 U collagenase I, 100 U collagenase II, 0.75 mg trypsin, 2% chicken serum for 15 min at 37°C to remove tissue debris and perichondrium. Tibiae were then digested with the same mixture, 4 × 40 min. Dissociation of cells was facilitated by repeated pipetting. Chondrocytes released by the four digestions were pooled, harvested by low speed centrifugation, and resuspended in culture medium.

Culture on Agarose (Anchorage-independent Growth). 2 ml chondrocyte suspension (1×10^5 cells per ml) was plated in 3-cm dishes coated with 1 ml 1% agarose in culture medium. Fresh medium was added after 3–4 d. Cells were passaged weekly by direct dilution in fresh medium without prior harvesting by centrifugation. In some experiments, and when large scale cultures were performed, chondrocytes were plated in bacteriological plastic dishes instead of agarose-coated dishes.

Culture in Plastic Tissue Culture Dishes (Anchorage-dependent Growth). Chondrocytes were plated at the same density as on agarose gel. Cells were passaged weekly 1:3/1:4 after trypsin digestion.

Labeling of the Cells and Isolation of Labeled Collagens

Cells were preincubated for 2 h in methionine-free medium containing ascorbic acid (50 μg/ml); [^{35}S]methionine was added at a concentration of 50 μCi/ml. After 2 h labeled collagens were isolated essentially as described in reference 26. Cells were combined with the medium, type I collagen was added as carrier at 30 μg/ml, and ammonium sulfate (35% of saturation) was added. After 16 h at 4°C the precipitate and the cells were collected by centrifugation, resuspended in 0.5 N acetic acid, and digested with pepsin (100 μg/ml) for 16 h at 4°C. In some experiments the labeled medium was collected and clarified by low speed centrifugation, and radioactive proteins were directly analyzed.

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SDS gel electrophoresis was performed as described by Laemmli (22) and modified by Bonatti and Descalzi-Cancedda (5). The concentration of acrylamide was 9%. Densitometric scannings of the autoradiography were obtained with a DU 8 spectrophotometer (Beckman Instruments Inc., Palo Alto, CA).

