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Retinoic Acid Stimulates Matrix Calcification and Initiates Type I Collagen Synthesis in Primary Cultures of Avian Weight-Bearing Growth Plate Chondrocytes

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Abstract The effect of retinoic acid (RA) on primary cultures of growth plate chondrocytes obtained from weight-bearing joints was examined. Chondrocytes were isolated from the tibial epiphysis of 6- to 8-week-old broiler-strain chickens and cultured in either serum-containing or serum-free media. RA was administered at low levels either transiently or continuously after the cells had become established in culture. Effects of RA on cellular protein levels, alkaline phosphatase (AP) activity, synthesis of proteoglycan (PG), matrix calcification, cellular morphology, synthesis of tissue-specific types of collagen, and level of matrix metalloproteinase (MMP) activity were explored. RA treatment generally increased AP activity, and stimulated mineral deposition, especially if present continuously. RA also caused a shift in cell morphology from spherical/polygonal to spindle-like. This occurred in conjunction with a change in the type of collagen synthesized: type X and II collagens were decreased, while synthesis of type I collagen was increased. There was also a marked increase in the activity of MMP. Contrasting effects of continuous RA treatment on cellular protein levels were seen: they were enhanced in serum-containing media, but decreased in serum-free HL-1 media. Levels of RA as low as 10 nM significantly inhibited PG synthesis and caused depletion in the levels of PG in the medium and cell-matrix layer. Thus, in these appendicular chondrocytes, RA suppressed chondrocytic (PG, cartilage-specific collagens) and enhanced osteoblastic phenotype (cell morphology, type I collagen, alkaline phosphatase, and mineralization). J. Cell. Biochem. 65:209–230.

Key words: retinoic acid; chondrocytes; weight-bearing joints; proteoglycan synthesis; proteoglycan depletion

Retinoic acid (RA), one of the active metabolites of vitamin A, has been identified as a key inducer governing skeletal development [Eichele, 1989; Roberts and Sporn, 1984]. RA is implicated in bone formation: either deficient or excessive levels of RA lead to abnormal bone growth [Pease, 1962]. Premature growth plate closure causing growth retardation can derive from chronic vitamin A intoxication [Paulsen, 1994]. Retinoids also are known to be important regulators of cell growth and differentiation of epithelial cells and to have many physiological roles [Lotan, 1980]. In hypervitaminosis A, a narrow proliferative zone and a wide hypertrophic zone are present in growth plate cartilage; in addition, precocious matrix mineralization and vascular invasion are obvious [Wolbach and Hegsted, 1953; Vasan and Lash, 1975]. In vitamin A deficiency (hypovitaminosis A), the growth plate has an ill-defined immature proliferative zone with little mitotic activity, and a narrow, undercalcified maturation/hypertrophic zone; however, there is an abundant proteoglycan (PG)-rich extracellular matrix [Howell and Thompson, 1967; Havivi and Wolf, 1967].

Many of the effects of RA on cartilage appear to be due to an alteration in the rate of matrix synthesis and/or changes in the pattern of gene expression [Benya and Padilla, 1986; Horton et al., 1987; Iwamoto et al., 1993a; Ballock et al., 1994]. RA at a level of 100 nM stimulates proliferation and terminal differentiation of cultured tibial chondrocytes from chicken embryos, but blocks synthesis of type X collagen and decreases ^{45}Ca incorporation into the cell layer [Takishita et al., 1990]. RA at a lower level of 10

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nM stimulates rabbit costal growth plate cartilage cell proliferation, but reversibly inhibits glycosaminoglycan synthesis in a medium containing vitamin A-deficient serum [Enomoto et al., 1990]. Similar studies on the inhibition of proteoglycan (PG) or PG core protein by RA have been reported in growth plate chondrocytes [Horton et al., 1987; Ballock et al., 1994; Iwamoto et al., 1993b; Shapiro and Poon, 1976] and in articular cartilage [Campbell and Handley, 1987; Von den Hoff et al., 1994].

