In Vitro Development of Hypertrophic Chondrocytes Starting from Selected Clones of Dedifferentiated Cells

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Abstract. Single cells from enzymatically dissociated chick embryo tibiae have been cloned and expanded in fresh or conditioned culture media. A cloning efficiency of ~13% was obtained using medium conditioned by dedifferentiated chondrocytes. A cloning efficiency of only 1.4% was obtained when conditioned medium from hypertrophic chondrocytes was used, and efficiencies of essentially 0 were found with fresh medium or medium conditioned by J2-3T3 mouse fibroblasts. Cell clones were selected by morphological criteria and clones showing a dedifferentiated phenotype (fibroblast-like) were further characterized. Out of 38 clones analyzed, 17 were able to differentiate to the hypertrophic chondrocyte stage and reconstitute hypertrophic cartilage when placed in the appropriate culture conditions. Cells from these clones expressed the typical markers of chondrocyte differentiation, i.e., type II and type X collagens. Clones not undergoing differentiation continued to express only type I collagen. Hypertrophic chondrocytes from differentiating clones were analyzed at the single cell level by immunofluorescence; all the cells were positive for type X collagen, while ~50% of them showed positivity for type II collagen.

DURING the organogenesis of the chick embryo tibiae, mesenchymal cells developing into hypertrophic chondrocytes pass through at least three distinct differentiation stages: (*a*) committed mesenchymal cells (20); (*b*) proliferating (stage I) chondrocytes (18, 20); and (*c*) hypertrophic (stage II) chondrocytes (6, 19, 24). Chondrocytes from Hamburger and Hamilton stage 28–30 (17) chick embryo tibiae dedifferentiate when cultured on plastic dishes. These cells, as well as dedifferentiated chondrocytes from another source (4), maintain the dedifferentiated phenotype as long as the conditions for the cell attachment to the plastic are kept. When the same cells are transferred into suspension culture, they resume the chondrocytic phenotype and mature to hypertrophic chondrocytes through a transient stage of cell aggregation (7). This process is accompanied by an increase in the length of the cell cycle and in the number of quiescent and degenerating cells (16), as well as by a modulation in the transcriptional activity of the collagen genes (9). We have recently reported the identification, purification, and characterization of a novel, low molecular weight protein, named Ch21, expressed and secreted by the in vitro differentiating chondrocytes at a late stage of development (11). When dedifferentiated cells are cultured in suspension in the constant presence of ascorbic acid, they organize their own extracellular matrix and develop into a tissue closely resembling calcifying hypertrophic cartilage (28, 29).

The heterogeneity of the dedifferentiated cell population did not allow us to definitely state whether the cells followed successive stages of differentiation or the culture conditions made a selection of cell subpopulations. To answer this important question we selected clones of dedifferentiated cells to follow the respective behavior of each clone in terms of morphology and collagen phenotype in the different culture conditions. We show that up to 45% of the cell populations obtained from single cloned dedifferentiated cells are still able to differentiate to hypertrophic chondrocytes and to reconstitute hypertrophic cartilage when transferred to the appropriate culture conditions. In addition, taking advantage of these cloned cell populations, we have investigated the correlation between type II and type X collagen appearance during in vitro differentiation of chondrocytes.

Materials and Methods

Cell Culture

Culture medium was Coon's modified Ham F-12 (1) with the addition of 10% FCS (Flow Laboratories, Irvine, Ayrshire, Scotland), 50 IU/ml penicillin, and 50 μg/ml streptomycin (Gibco Ltd., Paisley, Scotland); where indicated 50 μg/ml of ascorbic acid (Sigma Chemical Co., St. Louis, MO) were also added. Primary cultures, anchorage-dependent cultures, suspension cultures, and in vitro reconstitution of hypertrophic cartilage were performed as previously described (7, 28). Briefly, Hamburger and Hamilton stage 28–30 (17) chick embryo tibiae were removed, cleaned, washed in Ca^{2+}- and Mg^{2+}-free PBS, pH 7.2, and digested for 15 min at 37°C with 400 U/ml collagenase 1 (CooperBiomedical, Inc., Malvern, PA) and 0.25% trypsin (Gibco Ltd.). After sedimentation the supernatant, containing tissue debris and perichondrium, was discarded and the pellet was digested

for 45–60 min with the above dissociation buffer supplemented with 1,000 U/ml of collagenase II (CooperBiomedical, Inc.). Hypertrophic chondrocytes, grown in suspension culture, were digested with 1 mg/ml hyaluronidase (type III; Sigma Chemical Co.) in PBS for 15 min at 37°C before transfer to adherent culture.

Cloning

Freshly dissociated cells from chick embryo tibiae were seeded in 96-well tissue culture plates (Costar Data Packaging Corp., Cambridge, MA) at a 0.5 cell/well ratio. Cells were incubated in medium conditioned for 48 h by confluent cultures of chicken dedifferentiated chondrocytes, hypertrophic chondrocytes, or mouse fibroblast J2-3T3. Conditioned media were filtered (0.45 μm) and diluted 1:1 with fresh culture medium. Clones were scored under the microscope 7–10 d after plating and only clones showing a frank fibroblast-like morphology were selected and expanded for 3 wk before freezing. Cloning efficiency was expressed as follows:

$$\frac{\text{number of colonies obtained}}{\text{number of cells plated}} \times 100.$$

Metabolic Labeling

Cells were washed three times in methionine-free Coon's modified Ham F12 medium and incubated in the same medium for 2 h at 37°C. ^{35}S-Methionine (code SJ.235; Amersham, Buckingamshire, England) was added at a concentration of 100 μCi/ml together with 0.1% FCS, and the incubation was resumed for 2 h at 37°C. Supernatants were collected, clarified by low speed centrifugation, and processed for further analysis.