RNA Extraction and Cell-free Translation

For RNA preparation 10^6–10^7 cultured chondrocytes were lysed in 2.5 ml of 7

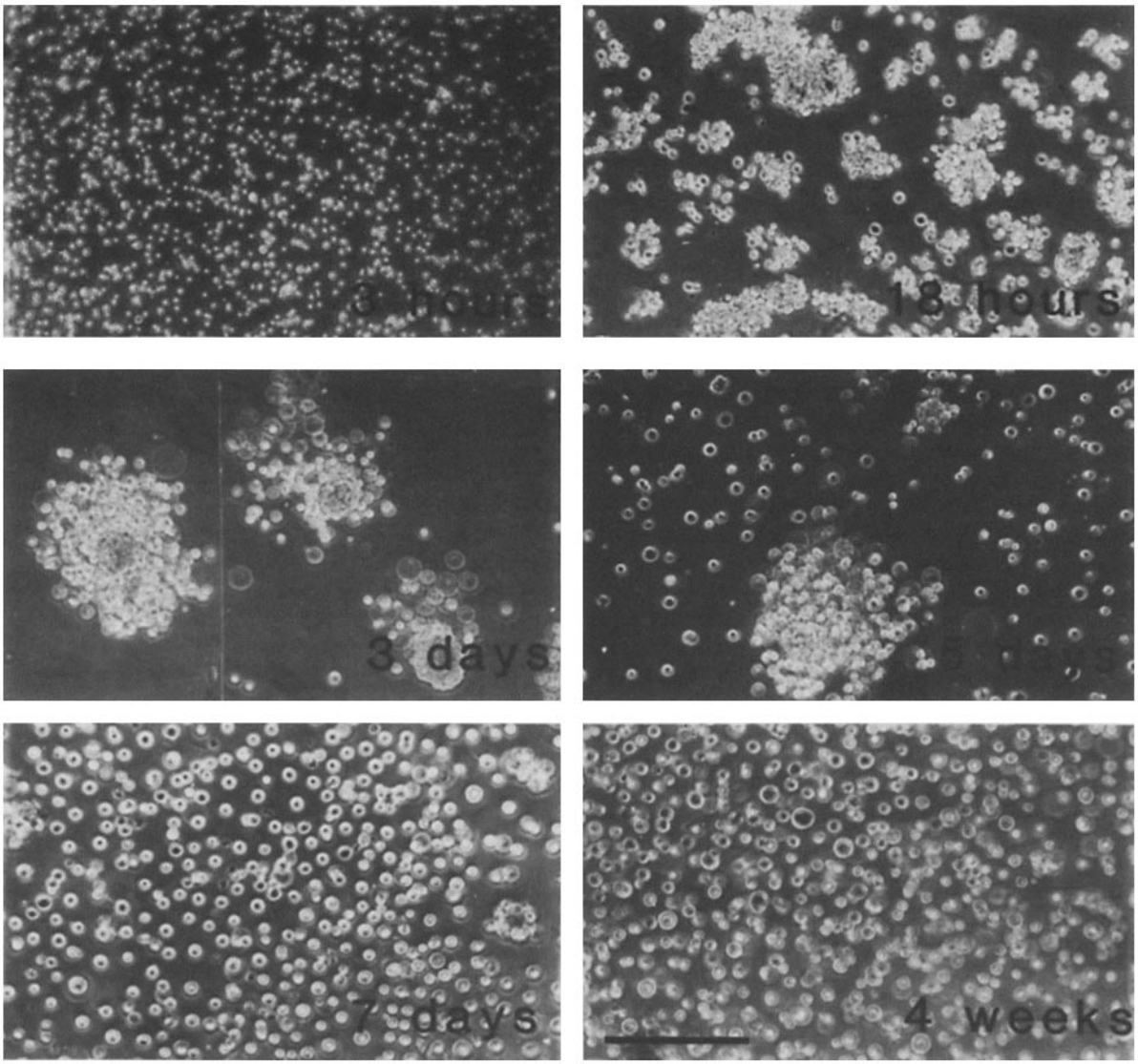

Figure 1. Chondrocytes cultured on agarose. Chondrocytes grown in agarose-coated dishes were observed by phase-contrast microscopy at different time intervals after the beginning of the culture. Bar, 200 μm.

M urea, 2% sarkosyl, 350 mM NaCl, 10 mM Tris-HCl, pH 7.9, 1 mM EDTA. The suspension was homogenized in a Dounce homogenizer (10 strokes), supplemented with 0.8 g CsCl, layered on top of 1.25 ml of 5.7 M CsCl, 100 mM EDTA, and centrifuged in a Beckman SW 50.1 rotor for 16 h at 36,000 rpm at 20°C. Pelleted RNA was dissolved in H_2O and reprecipitated with ethanol-0.3 M Na acetate, pH 5.2. The same procedure was followed to extract RNA from whole tibiae, previously digested for 30 min with collagenase and trypsin in order to remove tissue debris and perichondrium. Alternatively the guanidine extraction method described by Adams et al. (1) was used. The composition of the wheat germ cell-free translation mixture and the incubation conditions have already been described (7).

Results

Morphological Changes of Chondrocytes in Culture: Levels of Type X Collagen Synthesis

When freshly dissociated chondrocytes from tibiae of 29–31 stage embryos were plated in agarose-coated dishes, a condition under which cells cannot adhere and spread on the substratum, we constantly observed a characteristic sequence of events (Fig. 1). Within a few hours chondrocytes aggregated and formed compact clumps of small cells. From the second to the fifth day of culture the cells in the aggregates progressively increased in size and number and, at the same time, the aggregates started to flourish, decreasing their compactness and releasing isolated cells. By the seventh day the culture was formed mostly by isolated hypertrophic cells. These cells were passaged for 15 wk, and no significant morphological changes were observed. When the collagens synthesized by the chondrocytes during the culture were analyzed, synthesis of type X collagen was observed starting only 1 wk after the beginning of the culture (Fig. 2). Synthesis of type II collagen was observed at all culture times.

When freshly dissociated chondrocytes were plated in non-coated tissue culture dishes, the initially round cells attached to the plastic and progressively assumed the fibroblastic morphology characteristic of dedifferentiated chondrocytes (13, 28, 30, 36), athough some colonies of cells with an epithelial-like morphology were frequently present (Fig. 3). Dedifferentiated chondrocytes were weekly trypsinized and passaged for 12 wk. After two or three passages almost all cells presented the fibroblastic morphology. It has been shown by several laboratories that dedifferentiated chondrocytes have switched from the synthesis of type II (and other cartilage specific macromolecules) to that of type I collagen (reviewed in reference 25). In accordance with the literature, after 3–5 wk of culture, dedifferentiated chondrocytes with a fibroblastic morphology were positively stained by immunofluorescent antibodies against type I collagen (data not shown).

