Development 117, 245-251 (1993)
Printed in Great Britain © The Company of Biologists Limited 1993

Type VI collagen expression is upregulated in the early events of chondrocyte differentiation

Rodolfo Quarto[1], Beatrice Dozin[1], Paolo Bonaldo[2], Ranieri Cancedda[1] and Alfonso Colombatti[3,4,*]

[1]Laboratorio Differenziamento Cellulare, Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy
[2]Istituto di Istologia ed Embriologia, Universitá di Padova, Padova, Italy
[3]Divisione di Oncologia Sperimentale 2, Centro di Riferimento Oncologico, 33081 Aviano, Italy
[4]Dipartimento di Scienze e Tecnologie Biomediche, Universitá di Udine, Udine, Italy

*Corresponding author at Aviano

SUMMARY

Dedifferentiated chondrocytes cultured adherent to the substratum proliferate and synthesize large amounts of type I collagen but when transferred to suspension culture they decrease proliferation, resume the chondrogenic phenotype and the synthesis of type II collagen, and continue their maturation to hypertrophic chondrocyte (Castagnola et al., 1986, *J.Cell Biol.* 102, 2310-2317). In this report, we describe the developmentally regulated expression of type VI collagen in vitro in differentiating avian chondrocytes. Type VI collagen mRNA is barely detectable in dedifferentiated chondrocytes as long as the attachment to the substratum is maintained, but increases very rapidly upon passage of the cells into suspension culture reaching a peak after 48 hours and declining after 5-6 days of suspension culture. The first evidence of a rise in the mRNA steady-state levels is obtained already at 6 hours for the α3(VI) chain. Immunoprecipitation of metabolically labeled cells with type VI collagen antibodies reveals that the early mRNA rise is paralleled by an increased secretion of type VI collagen in cell media. Induction of type VI collagen is not the consequence of trypsin treatment of dedifferentiated cells since exposure to the actin-disrupting drug cytochalasin or detachment of the cells by mechanical procedures has similar effects. In 13-day-old chicken embryo tibiae, where the full spectrum of the chondrogenic differentiation process is represented, expression of type VI collagen is restricted to the articular cartilage where chondrocytes developmental stage is comparable to stage I (high levels of type II collagen expression). The steep rise in type VI collagen synthesis suggests that the transition of chondrocytes from a stage characterized by high levels of type I collagen to a later stage with a predominance of type II collagen (stage I chondrocytes) might be subdivided further in an early phase (stage Ia) characterized by a high and transient type VI collagen expression and a later phase (stage Ib) where type II collagen is predominant. These data might suggest a functional relationship between type VI collagen expression and the chondrogenic maturation process.

Key words: chondrocyte, differentiation, collagen type VI, in vitro

INTRODUCTION

In avian hypertrophic cartilage, the maturation pathway from mesenchymal prechondrogenic cells to fully differentiated hypertrophic chondrocytes proceeds through differentiation stages that can be defined by biochemical markers and several other parameters such as cell volume, cellular phenotype and growth kinetics. The chondrocyte differentiation process is divided into three schematic cellular compartments: (a) pre-chondrogenic mesenchymal cells, which are found first in the limb bud and later in the perichondrium (Kosher, 1983); these cells synthesize type I collagen, fibronectin and possibly basal levels of type II collagen; (b) stage I (proliferating) chondrocytes, which produce large amounts of type II collagen and cartilage-specific proteoglycans (Kosher, 1983; Hayashi et al., 1986; von der Mark et al., 1976); (c) stage II (hypertrophic) chondrocytes characterized by the production of type X collagen localized in hypertrophic calcifying cartilage (Capasso et al., 1984; Kielty et al., 1985; Schmid and Linsenmayer, 1985).

Chicken embryo chondrogenic cells maintained in vitro can differentiate and reproduce the main steps of hypertrophic cartilage maturation (Castagnola et al., 1986). Freshly dissociated chicken chondrocytes plated on plastic dishes dedifferentiate, stop type II and activate type I collagen synthesis. When transferred into suspension cultures, these cells readily aggregate and within few days cease synthesizing type I collagen while type II collagen secretion increases in the following days. After two weeks of sus-

