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Expression of type X collagen is transiently stimulated in redifferentiating chondrocytes pretreated with retinoic acid

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Growth of quail chrondrocytes in the presence of retinoic acid (RA) results in the suppression of the differentiated phenotype. RA-treated chondrocytes recover their differentiated phenotype if they are cultured for an additional 15 days in the absence of RA. A few days after removal from RA, treated chondrocytes acquire the polygonal morphology characteristic of chondrocytes growing as attached cells; they also gradually resume collagen II expression and synthesize a significantly higher proportion of collagen X mRNA than do untreated control cultures. The levels of collagen X mRNA decrease during the second week of culture in the absence of RA. Finally, at the end of 15 days, the absolute levels of collagen II and collagen X mRNAs are very similar in control and recovering chondrocytes.

INTRODUCTION

Retinoids are a group of compounds including retinoic acid (RA), retinol (vitamin A) and a series of natural and synthetic derivatives that together exert profound effects on development and differentiation in a wide variety of systems (Lotan, 1980; Roberts & Sporn, 1984; Chytil & Ong, 1984). Intracellular receptors for RA belong to the nuclear receptor superfamily, which includes receptors for steroid hormones and thyroid hormones (Petkovich *et al.*, 1987; Giguère *et al.*, 1987; Brand *et al.*, 1988; Benbrook *et al.*, 1988; Zelent *et al.*, 1989).

RA is known to have dramatic effects on the pattern of vertebrate limb development and regeneration, and a model has been proposed in which a gradient of RA serves as a morphogen to differentially supply positional information to a developing limb (Thickle *et al.*, 1982; Thaller & Eichele, 1987; Summerbell & Maden, 1990). Ide & Aono (1988) reported that low concentrations (100 nM) of RA promoted proliferation and chondrogenesis of the distal mesodermal cells from chick limb buds. Moreover, Paulsen *et al.* (1988) showed that physiological concentrations of RA (15 nM) selectively enhanced chondrogenesis in serum-free cultures of limb-bud mesenchymal cells.

Fully differentiated chondrocytes are characterized by the synthesis and the deposition of an extracellular matrix composed of cartilage-specific collagens and proteoglycans. Collagen II is referred to as the 'major' collagen, since it represents 80–90% of the collagens in cartilage (Mayne & Burgeson, 1987). Chondrocytes also synthesize and secrete several minor collagens, including collagens IX, X and XI (Miller & Gay, 1987). In cartilage, collagen X is restricted to hypertrophic zones and therefore may play a role in the transition of cartilage to bone (Schmid & Linsenmayer, 1987); collagens IX and XI seem to have a structural role (Mendler *et al.*, 1989).

Modulation of the differentiated phenotype occurs upon subculture (Mayne *et al.*, 1976), viral transformation (Okayama *et al.*, 1977; Pacifici *et al.*, 1977; Gionti *et al.* 1983, 1989) or treatment with a variety of agents, including RA (Takigawa *et al.*, 1982; Benya & Padilla, 1986; Yasui *et al.*, 1986; Horton & Hassel, 1986; Horton *et al.*, 1987*b*; Benya *et al.*, 1988). Horton *et al.* (1987*b*) observed that RA blocks the transcription of the collagen II gene and stimulates fibronectin synthesis in chick sternal chondrocytes.

Here we report that low concentrations (0.5 μM) of RA reversibly abolish the differentiated phenotype in quail embryo chondrocytes. Upon removal of RA, treated cultures gradually re-express type II collagen. When chondrocytes pretreated with RA were incubated in RA-free medium for 5 days, a strong stimulation of the expression of type X collagen was observed. Further culture in the absence of RA led to an increase in mRNA levels for collagen II and a concomitant decrease in mRNA for collagen X. By day 15, the ratio between type II and type X collagen mRNA levels was very similar to that exhibited by untreated control cultures.

EXPERIMENTAL

Cell culture

Chondrocytes were isolated from day 13 quail embryo tibiae as previously described (Gionti *et al.*, 1985). Floating cells were resuspended in Coon's modified F12 medium (Ambesi-Impiombato *et al.*, 1980) supplemented with 10% (v/v) foetal calf serum; after addition of RA, cells were plated at 5×10^4 cells/ml. all-*trans*-RA (Eastman–Kodak) was dissolved in 95% ethanol and stored at −80 °C in the dark. This solution was diluted with growth medium on day 0 and control cultures received an equivalent amount of ethanol. No ascorbate was added to the media because of its known antioxidant effect and ability to reversibly alter the chondrocyte phenotype (Daniel *et al.*, 1984). Chondrocytes were treated with RA for 72 h and subsequently cultured in normal medium for about 15 days. The experiments reported were performed on chondrocytes exposed to RA at 0.5 μM; no toxic effect was ever detected at this concentration in treated cultures.