In Vitro Translation of mRNAs Extracted from Chondrocytes

The messenger RNAs extracted from chondrocytes grown for 2 and 7 d in agarose-coated dishes were used to direct protein synthesis in a cell-free system (Fig. 4). As a control the RNAs extracted from whole tibiae of 29–31-stage embryos and epiphysis of 17-d embryos were used. As expected, type X collagen was not synthesized by the RNA from 29–31-stage tibiae. The RNA extracted from chondrocytes obtained by enzymatic dissociation of the same tissue and cultured for 2 d on agarose directed already detectable quantities of type X collagen. The RNA from chondrocytes cultured for 7 d directed the synthesis of type X collagen at levels comparable to levels of the synthesis directed by the mRNA from 17-d epiphysis.

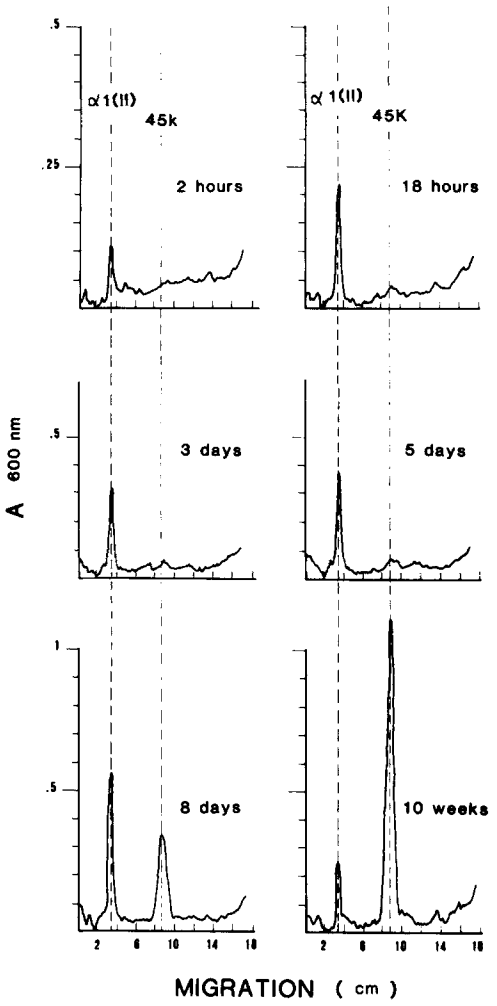

Figure 2. Densitometric scannings of collagens synthesized by chondrocytes cultured on agarose. Labeled collagens were pepsin extracted from combined culture medium and cells at different time intervals and analyzed on a 9% polyacrylamide gel. Densitometric scannings of the gel autoradiography were obtained with a Beckman DU8 spectrophotometer.

Reversion of Cell Phenotypes

When dedifferentiated chondrocytes passaged for several weeks in tissue culture dishes were transferred to agarose-coated dishes we observed a sequence of events similar to that described when freshly dissociated chondrocytes were plated directly on agarose-coated dishes (Fig. 5). It must be noted that, starting from dedifferentiated chondrocytes, the aggregation stage in the culture was always longer and that one extra week was necessary to reach the isolated cells stage. Fig. 5 refers to an experiment starting from dedifferentiated chondrocytes grown for 3 wk in tissue culture dishes. Identical results were obtained in experiments starting from dedifferentiated chondrocytes grown for 5 wk on tissue culture dishes.

On the contrary, when hypertrophic chondrocytes (derived from 7-d embryo tibial chondrocytes plated and grown for 1 wk on agarose) were transferred to tissue culture dishes they