pension culture virtually all cell aggregates loosen and release single hypertrophic chondrocytes. Cells at this stage synthesize high amounts of type II and type X collagens. Although the temporal changes in collagen levels in the cell culture system described above are settled, still few informations are available on the collagen genes expression during the early events of in vitro differentiating chondrocyte (Tacchetti et al., 1992). Therefore, we investigated the early events in terms of collagens expression and in particular of type VI. This collagen has some distinctive structural characteristics such as a short triple helix and individual chains of quite different M_r: 140,000 (α1 and α2) and >300,000 (α3) (Colombatti et al., 1987). Accordingly, the mRNAs for the different chains migrate at about 4.3 (α1 and α2) and 10.0 (α3) kb (Bonaldo et al., 1989, 1990; Koller et al., 1989). Type VI collagen is a component of 100 nm-long periodic microfilaments that have a widespread distribution in connective tissues (Timpl and Engel, 1987). Among the minor collagens of cartilage (types V, VI, IX, X and XI), type VI has received little attention although some recent reports have demonstrated immune reactivity with polyclonal antibodies to type VI collagen in bovine, canine and human articular cartilage (Ayad et al., 1984, 1989; Keene et al., 1988; Poole et al., 1988; Ronziere et al., 1990).

In this study, we provide evidence for a very early and transient upregulated expression of type VI collagen in in vitro differentiating avian chondrocytes. These results suggest that there might be an intermediate early stage in the developmental pathway of the chondrogenic lineage characterized by high expression of type VI collagen.

MATERIALS AND METHODS

Cell culture

Primary cultures and suspension cultures of dedifferentiated cells were performed from stage 28-30 (Hamburger and Hamilton, 1951) chick embryo tibiae as described previously (Castagnola et al., 1986). In brief, tibiae were isolated from embryos and subjected to enzymatic treatment with trypsin and collagenase, in order to remove perichondral tissues. Rudiments were then transferred to a fresh solution of trypsin/collagenase mixture and a suspension of single chondrocytes was recovered. Cells were maintained in Coon's Modified Ham F-12 medium (Ambesi-Impiombato et al., 1980) supplemented with 10% Fetal Calf Serum (FCS) (Flow Laboratories, Irvine, Scotland). Only dedifferentiated cultures containing a low percentage (5-6%) of contaminating differentiated chondrocytes were used as starting cell population for the present studies.

mRNA quantitation

The guanidinium isothiocyanate/CsCl method of Chirgwin et al. (1979) was used to extract total cellular RNA from chondrocytes.

Electrophoresis of the RNA was performed on 0.7% (w/v) agarose gel containing 2.3 M formaldehyde in 3-N-morpholinopropane sulfonic buffer for 8 hours at 150 V using 20 cm plates (Sambrook et al., 1989). RNA was then transferred onto nitrocellulose filters and hybridized with [α-^{32}P]dCTP (3000 Ci/mmol, Amersham International, Amersham UK) labeled cDNA probes at 68°C overnight in 5× NaCl/phosphate/EDTA (10 mM phosphate, 180 mM NaCl, 1 mM EDTA, pH 7.7) (SSPE) containing 5× Denhardt's solution, 50% (v/v) formamide, 0.5% (w/v) sodium dodecyl sulfate (SDS), and 100 μg/ml salmon sperm DNA. After washing in 2× SSPE for 5 minutes at room temperature, in 1× SSPE plus 1% (w/v) in SDS for 30 minutes at 65°C, and in 0.1× SSPE for 30 minutes at room temperature, the filters were exposed to β-max Hyperfilms (Amersham).

All values were corrected for the amount of RNA blotted as estimated by hybridization with a DNA probe for ribosomal RNA (rDNA). The cDNAs for chicken type I (α1), II (α1), X (α1), VI (α1,α2,α3) collagens used in the hybridization assays have been described elsewhere (Bonaldo et al., 1989, 1990; Koller et al., 1989; Castagnola et al., 1988). The genomic clone pXCR7 (*Hin*dIII-*Hin*dIII fragment of *Xenopus laevis* rDNA) was a gift from F Amaldi (Universitá Tor Vergata, Roma, Italy).

Metabolic labeling, immunoprecipitation and SDS-PAGE analysis

Dedifferentiated adherent chondrocytes and cells trypsinized and maintained in suspension culture in complete medium supplemented with 10% FCS, for 1, 2, 3, 7 or 14 days were metabolically labeled in methionine-free Dulbecco's modified minimum essential medium (Flow) containing 1% dialyzed FCS. Labeling was carried out for 4 hours with [^{35}S]methionine (800 Ci/mmol, Amersham) at 50-100 μCi/ml and in the presence of 50 μg/ml ascorbic acid. At the end of incubation, the medium was collected and brought to extraction buffer with the following final concentration: 20 mM Tris-HCl, pH 7.6, 100 mM NaCl, 1% (w/v) SDS, 25 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 5 mM N-ethylmaleimide, 1 mM paraaminobenzamidine. The media were clarified by centrifugation at 10,000 ***g*** and used for immunoprecipitation. 2 μl of rabbit polyclonal antiserum against the pepsin form of chicken type VI collagen (Colombatti et al., 1987) and 50 μl of Sepharose-Protein A were added, and the incubation continued for 4-6 hours. After extensive washing, the precipitates were dissolved in Laemmli sample buffer in the presence 5% (v/v) 2-mercaptoethanol. Immunoprecipitated material was resolved by SDS-PAGE on a 6% (w/v) polyacrylamide slab gel using the buffer system of Laemmli (1973).