RA has been shown to alter collagen expression of chondrocytes rapidly, although a consistent pattern has not been established. Type II collagen expression is decreased [Iwamoto et al., 1993a; Horton et al., 1987; Ballock et al., 1994; Freyria et al., 1995; Dietz et al., 1993], while in some cases type I or III collagen, or fibronectin, are induced [Benya and Padilla, 1986; Horton et al., 1987; Dietz et al., 1993; Hein et al., 1984; Oettinger and Pacifici, 1990], or type I is not induced [Benya and Padilla, 1986; Freyria et al., 1995]. Using immunofluorescence methods, type X collagen has been shown to increase after RA treatment of chondrocytes isolated from the cephalic portion of the sternum [Iwamoto et al., 1993b]. Other findings on the effect of RA on the expression of type X collagen indicate that transient increase [Iwamoto et al., 1993b; Oettinger and Pacifici, 1990; Iwamoto et al., 1994; Cockshutt et al., 1993; Pacifici et al., 1991], decrease [Dietz et al., 1993; Cancedda et al., 1992], or no change [Iwamoto et al., 1993a] may be observed, depending on the conditions of the cells.

While there are many reports on suppression of the differentiated phenotype of embryonic sternal chondrocytes by RA, little work has been done on its effects on mineralizing cultures of growth plate chondrocytes isolated from weight-bearing skeletal tissues. Furthermore, most previous work on the effects of RA on chondrocytes has been performed in culture systems without provision of ascorbate. In our tibial growth plate cultures from rapidly growing broiler chickens, ascorbate has been found to be essential for normal collagen synthesis and matrix mineralization [Wu et al., 1989]. Thus, in this study, we examined the effect of RA on cell growth, alkaline phosphatase activity, and mineral deposition in these cells. We also studied the inhibition by RA of the synthesis of matrix components such as the proteoglycans and type X and II collagens, as well as the switch to expression of type I collagen.

METHODS

Chondrocyte Cultures

Chondrocytes were isolated from epiphyseal growth plate cartilage of the tibiae of 6- to 8-week-old hybrid broiler-strain chickens and plated in 35-mm dishes as previously described [Wu et al., 1989, 1995]. For serum-containing DATP5 medium, cells were supplied with Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 8 amino acids found to be enriched in the extracellular fluid of the avian growth plate [Ishikawa et al., 1986], insulin-transferrin-selenite (ITS), and 1 mM Na_2HPO_4 [Ishikawa and Wuthier, 1992] from day 6 or 7 onward. For serum-free HL-1 medium, on day 6 or 7, cells were supplied with a medium composed of a 1:1 mixture of DMEM (containing 10% FBS) and serum-free HL-1 medium, and from day 10 onward, the cells were given only HL-1. The culture medium was changed every 3–4 days. Ascorbate was provided from day 3 onward at a concentration of 50 μg/ml. To prepare the stock solution, RA was dissolved in absolute ethanol at 1 mM and added to the treatment group after dilution to the desired concentration. Control culture dishes received the same volume of ethanol (2.0–2.5 μl/2 ml of medium); the ethanol concentration did not exceed 0.125%.

Cell Harvest

On the specified day, chondrocytes were harvested after removal of the medium. The cell layer was rinsed twice with 1 ml of TMS (50 mM Tris, pH 7.5, 1.5 mM $MgCl_2$, and 10% sucrose) and scraped from the culture dishes after addition of 2 ml of TMS. Scraping was repeated twice with 1 ml of TMS to ensure removal of all cell and matrix material. This suspension was sedimented at 3,000 rpm for 30 min and 1 ml of TMT (10 mM Tris, pH 7.5, 0.5 mM $MgCl_2$, 0.05% Triton X-100) was added and stored frozen at −20°C. The harvested material was dispersed by sonication, and used for the analyses described below.

Biochemical Analyses

All analyses were measured using a Labsystems IEMS microplate reader MF (Needham Heights, MA). Cellular and matrix protein content was analyzed using bovine serum albumin as a standard [Lowry et al., 1951]. Alkaline phosphatase (AP) activity was determined us-

ing p-nitrophenylphosphate as a substrate by incubating aliquots (20 µl) of the TMT sonicate with 250 µl of substrate at 37°C for 5–10 min [Wu et al., 1995]. Proteoglycan content of the cell-matrix layer was analyzed in aliquots (20 µl) of the TMT sonicate by mixing with 250 µl of dimethylmethylene blue reagent and measuring the absorbency difference at 595 and 520 nm [Chandrasekhar et al., 1987]. For determination of calcium and phosphate mineral content in the cell layer, the TMT sonicate was centrifuged at 3,000 rpm for 40 min and the TMT supernatant was removed. The resulting cell-matrix pellet was incubated in 0.1 N HCl (1 ml per dish) at 4°C overnight to dissolve the mineral [Wu et al., 1995]. The HCl extract obtained after centrifugation was used for Ca^{2+} and Pi analyses. Ca^{2+} was measured using *o*-cresol phthalein complexone [Baginski et al., 1973], and Pi was analyzed using the ammonium molybdate method [Ames, 1966]. Cellular DNA content was determined using bis-benzimidazole (Hoechst 33258) with aliquots of the TMT cell sonicate; the DNA standard curve was constructed using the same level of TMT vehicle [Rago et al., 1990]. For statistical analysis of the data, Student's *t*-tests were performed; significant differences were taken as $P < 0.01$.