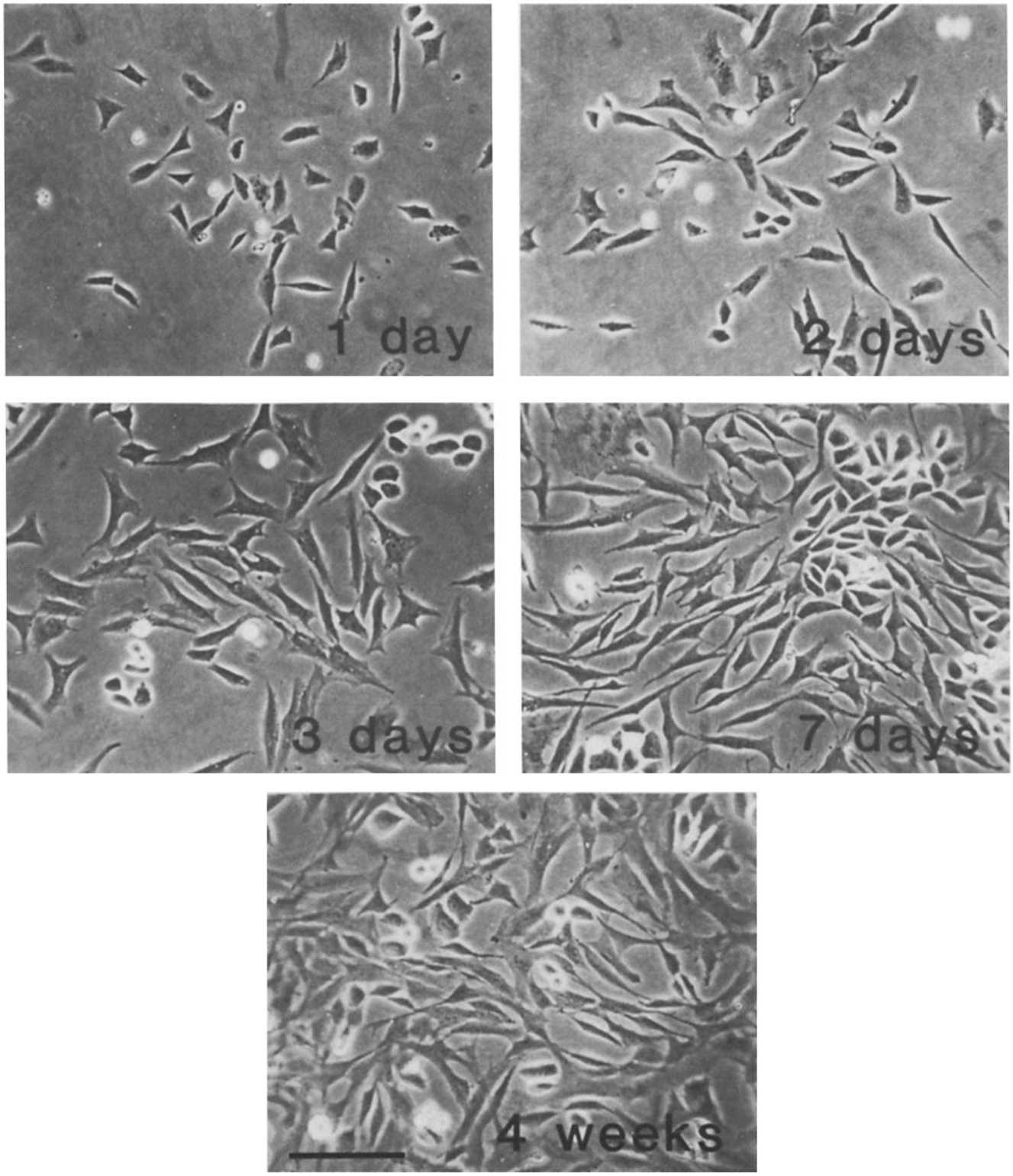

Figure 3. Chondrocytes cultured in tissue culture dishes. Cells were observed at different time intervals by phase-contrast microscopy after the beginning of the culture. Bar, 100 μm.

did not dedifferentiate but attached and assumed an epithelial-like morphology (Fig. 6). Cell attachment was facilitated by previous dissociation of the extracellular matrix by enzymatic digestion and repeated pipetting. Attached chondrocytes maintained the differentiated morphology for several weeks.

The radioactive proteins released in the medium by chondrocytes grown in tissue culture dishes and transferred to agarose, and vice versa, are analyzed in Fig. 7. Synthesis of type X collagen was observed when chondrocytes grown for 2 wk in tissue culture dishes were transferred for 1 wk to