Immunofluorescence

8 μm cryostat sections were cut from 13-day-old embryo chicken long bones and mounted on polylysine-coated glass slides.

Sections were pretreated for 30 minutes at 37°C with 1 mg/ml of testicular hyaluronidase (type 1S, Sigma Chemical Company, IL) and then incubated with a rabbit polyclonal antiserum against the pepsin form of chicken type VI collagen followed by rhodamine-conjugated goat anti-rabbit. Sections were mounted in glycerol and viewed with a Leitz microscope equipped for epifluorescence.

RESULTS

Early transient rise of type VI collagen mRNA levels during in vitro chondrocyte differentiation

The steady state levels of mRNA for the α1, α2 and α3 chains of type VI collagen were measured by Northern blot hybridization in chondrocytes grown in vitro. RNA was prepared from adherent dedifferentiated cells or from cells maintained in suspension for different lengths of time during the induction of the differentiation process.

Very little type VI collagen mRNA was detected in adherent cells. At day 3 after detachment, however, the levels of mRNA for all three chains of type VI collagen were strongly increased, and returned to nearly basal levels after 14 days (data not shown).

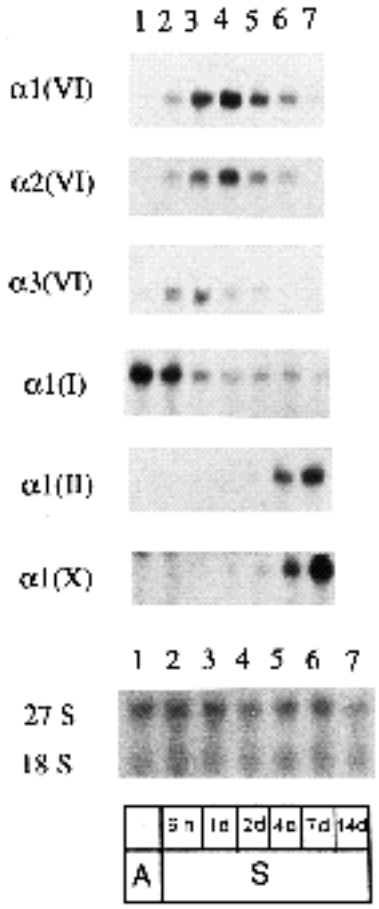

Fig. 1. Autoradiogram of a northern blot of total RNAs extracted from cultured chondrocytes maintained adherent (A, lane 1) or in suspension for different lengths of time (S, lanes 2-7) as indicated. The filters were hybridized with α-^{32}P-labeled cDNA probes specific for the different collagens chains or for ribosomal RNA. Only the relevant part of the autoradiograms are shown. Exposure times of the filters hybridized with the α1(VI), α2(VI) and α3(VI) probes were identical.

In a second series of experiments, we investigated in more detail the temporal changes in the expression of type VI collagen mRNA during the early phases of in vitro chondrocyte differentiation. As can be seen in Fig. 1, the levels of mRNA for the α1(VI) and α2(VI) chains increased between 6 and 48 hours. The values at 96 hours were already lower and decreased further after 7 and 14 days. Instead, expression of mRNA for the α3 (VI) chain was well evident at 6 hours,before any indication of differentiation of the mesenchymal cells, and reached its peak value already at 24 hours to decrease substantially at 96 hours.

Specific mRNA levels were quantitated by densitometric scanning of autoradiographic films of northern blots. The relative mRNA level of the three chains were in the range of 4- to 6-fold after 6 hours; those of α1(VI) mRNA increased up to about 25-fold after 48 hours (peak value), whereas the increase of α2(VI) mRNA was less dramatic since its highest values at 48 hours were about 15-fold above those detectable in adherent cells; the α3(VI) mRNA level increased up to 6-fold at 24 hours (peak value) with respect to adherent cells.

The expression of mRNA for the α1 chains of collagen types I, II and X was measured for comparison. mRNA for the α1(I) chain decreased substantially at 24 hours and remained nearly constant throughout. In accord with previous observations in the same experimental system (Castagnola et al., 1988), mRNAs for both α1(II)and α1(X) chains were detectable at day 4 and increased, especially for α1(X) up to day 14. Hybridization with a probe for rDNA showed that the RNA present in the different samples were equivalent confirming the significance of the progressive increase and the subsequent decrease of the steady-state levels of type VI collagen mRNA in the early stages of chondrocyte differentiation.