The amount of collagen produced in the presence or absence of RA was monitored in both the medium and cell-matrix fractions. For isolation of medium collagen, the media were pooled from four dishes, protease inhibitors were added (2.5 mM EDTA, 0.2 mM PMSF, and 1 mM benzamidine) and the mixture clarified by centrifugation. A 1.25-ml portion of the resulting supernatant was transferred to a microfuge tube and 0.54 ml of saturated $(NH_4)_2SO_4$ was added (30% saturation), incubating at 4°C for 16 h. The medium collagen was then sedimented at 16,000*g* for 20 min. In some instances, medium collagen was further digested with pepsin (150 µg/ml) in 0.5 M acetic acid at 4°C overnight. For isolation of the cell-matrix collagen, the cell layer was extracted with pepsin/acetic acid containing the above mentioned protease inhibitors [Wu et al., 1995; Miller and Rodes, 1982]. The extracted cell-layer collagen was obtained after centrifugation of the pepsin digest and was precipitated using 2.75 M NaCl in acetic acid.

Medium and cell-matrix layer proteins were analyzed by 7.5–15% gradient, or 8% acrylamide, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analyses [Laemmli, 1970]. Immunoblot analysis of type I collagen in the medium and cell-matrix layer proteins was carried out after SDS–PAGE by the method of Towbin and Gordon [1984]. Proteins were transferred from the gels onto nitrocellulose sheets and probed with rabbit antichicken type I collagen antibody (Biodesign, Kennebunk, ME), with antichicken type X collagen monoclonal antibody (gift from Dr. G. Balian), or with rabbit antihuman fibronectin (UBI, Lake Placid, NY).

Matrix metalloprotease (gelatinase) activity in the culture medium was assayed by zymography using 0.2% gelatin in SDS–PAGE [Herron et al., 1986] under nonreducing conditions in 10% acrylamide gels. After electrophoresing the samples, gels were soaked in a mixture of 2.5% Triton X-100, 50 mM Tris, 5 mM $CaCl_2$, 1 µM $ZnCl_2$, and 0.02% NaN_3, pH 7.6, for 1 h, then overnight in 1% Triton X-100, 50 mM Tris, 5 mM $CaCl_2$, 1 µM $ZnCl_2$, 0.02% NaN_3, pH 7.6, at 37°C. Gels were stained with Coomassie Brilliant Blue R-250.

Calcium deposits were stained with 0.5% alizarin red S solution, pH 4.0, for 5 min at room temperature [Chung et al., 1992]. Stained culture dishes were washed three times with water and ethanol. Densitometric image analyses of collagen bands were performed with a digital imaging system (Alpha Innotech, San Leandro, CA). For comparison with results of the effects of RA on collagen synthesis by the cell cultures, sections of growth plate cartilage from a 2-week-old chicken were stained with the van Gieson stain for localization of fibrillar (type I) collagen [Fullmer, 1979].

RESULTS

During the course of investigation of RA modulation of cultured growth plate chondrocytes, we discovered that levels of RA as low as 10 nM were potent enough to significantly decrease proteoglycan synthesis. RA at concentrations higher than 1 µM caused abundant cell floaters, loss of matrix, and cell death in serum-free medium, depending on the culture stage. Therefore, we focused studies within the range of 10–250 nM RA.

Effect of RA on Cellular Protein Synthesis and on Alkaline Phosphatase Activity

A complex pattern of multiple effects was revealed. As shown in Figure 1A,B, in DATP5