Immunoprecipitation and SDS-PAGE

Immunoprecipitation and SDS-PAGE were performed as previously described (16). Antibodies against chicken collagen type I and Ch21 have been described elsewhere (11, 22). Antisera to type II and type X collagens were raised in rabbits using electrophoretically pure proteins and tested for specificity by radioimmunoassay, immunofluorescence, and immunoprecipitation (28).

mRNA Quantitation

The guanidinium isothiocyanate/CsCl method of Chirgwin et al. (10) was used to extract total cellular RNA from chondrocytes. Liver total RNA from 17-d-old chick embryos was prepared according to Auffray and Rougeon (2). 0.8-μg RNA aliquots were denatured in 7.4% formaldehyde (30) and blotted on BA85 nitrocellulose membrane with a 96 well-manifold (Schleicher & Schuell, Inc., Keene, NH). Recombinant DNA for chicken type I, II, and X collagens were labeled with (α-^{32}P)dCTP using the random primed labeling system (Amersham Corp., Arlington Heights, IL), with final specific activities ranging from 0.5 to 2.0 $\times$ 10^9 cpm/μg DNA. The nitrocellulose filters were prehybridized and hybridized at 42°C for 16 and 48 h, respectively, in a medium containing 50% formamide, 2× Denhardt's solution (1× Denhardt's solution = 0.02% each of Ficoll, BSA, and polyvinylpyrrolidone), 5× SSPE (1× SSPE = 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA, pH 7.4), 0.1% SDS, 50 μg/ml polyadenosine, and 100 μg/ml salmon sperm DNA. The hybridization medium was supplemented with 1–5 $\times$ 10^7 cpm/ml of the appropriate cDNA probe. To decrease crosshybridization between collagen sequences, the concentration of formamide was raised to 60% in the hybridization using the cDNA for type II collagen. After hybridization, the filters were washed under stringent conditions, the last wash being in 0.2% SSC (1× SSC = 0.15 M NaCl and 0.015 M Na-citrate) and 0.1% SDS at 54°C for 30 min. The radioactivity retained on individual dots was then quantitated by β liquid scintillation counting. The cDNAs for chicken collagens used in the dot-blot hybridization assays were the pCOL3 clone for α1(I) (33), the pCS1 clone for α1(II) (34), and the pcIII8 clone for α1(X) (8). The rat albumin cDNA was an 1,800-nucleotide sequence subcloned in pUC13 (13). Only the cDNA inserts were used for random labeling after digestion with the appropriate restriction enzyme and purification on agarose gel/NA45 membrane (Schleicher & Schuell, Inc.).

Histology

Samples were processed as previously described (28) with minor variations. Briefly, samples were fixed for 20 min in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3, postfixed for 20 min in 1% osmium tetraoxide in the same buffer, en bloc stained with uranyl acetate, and embedded in Poly-bed 812. 1-μm-thick sections were stained with toluidine blue and photographed with a photomicroscope (Axiophot; Carl Zeiss, Inc., Thornwood, NY).

Immunofluorescence

Adherent hypertrophic chondrocytes from differentiating clones were grown on tissue culture chamber slides (Miles Laboratories, Inc., Naperville, IL) fixed for 5 min in 3.7% formaldehyde in PBS, washed, and permeabilized with 0.1% Triton X-100 in PBS. Samples were then challenged with rabbit antiserum to chick type X collagen (diluted 1:30) and/or with goat anti-chick type II collagen (a gift from Dr. K. von der Mark, Erlangen, FRG) (diluted 1:30) for 1 h at room temperature. Staining was performed with TRITC-labeled donkey anti-goat IgG (H+L) and/or FITC-labeled mouse anti-rabbit (H+L) (Jackson Immunochemical Research Laboratories, Inc., Avondale, PA) for 30 min at room temperature. All dilutions were made in PBS containing 4 mg/ml mouse gammaglobulin. Slides were mounted with 80% glycerol in PBS and photographed with a photomicroscope equipped with epifluorescence illumination (Axiophot; Carl Zeiss, Inc.).

Results

The Cloning Efficiency of Primary Cultures Is Dramatically Increased by the Use of Culture Media Conditioned by Dedifferentiated Chondrocytes

Dissociated chick embryo tibiae were seeded into 96-well tissue culture plates, and fresh culture medium or medium containing 50% medium conditioned by different cell types was added (Table I). A high cloning efficiency (13.7%) was obtained when cells were plated in medium conditioned by

Table I. Influence of Conditioned Medium on Cloning Efficiency

	Medium conditioned by			
	Dedifferentiated cells*	Hypertrophic chondrocytes‡	J2-3T3 cells§	Normal fresh medium‖
Cells plated	1,000	1,000	1,000	1,000
Colonies obtained	137	14	0	3
Colony phenotype				
Differentiated	54%	–	–	–
Dedifferentiated	36%	–	–	–
Mixed	10%	–	–	–

* Actual number of cells plated = 1,990 (10 experiments).
‡ Actual number of cells plated = 1,390 (8 experiments).
§ Actual number of cells plated = 700 (4 experiments).
‖ Actual number of cells plated = 600 (4 experiments).