Secretion of type VI collagen during the initial stages of in vitro chondrocyte differentiation

To investigate whether the rapid increase of type VI collagen mRNA was accompanied by the synthesis and secretion of corresponding polypeptides, adherent dedifferentiated and suspension-cultured chondrocytes were metabolically labeled with [^{35}S]methionine for 4 hours in the presence of ascorbic acid.

Adherent cells expressed a very low amount of type VI collagen (Fig. 2, lane 1). After 24 hours of suspension culture, the levels had increased reaching their peak at 72 hours and remained high up to day 7 to decrease at day 14 (Fig. 2, lanes 2-5). Therefore, the secretion of type VI collagen seems to be temporally up-regulated during in vitro chondrocytes differentiation in agreement with the levels of expression of the mRNAs for the different chains. On the basis of the intensity of the different bands, it seems that the α1(VI) chain undergoes the most pronounced increase. To exclude the possibility that the changes in type VI collagen expression depended only on the trypsin treatment, which has been shown to result in the upregulation of osteopontin mRNA steady state levels in similar cell cultures (Castagnola et al., 1991), we performed an immunoprecipitation from spent media of adherent dedifferentiated chondrocytes labeled after treatment for 48 hours with 2 μg/ml of the actin-disrupting drug cytochalasin D. This drug is known to affect the cell shape of adherent prechondrogenic cells (Fig. 3C) and to induce chondrogenic differentiation (Zanetti and Solursh, 1984).

Also this treatment increased the expression of type VI collagen at 48 hours (Fig. 4, lane 3) compared to adherent cells (Fig. 3A and Fig. 4, lane 1). Maintaining the cells for the same length of time in the solvent, DMSO, had no inducing effect on cell shape (Fig. 3B) and on type VI collagen expression (Fig. 4, lane 4). In this experiment, the basal levels of type VI collagen expression were higher than those detected above (Fig. 2). An induction of type VI collagen also was obtained when cells were transferred into suspension culture after mechanical detachment from the plastic culture dishes (data not shown).

Expression of type VI collagen in vivo

The above results suggest that during the process of in vitro chondrocyte differentiation a rapid rise of type VI collagen occurs at the very beginning of the chondrogenic process

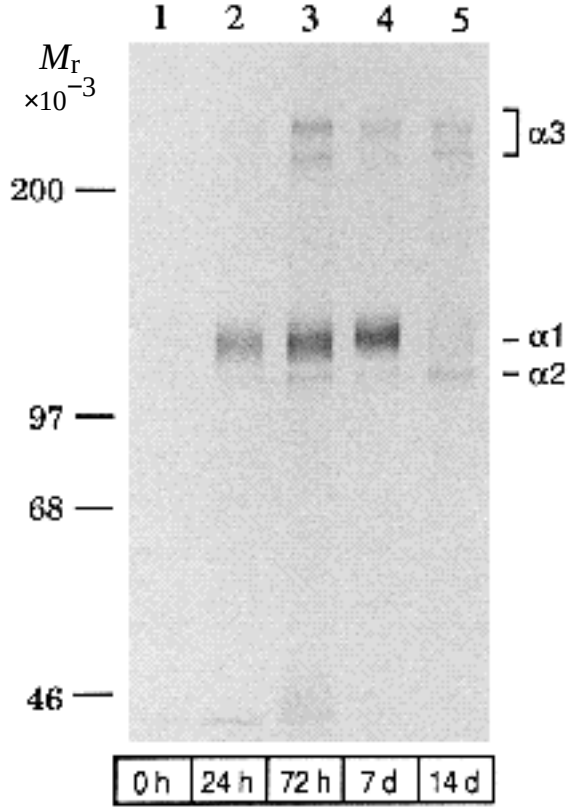

Fig. 2. Fluorogram of an SDS-PAGE analysis of type VI collagen immunoprecipitated from chicken chondrocytes. Adherent dedifferentiated chondrocytes were metabolically labeled in the presence of 50 μg/ml of ascorbic acid with 80 μCi/ml of [^{35}S]methionine for 4 hours. Trypsinized cells were maintained in suspension cultures for different times as indicated at the bottom and similarly labeled for 4 hours. At the end of the labeling, media were collected, immunoprecipitated with polyclonal antiserum to chicken type VI collagen and analyzed by electrophoresis in a 6% (v/v) polyacrylamide gel under reducing conditions. The migration of the different chains of type VI collagen is shown on the right and the migration of molecular weight standard markers is indicated on the left.