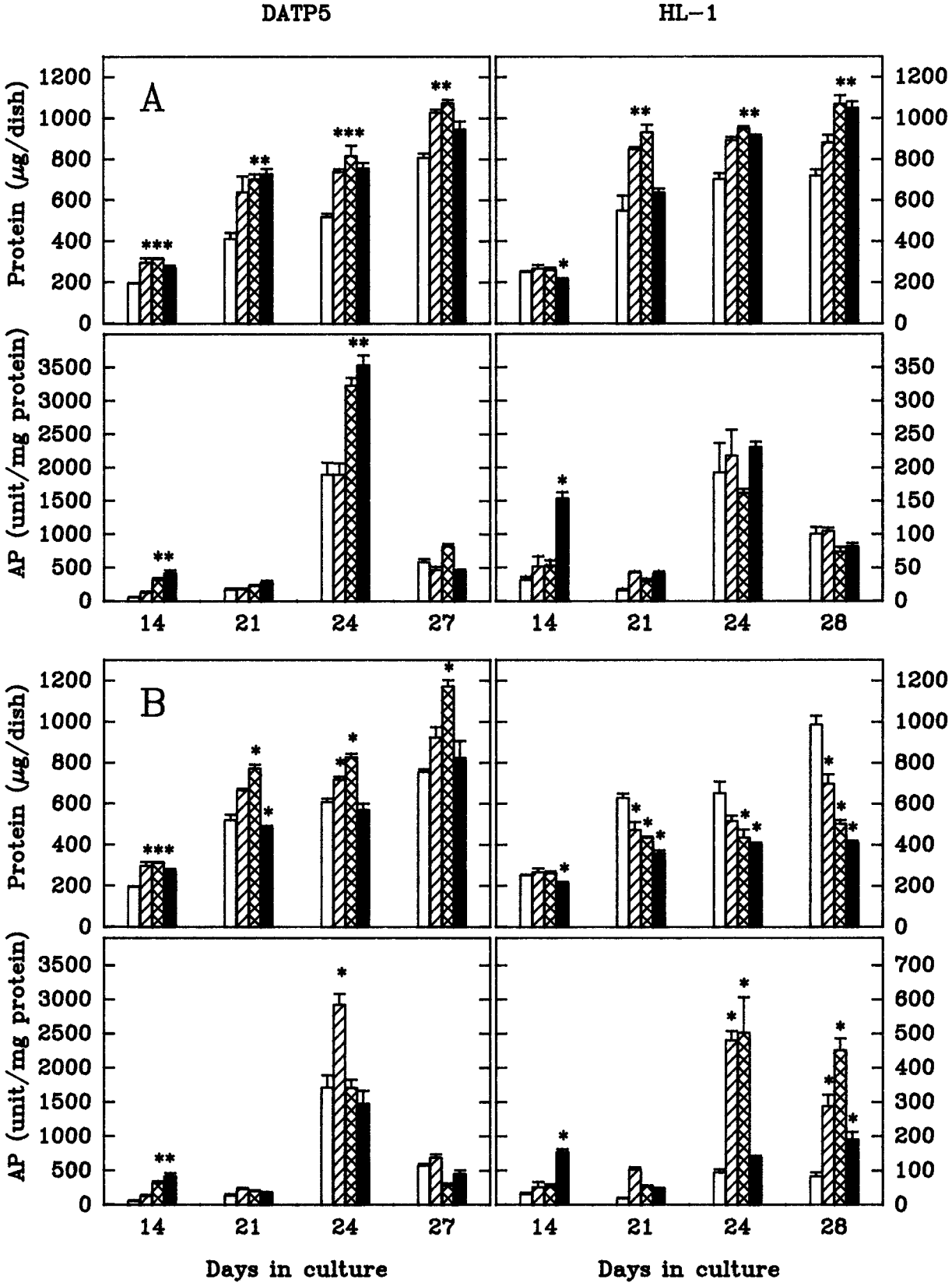


Fig. 1. Effect of all-trans retinoic acid (RA) on cell protein and alkaline phosphatase activity. Preconfluent primary cultures of avian growth plate chondrocytes were treated either transiently (**A:** days 7–14) or continuously (**B:** from day 7 onward) with varying levels of RA in either serum-containing (DATP5, *left*), or serum-free (HL-1, *right*) media. Values represent the mean ±SEM of four samples each. Open bars, control; hatched bars, RA 10 nM; cross-hatched bars, RA 50 nM; solid bars, RA 250 nM. Cellular protein, *top;* alkaline phosphatase, *bottom.* Asterisks (*) indicate values significantly different from the control; $P < 0.01$.

medium, synthesis of total cellular and matrix protein was stimulated by RA when cells were treated transiently from days 7–14, and then harvested on days 14, 21, and 27 (Fig. 1A, left). When DATP5 cells were treated continuously with RA (10 and 50 nM) from day 7 onward, synthesis of total cellular and matrix protein was stimulated (Fig. 1B, left). While in serum-free HL-1 medium, transient (days 7–14) RA treatment increased cellular and matrix protein synthesis (Fig. 1A, right); continuous treatment with RA was inhibitory (Fig. 1B, right). RA also caused a severalfold increase in alkaline phosphatase (AP) activity, depending on the dose and duration of treatment. An increase was noticeable as early as day 14, and some lasted until day 28 (Fig. 1A,B). Exhibition of AP activity is known to be cell stage dependent; stimulation of AP activity by RA also was found to be cell stage dependent. Thus, while the timing of the onset of induction of AP activity varied from one culture to another, stimulation of AP activity by RA appeared to be a general trend.