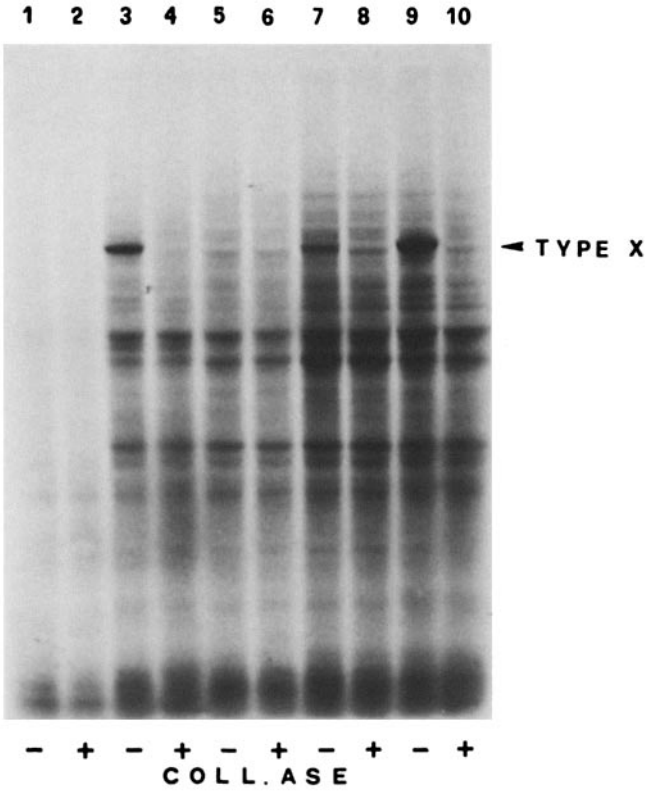

Figure 4. Cell-free translation of type X collagen. RNAs isolated from cultured chondrocytes or cartilage were translated in a cell-free wheat germ system in the presence of [^{35}S]methionine. Radioactive collagen was analyzed by gel electrophoresis. Lanes *1* and *2*, no RNA added; lanes *3* and *4*, RNA from epiphysis of 17-d-old tibiae; lanes *5* and *6*, RNA from 29–31 (6.5 d) stage whole tibiae; lanes *7* and *8*, RNA from chondrocytes grown for 2 d on agarose; lanes *9* and *10*, RNA from chondrocytes grown for 7 d on agarose. When indicated, an aliquot of the sample was collagenase digested before loading on the gel.

agarose (Fig. 7*b*) but not when they were maintained for 3 wk on tissue culture dishes (Fig. 7*e*). When chondrocytes grown for 2 wk on agarose were transferred for 1 wk to tissue culture dishes (Fig. 7*f*) they continued to synthesize levels of type X collagen comparable to those synthesized by chondrocytes grown for 3 wk on agarose (Fig. 7*c*). The maintenance of the synthesis of specific differentiation markers (i.e., type II collagen and cartilage proteoglycan protein) by chondrocytes grown on agarose and transferred to tissue culture dishes was also confirmed by immunofluorescence staining (unpublished results).

Correlation between Cell Growth and Type X Collagen Synthesis

The development of endochondral chondrocytes and the onset of type X collagen synthesis in culture may be the result of changes in the cell microenvironment or the phenotypic expression of a differentiation program that the already committed cell is following. To investigate these possibilities we set up chondrocyte cultures on agarose in the presence of 20 and 4% fetal calf serum. The cells in the presence of the higher serum concentration, after an initial lag of ~24 h, began to proliferate with a doubling time of 36–40 h. In the presence of 4% serum the lag period was longer and the cells began to proliferate at a very low rate after 3 d (Fig. 8). After 1 wk the chondrocytes had made more than two doublings in the 20% serum culture, and approximately the same number remained in the low serum culture; in both cases chondrocytes presented a hypertrophic morphology.

Collagens synthesized by chondrocytes grown in high and low serum concentrations were pepsin extracted from combined medium and cells and analyzed on polyacrylamide gel (Fig. 9). The ratio between the type X and the type II collagens was calculated and found to be 1.3 in the 20% serum culture and 0.5 in the 4% serum culture. In some experiments the doubling time of the chondrocyte culture was lowered to ~24 h by the addition to the medium of nine growth promoting factors (12). In this case after 1 wk of culture the calculated ratio between the type X and the type II collagens synthesized was 5.2.

Discussion

Tibial endochondral chondrocytes derive from limb bud undifferentiated mesenchymal cells. It is still debated which stimulus induces chondrogenesis (including the switch from type I to type II collagen). Certainly the loss of cell anchorage plays an important role. Previous reports indicated that limb mesenchymal cells undergo chondrogenesis when cultured in suspension (23, 42) or in a collagen gel (43), or when plated at very high density (11). All of these conditions interfere with cell anchorage and spreading. Cytochalasin D, a drug known to disrupt the actin cytoskeleton, also induced cartilage formation by cultures of limb bud cells (48).

Here we have shown that when the chondrocytes from 29–31-stage tibiae were grown under an anchorage-independent condition they expressed the differentiated phenotype and, more important, they seemed to continue their normal development. The cells acquired a hypertrophic morphology and initiated the synthesis of type X collagen that progressively became the major collagen. The analysis of collagens synthesized by cultured chondrocytes showed the presence of a large amount of type X collagen after ~1 wk of culture. The in vitro translation of the mRNAs purified from the cells showed high concentrations of the mRNA for the type X collagen in the RNA extracted from chondrocytes cultured for 7 d and only barely detectable amounts after 2 d of culture. This suggests that a transcriptional regulation step is involved. It has been shown that some hypertrophic chondrocytes are already present in the diaphysis region of 6.5-d tibiae (41), at the time we digested the tissue to establish our cultures. The type X collagen mRNA detected in the RNA extracted from cells cultured for 2 d is probably synthesized by those hypertrophic chondrocytes.