(stage I chondrocytes). Articular cartilage chondrocytes correspond to stage I chondrocytes, which are characterized by the production of type II collagen. Cryostat sections of 13-day-old embryo tibiae were examined for the presence of type VI collagen by immunofluorescence (Fig. 5). A very intense and defined staining with type VI collagen polyclonal antibody is present along the entire periarticular region. On the contrary, the deeper cartilage tissue is negative except for a very weak signal adjacent to the chondrocyte pericellular capsule.

DISCUSSION

The results of the present study indicate that the expression of type VI collagen is initiated immediately after in vitro dedifferentiated chicken embryo tibia cells are transferred into suspension culture and before the overt synthesis of cartilage-specific markers.

The increase and decrease of mRNA levels for the three type VI collagen chains are very peculiar and identify an early stage of chondrocyte differentiation (stage Ia) characterized by a rapid rise and fall of type VI collagen mRNA levels followed by a stage characterized by activation of the transcription of cartilage-specific collagen genes and synthesis and secretion of their products (stage Ib, Fig. 6). It is not known whether the changes detected in type VI collagen expression are causally involved in initiating chon-

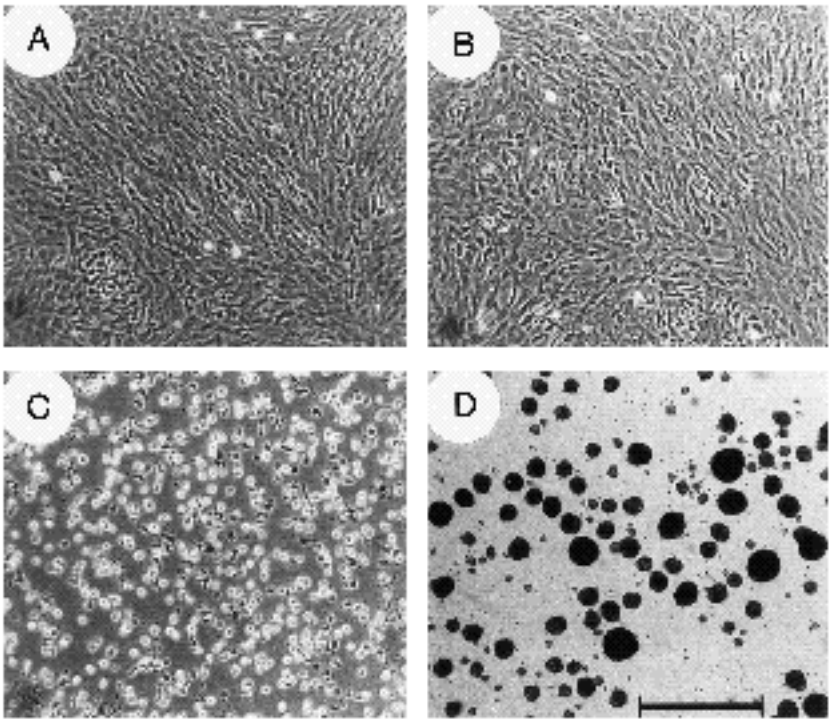

Fig. 3. Phase-contrast micrographs of chick embryo tibiae cultured chondrocytes maintained adherent for three weeks (A), in the presence of 0.02% DMSO (B) or 2 μg/ml of cytochalasin D in 0.02% DMSO for 48 hours (C) and in suspension for 48 hours (D). Bar, 150 μm.

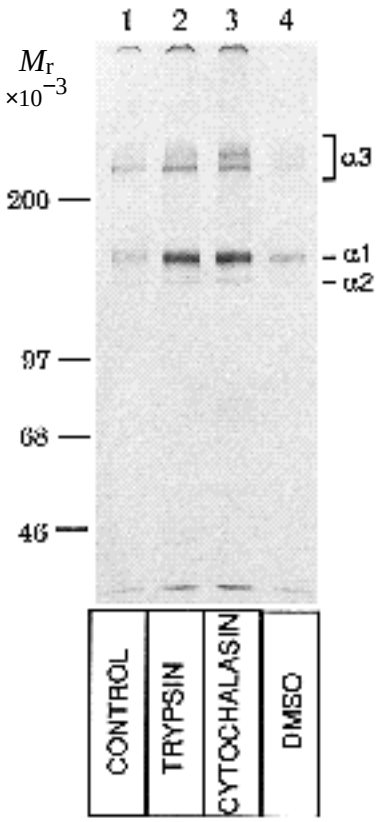

Fig. 4. Fluorogram of an SDS-PAGE analysis of type VI collagen immunoprecipitated form chicken chondrocytes. Untreated adherent dedifferentiated chondrocytes (lane 1), treated for 48 hours with 2 μg/ml of cytochalasin D in 0.02% DMSO (lane 3), or treated for 48 hours with 0.02% DMSO (lane 4) were metabolically labeled with 75 μCi/ml of [^{35}S]methionine for 4 hours in the presence of 50 μg/ml of ascorbic acid. In lane 2, dedifferentiated chondrocytes were trypsinized, maintained in suspension cultures for 48 hours and similarly labeled. At the end of the labeling, the media were collected, immunoprecipitated with polyclonal antiserum to chicken type VI collagen and analyzed by electrophoresis in a 6% (v/v) polyacrylamide gel under reducing conditions. The migration of the different chains of type VI collagen is shown on the right and the migration of molecular weight standard markers is indicated an the left.