Effect of RA on DNA Content of Chondrocyte Cultures

When grown in DATP medium, DNA content of the control cultures increased progressively from ~8 μg/dish at day 14 to ~18 μg/dish on day 24; when grown in serum-free HL-1 medium, cellular DNA levels were generally lower (Table I). Continuous administration of 50 nM RA from day 7 to day 21 nearly doubled DNA levels in the DATP chondrocytes but was somewhat less stimulatory in HL-1 medium. Removal of RA after day 14 and allowing the cells to continue in culture for 7 more days caused no major loss in DNA compared to cells continuously exposed to RA. Delay in the exposure of the cells to RA until confluency (day 14), with continued exposure to day 21, however, reduced the response to RA, especially in HL-1 medium. During this post-confluent period, RA caused no increase in total cellular and matrix protein synthesis in DATP5 medium and led to a decrease in these in serum-free HL-1 medium. Thus, RA generally stimulated cell division only in the preconfluent phase of the cultured chondrocytes.

Inhibition of Proteoglycan (PG) Synthesis by RA

In DATP5 cultures (Fig. 2, left) PG content of the cell-matrix layer was reduced at all time periods during RA treatment. In HL-1 cultures (Fig. 2, right), if cells were exposed to RA only transiently during the preconfluent stage (day 7–14), and the cell-matrix layer was examined on days 14, 17, 21, and 24 (Fig. 2A), inhibition of PG synthesis was observed only on days 14 and 17. Thus, PG production was restored in serum-free cultures upon removal of RA from the medium after the day 7–14 treatment. Recovery of PG synthesis was seen first with 10 nM RA-treated cells on day 17, and by days 21 and 24, all RA-treated cells were similar in PG content to the controls (Fig. 2A. right).

If RA treatment was continuous (Fig. 2B), there was pronounced inhibition of PG synthesis; levels of PG in the cell-matrix layer were

TABLE I. Effect of Timing and Length of Treatment With Retinoic Acid on DNA Content of Primary Cultures of Chicken Growth Plate Chondrocytes

Days of treatment/ day of harvest	DATP5 cultures		HL-1 cultures	
	Control	RA	Control	RA
	DNA content (μg/dish)			
7–14/14	8.5 ± 0.3	14.2 ± 1.6	7.4 ± 0.1	11.6 ± 0.4
7–17/17	10.5 ± 1.7	14.8 ± 0.9	10.7 ± 0.2	18.3 ± 0.9
7–14/21	12.6 ± 0.5	22.3 ± 1.6	8.6 ± 0.3	15.8 ± 0.5
7–21/21	12.6 ± 0.5	24.4 ± 1.3	8.6 ± 0.2	12.8 ± 1.0
7–24/24	18.1 ± 0.4	24.2 ± 0.6	14.0 ± 0.4	16.7 ± 0.6
14–21/21	11.1 ± 0.1	12.5 ± 0.9	10.2 ± 0.3	7.6 ± 0.3
	Lowry protein content (μg/dish)			
14–21/21	336.3 ± 9.4	350.8 ± 4.2	360.2 ± 8.0	201.0 ± 11.8

Cells were treated with RA (50 nM) beginning on the day indicated (mainly day 7) and continued as indicated. The cultures were harvested on the day indicated and analyzed for DNA content by the bis-benzimidazole method of Rago et al. [1990]. Protein analyses were by the Lowry [1951] method.