At the beginning of the culture chondrocytes spontaneously formed large aggregates; hypertrophic chondrocytes synthesizing type X collagen were mostly isolated. This may reflect a loss during endochondral development of membrane protein(s) responsible for cell-cell interactions or a modification in the composition of the extracellular matrix made by the chondrocytes. Studies are in progress to distinguish between these two possibilities. In vivo at embryo stage 22 an aggregation of limb bud mesenchymal cells precedes the onset of chondrogenesis and their differentiation into cartilage cells

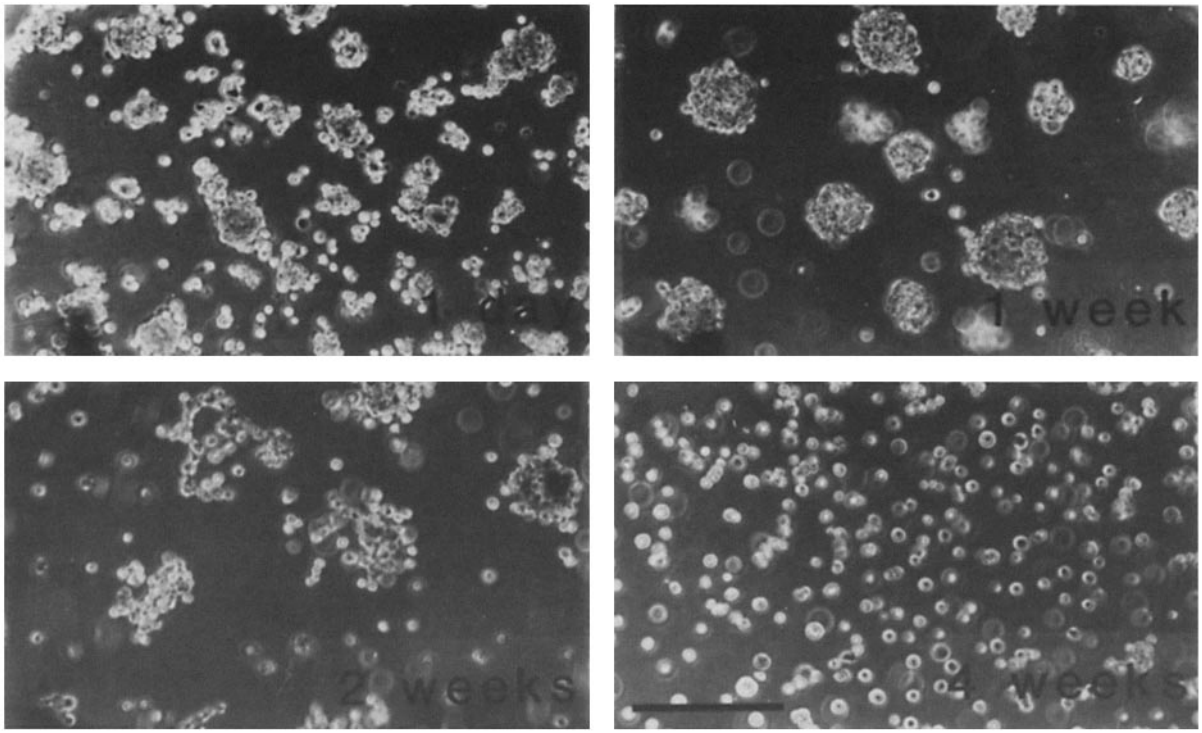

Figure 5. Reversion of the dedifferentiated chondrocyte phenotype. Dedifferentiated chondrocytes passaged for 3 wk in tissue culture dishes were transferred in agarose-coated dishes and observed by phase-contrast microscopy at different time intervals. Bar, 200 μm.