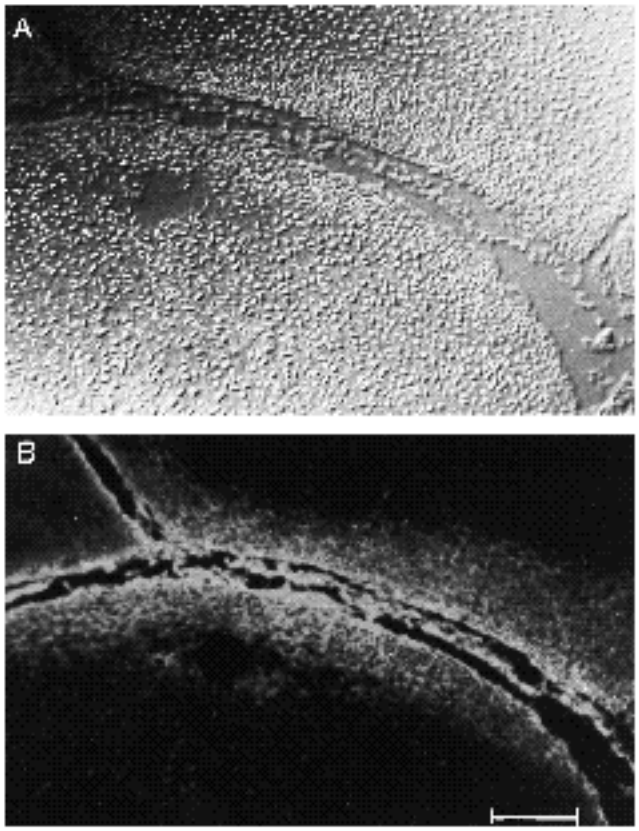

Fig. 5. Type VI collagen localization by indirect immunofluorescence on 13-day-old chicken embryo tibiae. Cryostat sections were viewed with Nomarski optics (A) or after incubation with a rabbit polyclonal antiserum to chicken type VI collagen followed by rhodamine-conjugated goat rabbit antiserum (B). Strong staining is prevailing in the extracellular matrix of periarticular cartilage. Bar, 100 μm.

drocyte differentiation and have a critical importance in the further regulation of cartilage-specific gene activity by known regulatory factors such as cAMP (Kosher, 1983; Rodgers et al., 1989), TGF-beta (Kulyk et al., 1989) and other factors diffusing through gap junctions (Coehlo and Kosher, 1991) that have been shown to play important roles in this process. Alternatively, the early expression of type VI collagen might be incidental and unrelated to the process of differentiation.

The presence of specific mRNA transcripts in cells that do not synthesize the corresponding protein has been observed previously. For example, α2 (I) collagen mRNA transcripts have been identified in vertebral chondrocytes in the absence of detectable amounts of the protein (Focht and Adams, 1984). This is not the case with type VI collagen, which is readily synthesized and secreted by suspension

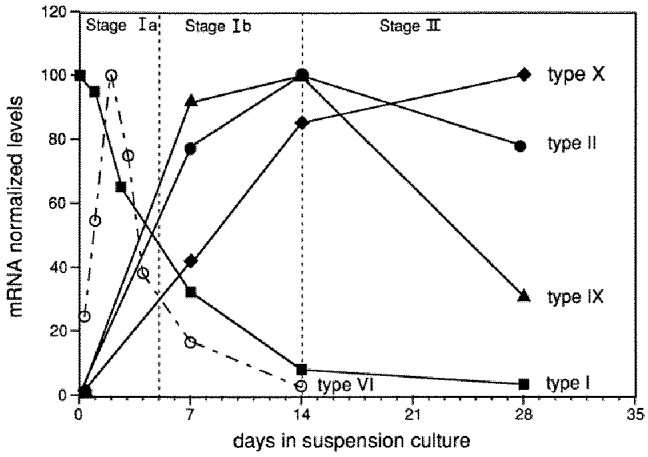

Fig. 6. Levels of mRNAs for collagen types I, II, VI, IX and X in dedifferentiated chondrocytes (time 0) and at different times after transfer to suspension culture. The values reported are from the quantitation of northern blots of Fig. 1 and from Castagnola et al. (1988). 100% expression corresponds to the highest relative values reached by the different collagen mRNAs. Stage Ia defines a stage characterized by expression of type VI collagen before the rise of cartilage-specific collagens.