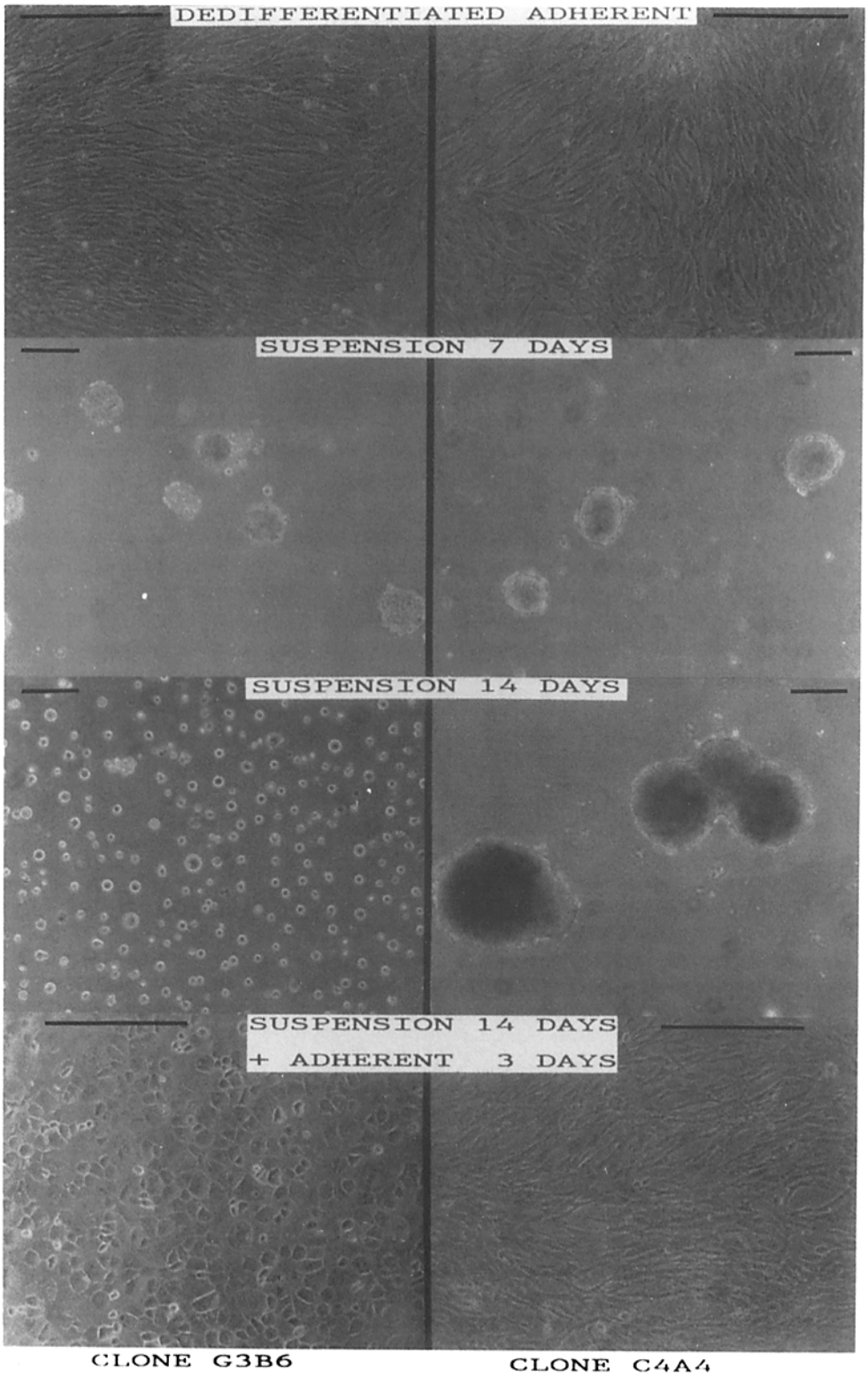

Figure 1. Cultures of two clones: G3B6 (subgroup 1) and C4A4 (subgroup 2). Cultured as dedifferentiated adherent cells, both clones show a frank fibroblastoid phenotype. After 7 d in suspension culture, G3B6 cells are still aggregated, but the aggregates are loose. At the same time, the aggregates formed by C4A4 cells are still tightly closed. After 14 d of suspension culture, cells from G3B6 clone appear as hypertrophic chondrocytes, while C4A4 cells remain aggregated. Plated as adherent after a mild enzymatic digestion, G3B6 hypertrophic chondrocytes assume the typical polygonal phenotype, while C4A4 cells retain the starting fibroblast-like phenotype. Bars, 300 μm.

dedifferentiated chondrocytes. On the contrary, efficiencies of 0–0.3% were obtained when either fresh medium or medium conditioned by the mouse fibroblast cell line J2-3T3 was used. Slightly higher efficiencies were obtained using medium conditioned by hypertrophic chondrocytes (1.4%). The morphology of the colonies was scored under the microscope. We observed that most of the colonies had a characteristic chondrocyte phenotype (5) and that the dedifferentiated phenotype was shown only by slightly more than one third of the colonies. The absolute number of the colonies formed in the presence of medium conditioned by hypertrophic chondrocytes was too low to have a statistically significant distribu-