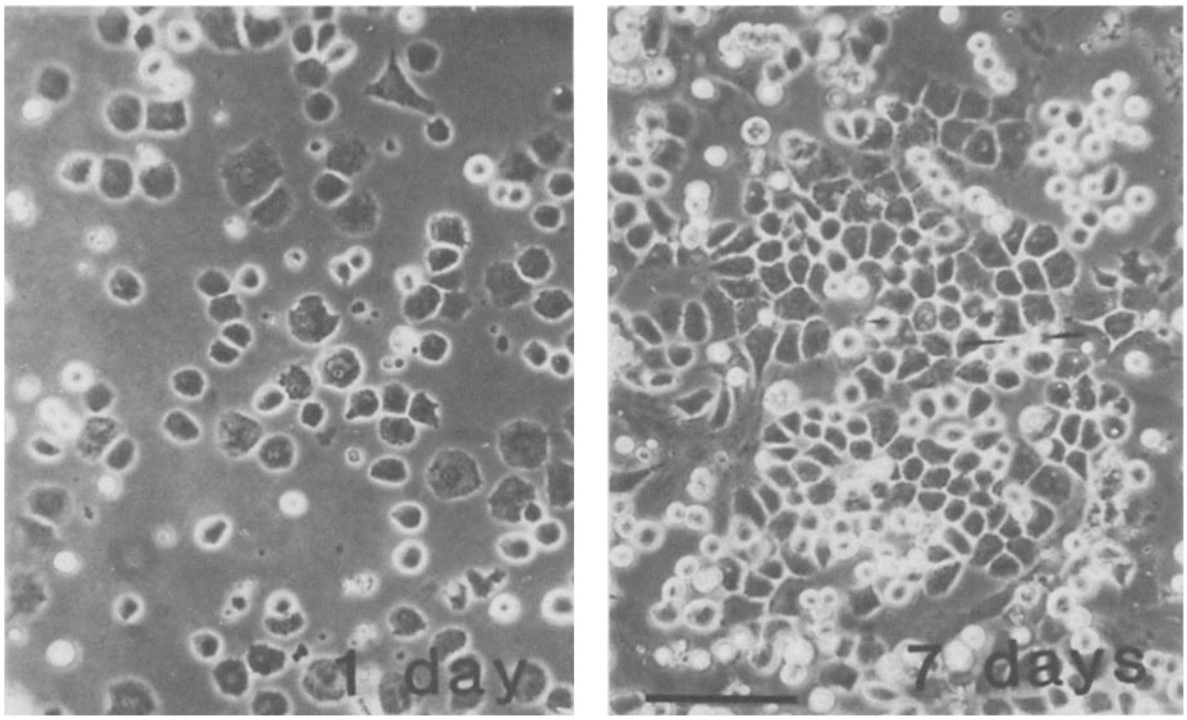

Figure 6. Maintenance of chondrocyte phenotype in tissue culture dishes. Chondrocytes grown for 1 wk in agarose-coated dishes were transferred in tissue culture dishes and observed by phase-contrast microscopy at different time intervals. Bar, 100 μm.

(21). Due to the deposition of new cartilage extracellular matrix chondrocytes progressively break off. Although this is not positive evidence, the aggregation of stage I chondrocytes after their extracellular matrix digestion may involve the same membrane proteins responsible of limb bud mesenchymal cells aggregation.

Which stimulus induces chondrocyte hypertrophy and type X collagen synthesis? The data presented suggest that the number of cell divisions is probably important. At the same time, modifications of the extracellular microenvironment,

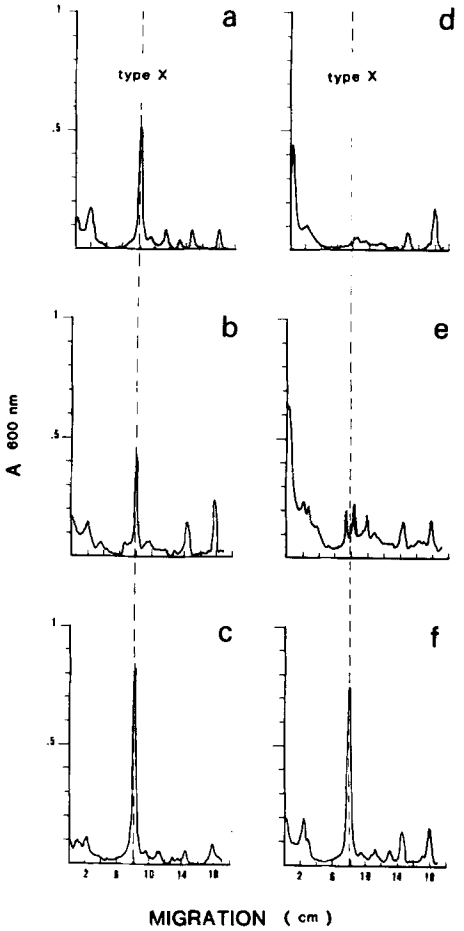

Figure 7. Densitometric scanning of proteins released by different cell phenotypes. Labeled proteins released in the medium by the cells were directly analyzed on polyacrylamide gel. (*a*) Cells grown for 1 wk on agarose. (*b*) Cells passaged for 2 wk in tissue culture dishes and then transferred for 1 wk to agarose. (*c*) Cells passaged for 3 wk on agarose. (*d*) Cells grown for 1 wk on tissue culture dishes. (*e*) Cells passaged for 3 wk on tissue culture dishes. (*f*) Cells passaged for 2 wk on agarose and transferred for 1 wk to tissue culture dishes. Approximately the same amount of counts was applied to each lane in the polyacrylamide gel.

due to the enzymatic dissociation of the cartilage during the preparation of the culture or the presence of some specific factor in the serum added to the culture medium, should be taken into account. In the embryo tibia the chondrocytes synthesizing type X collagen are localized in regions characterized both by big changes in the extracellular matrix and by the onset of the vascularity (10, 39, 40).