Protein labelling and PAGE

Proteins secreted in the medium were labelled at the time points indicated in the relevant Figure. Labelling and immunoprecipitation were performed as already described (Capasso *et al.*, 1982). Samples were digested with bacterial

Abbreviation used: RA, retinoic acid.

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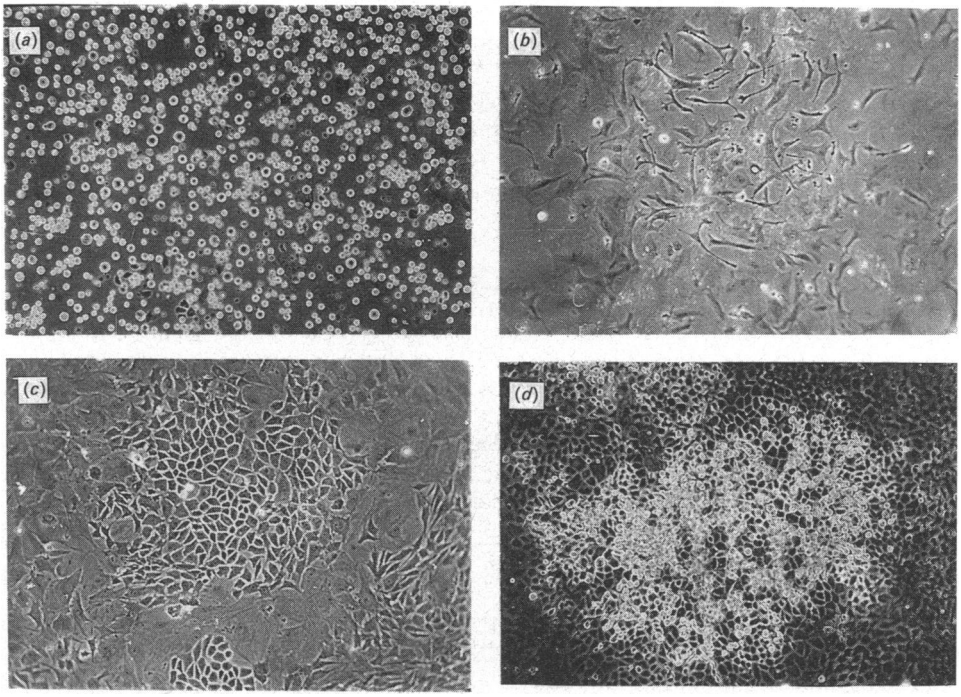

Fig. 1. Morphology of control and RA-treated quail chondrocytes

(*a*) Control chondrocytes after 30 days in culture; (*b*) chondrocytes grown in the presence of RA (0.5 μM) for 3 days; chondrocytes were then withdrawn from RA and cultured in normal medium for an additional 5 days (*c*) or 15 days (*d*). Cells were photographed under phase contrast, magnification × 75.

collagenase (Advanced Biofactures), as previously described (Gionti *et al.*, 1989).

Labelled media were immunoprecipitated with rabbit anti-(chick fibronectin) antiserum or with rabbit anti-(collagen II) IgG, generously provided by Dr. G. Tarone (University of Turin, Turin, Italy) and Dr. B. Vertel (Syracuse University, Syracuse, NY, U.S.A.) respectively. Rabbit IgG against chick collagen I was purchased from the Institut Pasteur de Lyon. SDS/PAGE was performed according to Laemmli (1970), with a polyacrylamide concentration of 7%.

DNA probes

The α1(II) probe used was BC7, a 1000 bp cDNA fragment coding for the C-propeptide domain of bovine type II collagen. This clone has been shown to contain sequences exhibiting 85% sequence similarity with the chicken pro α1(II) collagen C-propeptide (Sangiorgi *et al.*, 1985). pCOL3 was an 800 bp cDNA fragment coding for the C-propeptide of chick pro α1(I) collagen (Adams *et al.*, 1979). pYN 1738 was a 3200 bp cDNA coding for the chicken α1(IX) collagen chain (Ninomiya & Olsen, 1984). pYN 3116 (Ninomiya *et al.*, 1986) was a 600 bp cDNA fragment coding for part of the non-collagenous sequence at the *C*-terminus of the chicken α_1(X) collagen chain. The GAPDH probe was a full-length cDNA clone encoding rat glyceraldehyde-3-phosphate dehydrogenase (Fort *et al.*, 1985).