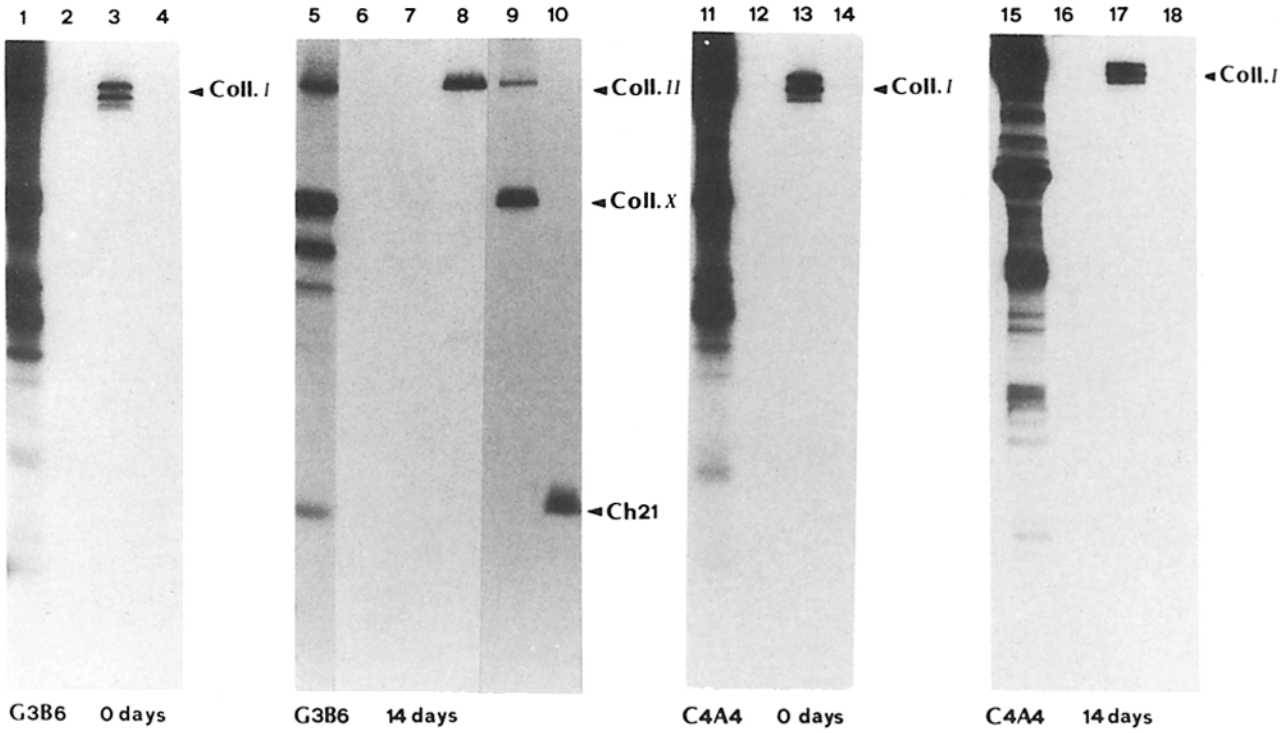

Figure 2. ^{35}S-proteins synthesized in vitro by subgroup 1 (G3B6) and subgroup 2 (C4A4) clones. Proteins were immunoprecipitated and analyzed in reducing conditions on 12.5% SDS-PAGE as described in Materials and Methods. (Lanes *1–4* and *11–14*) Proteins released in the medium by clones as dedifferentiated cells. (Lanes *5–10* and *15–18*) Proteins released in the medium by clones cultured for 14 d in suspension. (Lanes *1*, *5*, *11*, and *15*) Total proteins. (Lanes *2*, *6*, *12*, and *16*) Proteins reacted with normal rabbit serum. (Lanes *3*, *7*, *13*, and *17*) Proteins reacted with rabbit antiserum against chicken type I collagen. (Lanes *4*, *8*, *14*, and *18*) Proteins reacted with rabbit antiserum against chicken type II collagen. (Lanes *9* and *10*) Proteins reacted with rabbit antisera against chicken type X collagen and Ch21, respectively. Antibodies against type X collagen show cross-reactivity with type II procollagen.

tion according to the different phenotype. The cell doubling time of the differentiated colonies was always too low to allow the expansion of these clones. The cell doubling in the dedifferentiated colonies was more variable. We selected and expanded only clones having a frank dedifferentiated phenotype and an approximate doubling time of 24 h. When the number of cells reached ~20 × 10^6, half of the cells were frozen and stored in liquid nitrogen and the remaining half further expanded and used. Out of 10 experiments performed, a total of 38 clones of dedifferentiated chondrocytes were eventually available.

Based on Their Properties, Clones Can Be Divided in Two Subgroups

Clones were characterized within 4 wk from the primary culture. 38 clones were analyzed. All of them maintained the fibroblast-like phenotype when cultured as adherent cells (Fig. 1). After transferring in suspension culture they aggregated within 24 h, but only 17 (~45%) of them began to "flourish" after 1 wk. After 2 wk these same clones gave rise to a population of single isolated hypertrophic chondrocytes (subgroup 1), while the cells of the other clones still remained tightly aggregated (subgroup 2) (Fig. 1). As expected, the hypertrophic chondrocytes from subgroup 1 clones, when replated on plastic dishes after mild digestion of extracellular matrix (Fig. 1), presented the characteristic epithelial-like morphology (5). The cells from the subgroup 2 clones, when replated, presented the same morphology of the starting dedifferentiated cell population (Fig. 1). It must be noted that a clone of dedifferentiated chondrocytes could not be included in one of the 2 subgroups before it was tested in suspension culture. The morphologies of the 2 subgroups of clones grown as adherent cells were essentially indistinguishable; the calculated doubling times ranged for all clones between 20 and 28 h. Immunoprecipitations of radiolabeled proteins secreted by these cells are shown in Fig. 2. Cells from both subgroups at the dedifferentiated stage secreted exclusively type I collagen in the culture medium (lanes *3* and *13*). No type II (lanes *4* and *14*) was immunologically detected at this stage. After 2 wk of suspension culture, the protein pattern of the two cell subgroups was dramatically different. Clones from subgroup 1 ceased secreting type I collagen (lane *7*), and actively synthesized type II and type X collagen (lanes *8* and *9*) and Ch21 (lane *10*). Clones from subgroup 2 still expressed only type I collagen (lane *17*) and no type II collagen (lane *18*) was detected. Type X collagen and Ch21 were not found by immunoprecipitation in the culture medium of both subgroups of clones as dedifferentiated cells, and in the medium of subgroup 2 clones after 2 wk of suspension culture (data not shown).

mRNA Analysis

The steady state levels of the mRNAs encoding type I, II, and X collagens were quantitated by dot-blot hybridization assays in cells either maintained dedifferentiated, or grown in suspension for 2 wk. For each time point, the assay was per-