In this paper we have also shown that chondrocytes obtained from tibiae of 29–31-stage chick embryos (stage I chondrocytes) plated on anchorage-permissive dishes do not continue their development and dedifferentiate. A variable degree of dedifferentiation of chondrocytes in standard culture conditions (i.e., on plastic tissue culture dishes) has been seen by most laboratories that have cultured chondrocytes (13, 28, 30, 36). Dedifferentiation occurs spontaneously but is accelerated by bromodeoxyuridine (22, 36), by the carcinogen phorbol-myristate-acetate (24, 31), and by viral transformation (2, 18, 47). It is interesting that fibronectin, a protein known to promote cell adhesion, also enhances chondrocyte dedifferentiation (46). In the attempt to determine the percentage of dedifferentiated cells in our culture, we have analyzed by Northern blot with probes for α 1 (I) and α 1 (II) mRNAs the RNAs extracted from cultured chondrocytes. We have observed the appearance of high levels of α 1 (I) mRNA and the decrease of α 1 (II) mRNA to the detection limit after 3–5 wk culture in anchorage-permissive dishes (Castagnola, P., G. Moro, and R. Cancedda, manuscript in preparation). In this paper we have shown that, after several passages, fibroblastic dedifferentiated chondrocytes when transferred to suspension culture can reinitiate chondrogenesis, as if they were limb mesenchymal cells. Similarly, rabbit articular dedifferentiated chondrocytes can reexpress the differentiated collagen phenotype when cultured in agarose gels (4).

Stage II hypertrophic chondrocytes plated on anchorage-permissive dishes maintained the differentiated phenotype and continued the synthesis of type X collagen. Therefore, at variance with stage I chondrocytes, stage II chondrocytes show a phenotypic stability. They can probably revert upon transformation with Rous sarcoma virus; we have reported dedifferentiation by viral transformation in a chondrocyte population that we consider equivalent to stage II chondrocytes (18).

In our definition stage I chondrocytes are small chondrocytes derived from presumptive calcification regions of cartilage. These cells, synthesizing predominantly type II collagen,

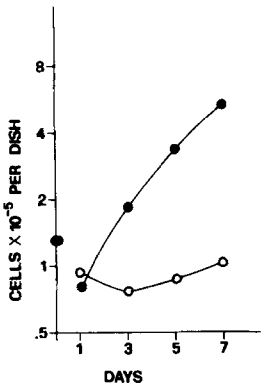

Figure 8. Growth of chondrocytes in the presence of high and low serum concentrations. Cells were seeded at 1.3×10^5 cells per 30-mm agarose-coated dish. Cell number per dish at each indicated time represents the mean value of duplicate cultures. ●, 20% serum; ○, 4% serum.

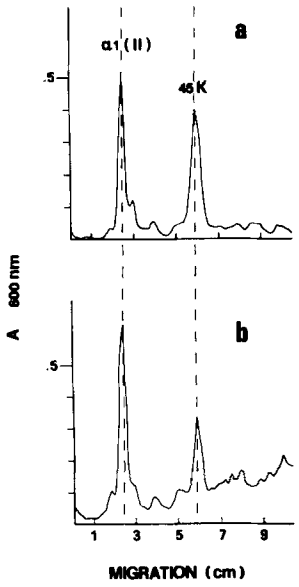

Figure 9. Densitometric scannings of collagens synthesized by chondrocytes grown in the presence of high and low serum concentrations. Collagens were pepsin extracted from combined culture medium and cells grown for 1 wk on agarose. (*a*) Chondrocytes grown in the presence of 20% fetal calf serum. (*b*) Chondrocytes grown in the presence of 4% fetal calf serum.

can undergo hypertrophy and initiate the synthesis of type X collagen that, progressively, becomes the major collagen. Stage I chondrocytes must probably be distinguished from those derived from permanent cartilaginous regions that, according to references 14 and 15, when cultured do not undergo hypertrophy and remain unable to synthesize detectable levels of type X collagen.

This work was supported by grants from Progetti Finalizzati "Oncologia" and "Ingegneria Genetica e Basi Molecolari delle Malattie Ereditarie," Consiglio Nazionale delle Ricerche, Rome, and by funds from Ministero Pubblica Istruzione, Italy.

Received for publication 14 November 1985, and in revised form 14 February 1986.

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