RNA extraction and Northern blot hybridization

Total cellular RNA, extracted by the method of Chomczynski & Sacchi (1987), was denatured, fractionated on formaldehyde/agarose gels (Lehrach *et al.*, 1977) and blotted on to an Amersham Hybond N nylon membrane.

Hybridization was performed according to the manufacturer's (Amersham) directions, with the following modifications: the hybridization temperature was 45 °C; filters were washed twice in 2 × SSPE/0.1% SDS [20 × SSPE is 3.6 M-NaCl/0.2 M-sodium phosphate buffer (pH 7.4)/20 mM-Na_2EDTA] for 5 min at room temperature and then five times in 0.2 × SSPE/0.1% SDS at 55 °C. For experiments requiring more stringent conditions (see Fig. 3) hybridization and washings were performed at 65 °C. Washed filters were dried and exposed to Fuji films with intensifying screens at −80 °C. Autoradiographs were scanned with a 2202 Ultroscan Laser Densitometer (LKB).

High specific activity nick-translated probes were prepared using the Boehringer nick-translation kit as specified by the supplier.

RESULTS AND DISCUSSION

Cultured quail embryo chondrocytes display two morphological phenotypes: polygonal epithelial-like cells and floating cells (Gionti *et al.*, 1985). When floating chondrocytes were exposed to RA for 3 days, there was a dramatic change in morphology. At 1 day after RA addition, 30% of chondrocytes had adhered to the substrate and, after 3 days of treatment, adhesion to the culture dish was complete. Attached cells became elongated, displaying a 'dedifferentiated morphology' (Fig. 1*b*). Upon removal of RA, chondrocytes promptly reverted to the typical polygonal morphology of quail chondrocytes, which grew attached to the culture dish (Fig. 1*c*). At the end of 15 days, RA-treated chondrocytes continued to grow as attached cells and displayed a minimal floating cell production (Fig. 1*d*), whereas the majority of the untreated control chondrocytes still grew in suspension as floating cells (Fig. 1*a*).

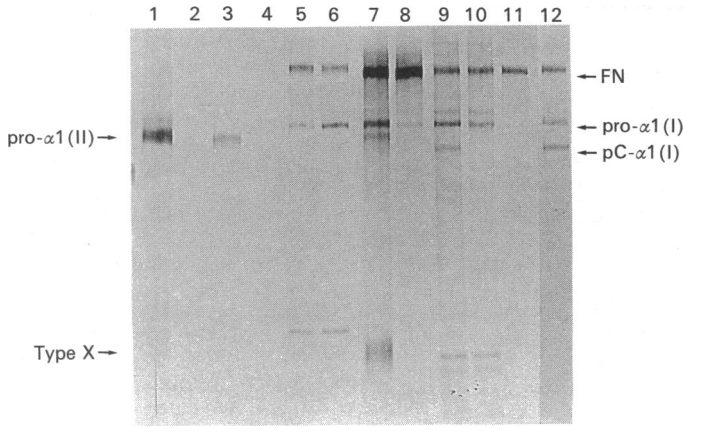

Fig. 2. Secreted proteins from quail embryo fibroblasts (QEF) and quail embryo chondrocytes (QEC)

Lanes 1–4, proteins from QEC before (lane 1) and after (lane 2) collagenase digestion, or after immunoprecipitation with rabbit antibodies against chicken collagen II (lane 3) and chicken fibronectin (lane 4). Lanes 5–6; QEC, treated with RA at 0.5 μM for 3 days, before (lane 5) and after (lane 6) collagenase digestion. Lanes 7–8, proteins from QEC, withdrawn from RA and cultured for an additional 5 days, before (lane 7) and after (lane 8) collagenase digestion. Lanes 9–12, proteins from control fibroblasts before (lane 9) and after (lane 10) collagenase digestion, or after immunoprecipitation with rabbit antibodies against chicken fibronectin (lane 11) and chicken collagen I (lane 12). FN, fibronectin; pro-α1(II), α1(II) procollagen chain; pro-α1(I), α1(I) procollagen chain; pC-α1(I), α1(I) C-propeptide chain; type X, α1(X) collagen. Note that an unidentified collagenase-resistant band co-migrates with the pro-α1(I) collagen chain. All samples were reduced and alkylated before electrophoresis.