Table II. Collagen mRNA Levels in Cloned Cells

Clone	Days of suspension culture	Type I collagen		Type II collagen		Type X collagen		Albumin
		cpm	*norm*	*cpm*	*norm*	*cpm*	*norm*	*cpm*
Experiment I								
C4A4	0	5,376	1.0	256	1.0	42	1.0	
C4A4	14	3,514	0.65	185	0.72	74	1.76	
R15	0	5,186	1.0	241	1.0	41	1.0	
R15	14	2,662	0.50	2,995	12.3	319	7.7	
Experiment II								
R33	0	1,725	1.0	265	1.0	321	1.0	ND
R33	14	1,152	0.65	242	0.92	530	1.65	ND
G3B6	0	3,140	1.0	674	1.0	663	1.0	0
G3B6	14	2,186	0.69	26,005	38.6	7,140	11.2	0
Liver	–	1,382		0		11		466

R15–G3B6, subgroup 1 clones; C4A4–R33, subgroup 2 clones. *cpm*, mean of duplicates corrected for cpm background (50–80 cpm); *norm*, ratio vs. value in dedifferentiated cells.

formed on two types of cloned cell populations, one from subgroup 2 (C4A4 and R33) and the other one from subgroup 1 clones (R15 and G3B6). The data are presented in Table II. For all clones, type I collagen mRNA was highly represented in the dedifferentiated cells and would decrease after transfer of the cells to suspension culture. A detectable level of type II collagen mRNA was observed in the dedifferentiated cells of all clones. This level was highly enhanced in subgroup 1 clones grown in suspension, while no increase was observed in those from subgroup 2. The dedifferentiated cells of all clones expressed variable basal levels of type X collagen mRNA as well. In the suspension culture, the expression of this mRNA increased significantly in the differentiating clones (subgroup 1) to reach an 8–11-fold enhanced level after 2 wk. The cells of the nondifferentiating clones (subgroup 2) transferred to suspension culture showed only a faint accumulation of type X collagen mRNA (~1.7-fold increase).

Hypertrophic Cartilage Can Be Reconstituted In Vitro Only by Differentiating Clones

To assess the influence of an organized extracellular matrix on the differentiation of dedifferentiated cells, clones from both subgroups were cultured in suspension in the presence of ascorbic acid. In cultures of subgroup 1 clones, cells readily aggregated. These aggregates progressively increased in volume, but never released single isolated cells. In cultures of subgroup 2 clones, cells also aggregated, but the size of the aggregates did not change with time. After 10 d, aggregates from both cultures were fixed, embedded, and stained as described in Materials and Methods. The aggregates of subgroup 1 clones displayed an extremely well-organized structure closely resembling hypertrophic cartilage (Fig. 3 *A*). All the cells were contained in lacunae, no gradient of maturation from the periphery to the center was detectable, and no layer of elongated cells surrounded the aggregates. This degree of differentiation was even more pronounced than the morphology of in vitro–reconstituted hypertrophic cartilage obtained from an uncloned population of dedifferentiated cells which show varying degrees of cellular differentiation (28). Aggregates from cultures of subgroup 2 clones showed no evidence of extracellular matrix organization and cell differentiation (Fig. 3 *B*).

Some Hypertrophic Chondrocytes Synthesizing Type X Collagen Do Not Synthesize Type II Collagen

Hypertrophic chondrocytes derived from subgroup 1 clone R36, grown in suspension for 3 wk, were replated as adherent cells. After an additional week in culture, cells were double labeled with antibodies directed against type X and type II collagens. The cytoplasms of all the cells were positively stained by the antibodies against type X collagen. 200 type II collagen–positive cells were scored and compared to the total number of cells present in the same microscopic fields. The antibodies directed against type II collagen positively stained only ~50% of the cell population, indicating that some cells synthesized type X collagen in the absence of the type II. In Fig. 4, *A* and *B*, we show selected fields of double-labeled cells.

Discussion

We have previously described a cell culture system that allows the in vitro differentiation of hypertrophic chondrocytes starting from dedifferentiated cells (6). This culture system has proved to be very useful in elucidating several aspects of the differentiation process, including changes in the kinetic properties (16), modulation of the collagen gene expression (9), and the search for new differentiation markers (11). Investigations on the molecular mechanisms of cell differentiation could be better performed with a more homogeneous cell population. Cloned cell populations from primary cultures have already been used in other systems to answer questions of otherwise difficult interpretation (3, 15, 27). In the present study the availability of clones of prechondrogenic cells allowed us to assess that the phenomena observed during the "in vitro" differentiation were really due to changes in the differentiation state of the cells and not to a selection of a cell subpopulation.

Starting from primary cultures, clones of both differentiated and dedifferentiated cells were obtained. The possibility that some of the clones derived from undifferentiated peri-