cultures of differentiating chondrocytes. Strong positivity for type VI collagen is restricted to the articular cartilage of 13-day-old (this report) or younger (A. Colombatti et al., in preparation) embryo tibiae since little, or no, type VI collagen is detectable in the remaining areas of the cartilage. The articular cartilage corresponds to areas of poor proliferation and initiation of chondrogenesis and this suggests that type VI collagen may be fullfilling some as yet undefined functions in promoting chondrogenesis rather than being involved in maintaining the cartilage phenotype. The rise in the expression of type VI collagen is transient and the reason for its disappearance in cultured chondrocytes is not known. The contradiction between the transient high expression in vitro and the continuous expression in the articular cartilage is only apparent: in fact, during the in vitro differentiation process chondrocytes undergo rapid and dramatic changes from a dedifferentiated phenotype to the full expression of the hypertrophic cartilage, whereas articular chondrocytes in vivo are in a resting state (stage I).

It is worth mentioning that *v-myc*-infected quail chondrocytes, which represent a long-term cell culture system constituted by a cell population with a phenotype typical of stage I immature chondrocytes but unable to progress further to stage II hypertrophic chondrocytes (Quarto et al., 1992), express constitutively high levels of type VI collagen both in adherent and in suspension cultures (Quarto et al., unpublished).

The dramatic increase within few hours and the subsequent rapid fall of the mRNA levels for the three type VI collagen chains suggest a strong transcriptional control. It was recently found that the promoter regions of chicken α2(VI) (Koller et al., 1991) and murine α1(VI) (P. Bonaldo et al., unpublished data) genes are quite peculiar among collagen genes and it is very likely that they are regulated in a different manner compared to the promoters of the other collagen genes. Furthermore, given the slightly different kinetics of α3(VI) mRNA versus α1(VI) and α2(VI) mRNA, it will be of interest to determine whether a common set of transcription factors or unique regulatory elements are involved in the rapid changes in the expression of the three type VI collagen genes that occur before the onset of overt chondrogenesis.

We thank Maria Teresa Mucignat for her knowledgeable help with Northern blotting and Dr B. Trüeb, ETH Zurich, for providing chicken α2(VI) cDNA clones. This work was supported by grants from 'Progetti Finalizzati: Ingegneria Genetica and Applicazioni Cliniche della Ricerca oncologica' CNR Rome and by the Associazione Italiana per la Ricerca sul Cancro.

REFERENCES

Ambesi-Impiombato, F. S., Parks, L. A. and Coon, H. G. (1980). Culture of hormone-dependent functional epithelial cells from rat thyroids. *Proc. Natl. Acad. Sci. USA* **77,** 3455-3459.

Ayad, S., Evans, H., Weiss, J. B. and Holt, L. (1984). Type VI collagen but not type V collagen is present in cartilage. *Coll. Rel. Res.* **4,** 165-168.

Ayad, S., Marriott, A., Morgan, K. and Grant, M. E. (1989). Bovine cartilage types VI and IX collagens. *Biochem. J.* **262,** 753-761.

Bonaldo, P., Russo, V., Bucciotti F., Doliana R. and Colombatti, A. (1989). α1 chain of chick type VI collagen: the complete cDNA sequence reveals a hybrid molecule made of one short collagen and three von Willebrand factor type A-like domains. *J. Biol. Chem.* **264,** 5575-5580.

Bonaldo, P., Russo V., Bucciotti, F., Doliana, R. and Colombatti, A. (1990). Structural and functional features of the chicken α3 chain indicate a bridging role for collagen VI in connective tissues. *Biochemistry* **29,** 1245-1254.

Capasso, O., Tajana, G. and Cancedda R. (1984). Location of 64K collagen producer chondrocytes in developing chicken embryo tibiae. *Mol. Cell Biol.* **4**, 1163-1168.

Castagnola, P., Bet, P., Quarto, R., Gennari, M. and Cancedda, R. (1991). cDNA cloning and gene expression of chicken osteopontin. *J. Biol. Chem.* **266,** 9944-9949.

Castagnola, P., Dozin, B., Moro, G. and Cancedda R. (1988). Changes in the expression of collagen genes show two stages in chondrocyte differentiation in vitro. *J. Cell Biol.* **106,** 461-467.