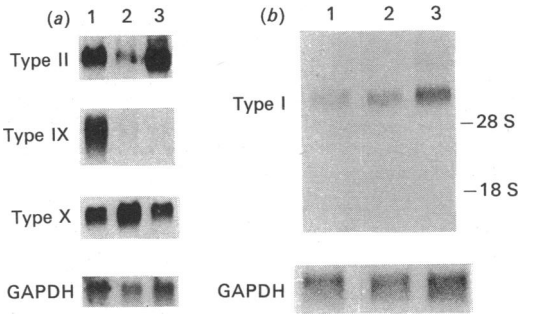

Fig. 3. Expression of collagen mRNAs in RA-treated chondrocytes

(*a*) Steady-state levels of pro-α1(II), -α1(IX) and -α1(X) collagen mRNAs in quail chondrocytes treated with RA at 0.5 μM. (*b*) Steady-state levels of pro-α1(I) collagen mRNA in quail chondrocytes treated with RA at 0.5 μM. Lane 1, control cultures; lanes 2 and 3, chondrocytes treated with RA for 3 days and cultured for an additional 5 days (2) or 15 days (3) in normal media. Probes were removed and filters were subsequently reprobed with glyceraldehyde-3-phosphate dehydrogenase (GADPH) to check uniform loading. To avoid cross-hybridization to pro-α1(II) collagen mRNA, hybridization and washings were performed at 65 °C for the experiment shown in (*b*).

We examined by PAGE the effect of RA on extracellular matrix protein synthesis (Fig. 2). The identification of distinct collagen types was based on their electrophoretic mobilities and collagenase sensitivities. Pro-α1(II) collagen chains from untreated control chondrocytes as well as α1(I) collagen chains and fibronectin from fibroblasts were identified by immunoprecipitation with specific antibodies (Fig. 2, lanes 1–4 and 9–12). As already shown by Horton *et al.* (1987*b*), we detected a marked suppression of α1 chain of collagen II and the induction of fibronectin synthesis in chondrocytes exposed for 3 days to RA (Fig. 2, lanes 5 and 6). At 3–5 days after removal from RA, treated cells had partially resumed the ability to synthesize α1(II) collagen chain; fibronectin and α1(X) collagen expression were markedly stimulated (Fig. 2, lanes 7 and 8). At 15 days after RA treatment, the synthesis of the α1 chain of collagen X was no longer detectable; fibronectin was still produced, although at lower rates (results not shown). This RA treatment induces fibronectin expression and, concomitantly, the conversion of chondrocytes from spherical floating cells to adherent cells.

To determine whether the RA-induced changes in the composition of the extracellular matrix reflected a comparable change in the steady-state levels of collagen mRNAs, total RNA from control chondrocytes, RA-treated chondrocytes and recovering chondrocytes (5 and 15 days after removal from RA) were analysed by Northern blot hybridization. By this type of analysis we examined the steady-state levels of collagen types I, II, IX and X mRNAs, and the results are presented in Fig. 3 and Table 1. RNA extracted from control chondrocytes contained a single 5.0 kb species corresponding to α1(II) collagen and a 2.5 kb species corresponding to α1(X) collagen mRNAs, while a broad band was detectable using an α1(IX) collagen probe (Fig. 3*a*). With this probe, Ninomiya & Olsen (1984) have detected in cartilage RNA two bands, 4.2 kb and 3.5 kb long. We observed a marked decrease in mRNA levels for every collagen examined in chondrocytes undergoing RA treatment (see below). Upon removal, we observed a gradual increase in α1(II) collagen mRNA. We also observed a transient alteration of the proportion of collagen type II to type X mRNA, since α1(X) collagen mRNA sharply increased within the first week of culture in the absence of RA. The low levels of collagen type II mRNA and the high levels of collagen type X mRNA correlated with the low rate of synthesis of α1(II) chains and the high rate of synthesis of α1(X) chains that we detected by SDS/PAGE in parallel cultures (Fig. 2, lanes 7 and 8). The levels of α1(X) collagen mRNA decreased during the second week of culture in the absence of RA and finally, at the end of 15 days, the absolute levels of collagen II and collagen X mRNAs were quite similar in control and recovering chondrocytes. Treatment of chondrocyte cultures for 3 days with RA was sufficient to produce a long-lasting inhibition of α1(IX) collagen expression. In another experiment, presented in Table 1, a densitometric analysis was also performed. We detected a dramatic decrease in the steady-state levels of α_1 chain mRNAs for types II, IX and X collagens in chondrocytes undergoing RA treatment. These negative results are consistent with the absence of detectable collagen chain synthesis observed by SDS/PAGE (Fig. 2, lanes 5 and 6), and are in agreement with the earlier findings of Horton *et al.* (1987*b*), who detected a strong suppression of transcription of the type II collagen gene in chick chondrocytes undergoing RA treatment. The expression of the α1(X) chain was strongly stimulated (3.6-fold) in chondrocytes recovering from RA at 5 days, and thereafter began to decrease, whereas the increase in α1(II) collagen mRNA was gradual and continuous.