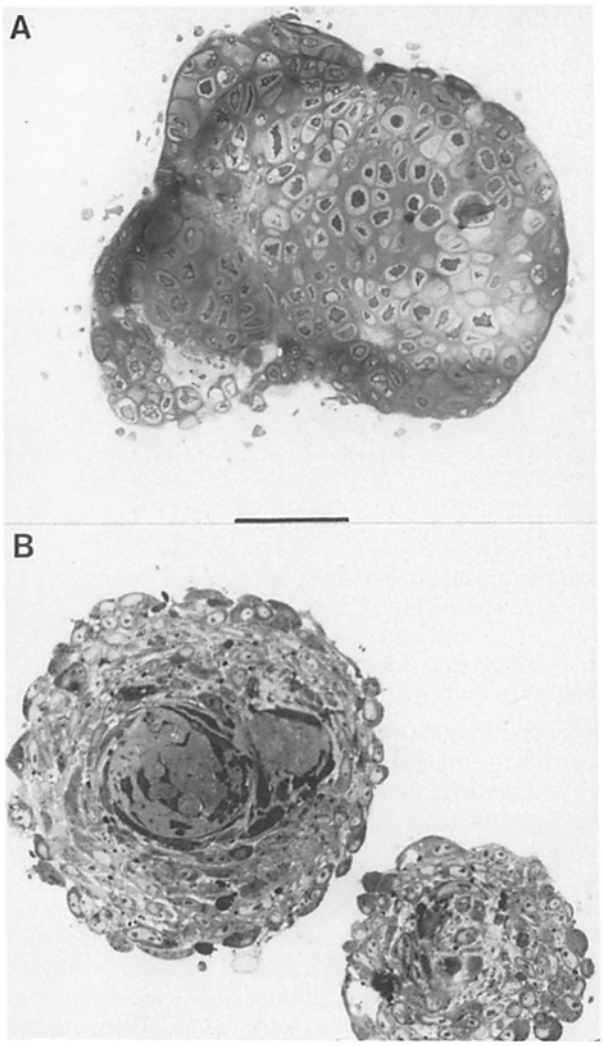

Figure 3. Sections of aggregates from cloned dedifferentiated cells cultured for 10 d in suspension in the presence of ascorbic acid. (*A*) Clone G3B6 (subgroup 1) shows an organized structure resembling hypertrophic cartilage; all the cells are contained in lacunae and an abundant extracellular matrix is deposited. No elongated cells are present at the periphery of the aggregate. (*B*) Clone R1 (subgroup 2) does not show any organization of the extracellular matrix and the cells in the aggregate present an elongated phenotype. Bars: (*A*) 136 μm; (*B*) 80 μm.

chondrial prechondrogenic cells must also be considered. We focused our attention only on clones formed by cells that presented a frank fibroblast-like morphology. Up to 45% of these clones (subgroup 1) underwent differentiation to hypertrophic chondrocytes when transferred into suspension culture. The general characteristics of the differentiation process were comparable, with minor differences, to the differentiation of the uncloned starting cell population. The percentage of clones with a chondrocyte phenotype was relatively high, compared to the almost homogeneous population of dedifferentiated cells obtained when the cells of the primary cultures are plated and passaged at higher densities. A possible explanation is the fact that when the primary cultures are plated at higher densities, the dedifferentiated cells, having a doubling time lower than differentiated cells (16), could be selected for during the culture. Furthermore, in culture, differentiated cells tend to detach from the culture dishes (5) and therefore could be lost during the changes of medium. It has to be taken into consideration that the origin of the nondifferentiating clones (subgroup 2) may be different. Since these cells in fact do not differentiate into hypertrophic chondrocytes in the standard conditions, at least some of them could be true fibroblast clones. On the other hand, in some of these clones (i.e., Table II, experiment II, clone R33) cultured attached as fibroblast-like cells, we found very low levels of mRNA for type II and type X collagen, but clearly over the mRNA signal found in an unrelated tissue (i.e., chicken liver). Therefore, we believe that at least some nondifferentiating clones belong to the chondrogenic lineage.

Here we have shown that the cloning efficiency is dramatically increased by culture medium conditioned by dedifferentiated cells, but not by culture medium conditioned by hypertrophic chondrocytes and mouse fibroblasts. Other authors (25) have already shown the presence in "cartilage-conditioned medium" of an activity enhancing the colony formation and the chondrocytic phenotype of stage 21–28 chick embryo limb bud cells. Although no formal proof can be given, it is tempting to postulate that dedifferentiated chondrocytes produce a factor capable of inducing and/or promoting their own growth.

When we compared the mRNA levels for type I, II, and X collagens with the synthetic rates of the corresponding proteins, a discrepancy was observed in the cloned cells at the beginning and at the end of the differentiation pathway. In dedifferentiated cells from subgroup 1 (differentiating) clones, evident levels of protein corresponded to high levels of type I collagen mRNA. After 2 wk of suspension culture, in these clones, an averaged 60% decrease of the type I collagen mRNA was accompanied by the complete disappearance of the corresponding protein, and a significant increase of type II and type X collagen mRNAs was paralleled by the synthesis of the two collagens. During the suspension culture, in the clones from subgroup 2 (nondifferentiating) the level of the type I collagen mRNA would decrease about one third, but variations in the levels of the type II and type X collagen mRNAs comparable to the ones of the other subgroup of clones were never observed. Still the cells synthesized only type I collagen. Our data, in agreement with findings of other groups (12, 14, 21, 23), might suggest a translational regulation of the type I collagen expression during the in vitro differentiation process of chondrocytes from dedifferentiated cells.

It has been reported that during differentiation of limb mesenchyme to cartilage, the cells switch from type I to type II collagen synthesis (31) and that rarely (<2%) a single cell expresses type I and type II collagen simultaneously (32). In this paper we have investigated the correlation between type II and type X collagen appearance and found that type X collagen is produced by hypertrophic cells whether or not type II collagen is expressed. Although the possibility that some cells are able to activate the synthesis of type X collagen without passing through a stage of type II collagen production cannot be ruled out, it seems to be at least unlikely given the evidence of Solursh et al. (26) on earlier stage chondrocytes. Our data, in fact, suggest that the appearance of type X collagen occurs in cells producing type II collagen, and that an arrest in the production of type II collagen takes place only at a later stage of differentiation.