Castagnola, P., Moro, G., Descalzi-Cancedda, F. and Cancedda R. (1986). Type X collagen synthesis during in vitro development of chick embryo tibial chondrocytes. *J. CellBiol.* **102,** 2310-2317.

Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. and Rutter, W. J. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**, 5294-5299.

Coelho, C. N. D. and Kosher, R. A. (1991). Gap junctional communication during limb cartilage differentiation. *Dev. Biol.* **144,** 47-53.

Colombatti, A., Bonaldo, P., Ainger, K., Bressan, G. M. and Volpin, D. (1987). Biosynthesis of chick type VI collagen. (I). Intracellular assembly and molecular structure. *J. Biol. Chem.* **262,** 14454-14460.

Focht, R. J. and Adams, S. L. (1984). Tissue specificity of type I collagen gene expression is determined at both transcriptional and post-transcriptional levels. *Mol. Cell. Biol.* **4**, 1843-1852.

Hamburger, V. and Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morph.* **88,** 49-92.

Hayashi, M., Ninomiya, Y., Parsons, J., Hayashi, K., Olsen, B. R. and Trelstad, R. (1986). Differential localization of mRNAs 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.

Keene, D. R., Engvall, E. and Glanville, R. W. (1988). Ultrastructure of type VI collagen in human skin and cartilage suggests an anchoring function for this filamentous network. *J. Cell Biol.* **107,** 1995-2006.

Kielty, C. M., Kwan, A. P. L., Holmes, D. F. Schor, S. L. and Grant, M. E. (1985). Type X collagen, a product of hypertrophic chondrocytes. *Biochem. J.* **27,** 545-554.

Koller, E., Hayman, A. R. and Trüeb, B. (1991). The promoter of the chicken α2(VI) collagen gene has features characteristic of housekeeping genes and of proto-oncogenes. *Nucleic Acids Res.* **19,** 485-491.

Koller, E., Winterhalter, K. H. and Trüeb, B. (1989). The globular domains of type VI collagen are related to the collagen-binding domains of cartilage matrix protein and von Willebrand factor. *EMBO J.* **8**, 1037-1077.

Kosher, R. A. (1983). The chondroblast and the chondrocyte. In *Cartilage*. Vol I (ed. B. K. Hall), pp. 59-85. New York: Academic Press.

Kulyk, W. M., Rodgers, B. J., Greer, K. and Kosher, R. A. (1989). Promotion of embryonic chick limb cartilage differentiation by transforming growth factor-β. *Dev. Biol.* **135,** 424-430.

Laemmli, U. K. (1973). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Poole, C. A., Ayad, S. and Schofield, J. R. (1988). Chondrons from articular cartilage. (I) Immunolocalization of type VI collagen in the pericellular capsule of isolated canine tibial chondrons. *J. Cell Sci.* **90,** 635-643.

Quarto, R., Dozin, B., Tacchetti, C., Robino, G., Zenke, M., Campanile, G. and Cancedda R. (1992). Constitutive myc expressions impairs hypertrophy and calcification in cartilage. *Dev. Biol.* **149,** 168-176.

Rodgers, B. J., Kulyk, W. M. and Hosher, R. A. (1989). Stimulation of limb cartilage differentiation by cyclic AMP is dependent on cell density. *Cell Differ. Development* **28**, 179-188.

Ronziere, M-C., Richard-Blum, S., Tiollier, J., Hartmann, D. J., Garrone, R. and Herbage, D. (1990). Comparative analysis of collagens solubilized from human foetal, and normal and osteoarthritic adult articular cartilage, with emphasis on type VI collagen. *Biochem. Biophys. Acta* **1038**, 222-230.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). In *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Schmid, T. M. and Linsenmayer, F. (1985). Developmental acquisition of

type X collagen in the embryonic chick tibiotarsus. *Dev. Biol.* **107,** 373-381.

Tacchetti, C., Tavella, S., Dozin, B., Quarto, R., Robino, G. and Cancedda, R. (1992). Cell condensation in chondrogenic differentiation. *Exp. Cell Res.* **200,** 26-33.

Timpl, R. and Engel, J. (1987). Type VI collagen. In *Structure and Function of Collagen Types*, Vol. III (ed. R. Mayne, R. E. Burgeson and R. P. Mecham), pp. 105-143. New York: Academic Press.

von der Mark, H., von der Mark, K. and Gay, S. (1976). Study of differential collagen synthesis during development of the chick embryo by immunofluorescence. (II) Localization of type I and type II collagen during long bone development. *Dev. Biol.* **53,** 153-170.

Zanetti, N. C. and Solursh, M. (1984). Induction of chondrogenesis by limb mesenchymal cultures by disruption of the actin cytoskeleton. *J. Cell Biol.* **99,** 115-123.

(Accepted 30 September 1992)