During the recovery from RA treatment, no significant changes were detected by hybridization to pCOL3, a cDNA probe specific for pro-α1(I) collagen, which is usually highly expressed in de-differentiated chondrocytes (Fig. 3*b*). A dramatic decrease in pro-α1(I) collagen mRNA was detected in chondrocytes undergoing RA treatment (results not shown).

After confirming the RA-induced inhibition of chondrocyte differentiation, here we report that the steady-state levels of type X collagen mRNA transiently increased in RA-treated

Table 1. Relative levels of specific collagen mRNAs in control and RA-treated chondrocytes

Collagen mRNA levels, calculated from densitometric scannings, were normalized to glyceraldehyde-3-phosphate dehydrogenase expression, and the expression in control chondrocytes was set at 1. N.D., not detectable.

		Relative mRNA level		
Cell type	Collagen ...	α1(II)	α1(X)	α1(IX)
Control		1	1	1
Treated with RA for 3 days		N.D.	N.D.	N.D.
5 days after RA removal		0.13	3.6	0.05
15 days after RA removal		1.7	1.5	0.06

chondrocytes recovering their differentiated phenotype. This observation correlates with the transient stimulation of collagen X protein synthesis that we detected in parallel cultures, suggesting that the expression of this collagen could be under transcriptional and post-transcriptional control. In fact, control chondrocytes contain mRNA specific for this collagen, but do not synthesize collagen α1(X) chains. In contrast, in sternal chondrocytes the expression of type X collagen is regulated by changes in transcript synthesis and is correlated with chondrocyte hypertrophy (Ninomiya *et al.*, 1986; Castagnola *et al.*, 1987; Lu Valle *et al.*, 1989).

Type X collagen is a transient, developmentally regulated, collagen in vertebrates. It is not synthesized by actively growing chondrocytes but its expression appears to be restricted to the zone of hypertrophic cartilage (Gibson *et al.*, 1982, 1984; Schmid & Conrad, 1982; Capasso *et al.*, 1982; Schmid & Linsenmayer, 1985; Gionti *et al.*, 1985; Reginato *et al.*, 1986; Solursh *et al.*, 1986). The role of type X collagen in the transition of cartilage to bone is unknown. It would be interesting to check whether osteogenic differentiation markers can be induced by repeated exposure to RA of chondrocytes undergoing stimulation of type X collagen.

Although inhibition by RA of chondrocyte phenotype has been reported by several laboratories, the mechanisms involved are still poorly understood. Retinoid-binding proteins have been detected in several cell types (Chytil & Ong, 1984) and their DNA and genes have been cloned (Colantuoni *et al.*, 1985; Shubeita *et al.*, 1987). It has been proposed that RA could be translocated to nuclei via its binding protein, the cellular RA-binding protein. Nuclear receptors that bind both RA and DNA have been identified (Petkovich *et al.*, 1987; Giguère *et al.*, 1987; Brand *et al.*, 1988; Benbrook *et al.*, 1988; Zelent *et al.*, 1989). However, it is still unclear how RA complexed with receptors directly or indirectly controls the expression of developmentally regulated genes.

Horton *et al.* (1987*a*) identified an enhancer element within the first intron of the rat collagen II gene. They also demonstrated that RA suppresses the enhancer activity of this element on the transcription of chloramphenicol acetyltransferase gene driven by collagen II promoter following transfection of such a chimaeric gene in chick chondrocytes. Identification of such cis-acting elements in other cartilage collagen genes will be helpful in understanding the mechanisms involved in the modulation of chondrocyte phenotype by RA.

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