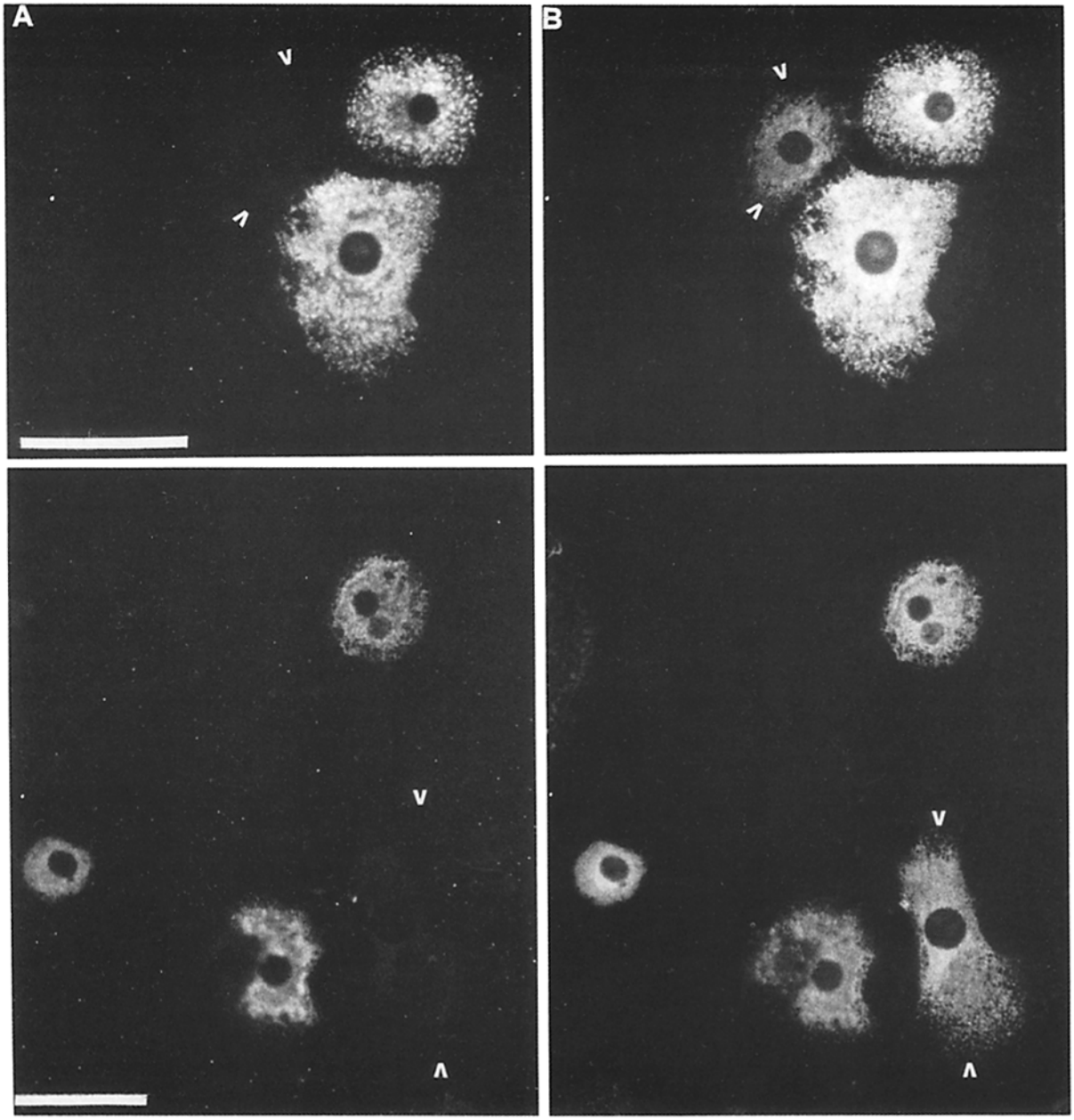

Figure 4. Double immunofluorescence showing type II (*A*) and type X (*B*) collagen intracellular staining. Cells from clone R36 (subgroup 1) were grown for 3 wk in suspension and replated as adherent cells 1 wk before staining. Arrowheads point to cells positive for type X collagen and negative for type II collagen. Bars, 10 μm.

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References

1. Ambesi-Impiombato, F. S., L. A. Parks, and H. G. Coon. 1980. Culture of hormone-dependent functional epithelial cells from rat thyroids. *Proc. Natl. Acad. Sci. USA.* 77:3455–3459.
2. Auffray, C., and F. Rougeon. 1980. Purification of mouse immunoglobulin heavy chain messenger RNAs from total myeloma tumor RNA. *Eur. J. Biochem.* 107:303–314.
3. Barrandon, Y., and H. Green. 1987. Three clonal types of keratinocytes with different capacities for multiplication. *Proc. Natl. Acad. Sci. USA.* 84:2302–2306.
4. Benya, P. D., and J. D. Shaffer. 1982. Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. *Cell.* 30:215–224.
5. Capasso, O., E. Gionti, G. Pontarelli, F. S. Ambesi-Impiombato, L. Nitsch, G. Tajana, and R. Cancedda. 1982. The culture of chick embryo chondrocytes and the control of their differentiated function in vitro. I. Characterization of the chondrocyte's specific phenotype. *Exp. Cell Res.* 142:197–206.
6. Capasso, O., G. Tajana, and R. Cancedda. 1984. Location of 64K collagen producer chondrocytes in developing chicken embryo tibiae. *Mol. Cell. Biol.* 4:1163–1168.
7. Castagnola, P., G. Moro, F. Descalzi-Cancedda, and R. Cancedda. 1986. Type X collagen synthesis during in vitro development of chick embryo

tibial chondrocytes. *J. Cell Biol.* 102:2310–2317.
8. Castagnola, P., G. Torella, and R. Cancedda. 1987. Type X collagen synthesis by cultured chondrocytes derived from permanent cartilaginous region of chicken embryo sternum. *Dev. Biol.* 123:332–337.
9. Castagnola, P., B. Dozin, G. Moro, and R. Cancedda. 1988. Changes in the expression of collagen genes show two stages in chondrocyte's differentiation in vitro. *J. Cell Biol.* 106:461–467.
10. Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry.* 18:5294–5299.
11. Descalzi Cancedda, F., P. Manduca, C. Tacchetti, P. Fossa, R. Quarto, and R. Cancedda. 1988. Developmentally regulated synthesis of a low molecular weight protein (Ch21) by differentiating chondrocytes. *J. Cell Biol.* 107:2455–2463.
12. Devlin, C. J., P. M. Brickell, E. R. Taylor, A. Hornbruch, R. K. Craig, and L. Wolpert. 1988. In situ hybridization reveals differential spatial distribution of mRNAs for type I and type II collagen in the chick limb bud. *Development (Camb.).* 103:111–118.
13. Dozin, B., J. E. Rall, and V. M. Nikodem. 1986. Tissue-specific control of rat malic enzyme activity and messenger RNA levels by a high carbohydrate diet. *Proc. Natl. Acad. Sci. USA.* 83:4705–4709.
14. Focht, R. J., and S. L. Adams. 1984. Tissue specificity of type I collagen gene expression is determined at both transcriptional and post-transcriptional levels. *Mol. Cell. Biol.* 4:1843–1852.
15. Furie, M. B., E. B. Cramer, B. L. Naprstek, and S. C. Silverstein. 1984. Cultured endothelial cell monolayers that restrict the transendothelial passage of macromolecules and electrical current. *J. Cell Biol.* 98:1033–1041.
16. Giaretti, W., G. Moro, R. Quarto, S. Bruno, A. Di Vinci, E. Geido, and R. Cancedda. 1988. Flow cytometric evaluation of cell cycle characteristics during in vitro differentiation of chick embryo chondrocytes. *Cytometry.* 9:281–290.
17. Hamburger, V., and H. L. Hamilton. 1951. A series of normal stages in the development of the chick embryo. *J. Morphol.* 88:49–92.
18. Hayashi, M., Y. Ninomiya, J. Parsons, K. Hayashi, B. R. Olsen, and R. L. Trelstad. 1986. Differential localization of mRNA of collagen types I and II in chick fibroblasts, chondrocytes, and corneal cells by in situ hybridization using cDNA probes. *J. Cell Biol.* 102:2302–2309.
19. Kielty, C. M., A. P. L. Kwan, D. F. Holmes, S. L. Schor, and M. E. Grant. 1985. Type X collagen, a product of hypertrophic chondrocytes. *Biochem. J.* 27:545–554.
20. Kosher, R. A. 1983. The chondroblast and the chondrocyte. *In* Cartilage. Vol. 1. B. K. Hall, editor. Academic Press Inc., New York. 59–85.
21. Kosher, R. A., W. M. Kulyk, and S. W. Gay. 1986. Collagen gene expression during limb bud cartilage differentiation. *J. Cell Biol.* 102:1151–1156.
22. Mauger, A., M. Demarchez, D. Herbage, J. A. Grimaud, M. Druguet, D. Hartmann, and P. Sengel. 1982. Immunofluorescent localization of collagen types I and III, and of fibronectin during feather morphogenesis in the chick embryo. *Dev. Biol.* 94:93–105.
23. Saxe, S. A., L. N. Lukens, and P. J. Pawlowski. 1985. Changes in the nuclear and cytoplasmic levels of type I and type II collagen RNAs during growth of chondrocytes in 5-Bromo-2′-deoxyuridine. *J. Biol. Chem.* 260: 3812–3819.
24. Schmid, T. M., and T. F. Linsenmayer. 1985. Developmental acquisition of type X collagen in the embryonic chick tibiotarsus. *Dev. Biol.* 107: 373–381.
25. Solursh, M., and R. S. Reiter. 1975. The enhancement of in vitro survival and chondrogenesis of limb bud cells by cartilage conditioned medium. *Dev. Biol.* 44:278–287.
26. Solursh, M., K. L. Jensen, R. S. Reiter, T. M. Schmid, and T. F. Linsenmayer. 1986. Environmental regulation of type X collagen production by cultures of limb mesenchyme, mesectoderm, and sternal chondrocytes. *Dev. Biol.* 117:90–101.
27. Streeten, E. A., R. Ornberg, F. Curcio, K. Sakaguchi, S. Marx, G. D. Aurbach, and M. L. Brandi. 1989. Cloned endothelial cells from fetal bovine bone. *Proc. Natl. Acad. Sci. USA.* 86:916–920.
28. Tacchetti, C., R. Quarto, L. Nitsch, D. J. Hartmann, and R. Cancedda. 1987. In vitro morphogenesis of chick embryo hypertrophic cartilage. *J. Cell Biol.* 105:999–1006.
29. Tacchetti, C., R. Quarto, G. Campanile, and R. Cancedda. 1989. Calcification of in vitro-developed hypertrophic cartilage. *Dev. Biol.* 132:442–447.
30. Thomas, P. S. 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc. Natl. Acad. Sci. USA.* 77: 5201–5205.
31. Von der Mark, H., K. Von der Mark, and S. Gay. 1976. Study of differential collagen synthesis during development of the chick embryo by immunofluorescence. I. Preparation of collagen type I and type II antibodies and their applications to early stages of the chick embryo. *Dev. Biol.* 48:237–249.
32. Von der Mark, K., V. Gauss, H. von der Mark, and P. Muller. 1977. Relationship between cell shape and type of collagen synthesized as chondrocytes lose their cartilage phenotype in culture. *Nature (Lond.).* 267: 531–532.
33. Yamamoto, T., M. E. Sobel, S. L. Adams, V. E. Avvedimento, R. Di Lauro, I. Pastan, B. de Crombrugghe, A. Showalter, D. Pesciotta, P. Fietzek, and B. R. Olsen. 1980. Construction of a recombinant bacterial plasmid containing pro-α1(I) collagen DNA sequences. *J. Biol. Chem.* 255:2612–2615.
34. Young, M. F., G. Vogeli, A. M. Nunez, M. P. Fernandez, M. Sullivan, and M. Sobel. 1984. Isolation of a cDNA and genomic DNA clones encoding type II collagen. *Nucleic Acids Res.* 12:4207–4228.