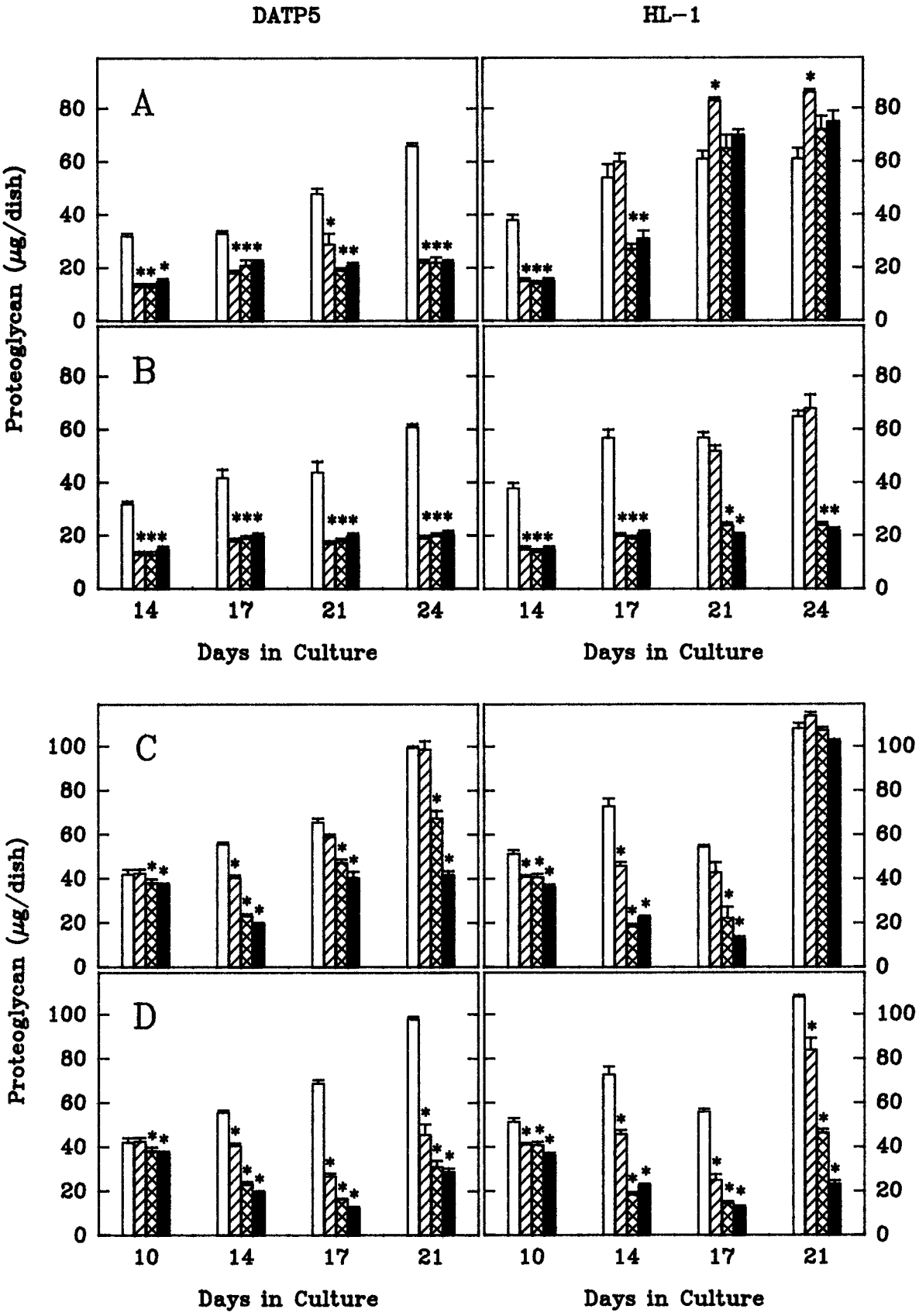


Fig. 2. Effect of all-trans retinoic acid (RA) on proteoglycan synthesis. Preconfluent primary cultures of avian growth plate chondrocytes were treated either transiently (**A,C:** days 7–14) or continuously (**B,D:** from day 7 onward) with varying levels of RA in either serum-containing (DATP5, *left*), or serum-free (HL-1, *right*) media. Values represent the mean ±SEM of four samples each. **A,B:** Cell layer proteoglycan. **C,D:** Media proteoglycan. Open bars, control; hatched bars, RA 10 nM; cross-hatched bars, RA 50 nM; solid bars, **A,B:** RA 250 nM, **C,D:** RA 100 nM. Asterisks (*) indicate values significantly different from the control; $P < 0.01$.

decreased more than 50% in both DATP5 (left) and HL-1 cultures (right).

Assays of PG content of the medium in the presence or absence of RA were also conducted (Fig. 2C,D). Again, PG content of the medium, after transient (days 7–14; Fig. 2C) or continuous (days 7–21; Fig. 2D) exposure to RA, was measured on days 10, 14, 17, and 21. In general, synthesis of PG into the medium by the cells was progressively inhibited by increasing levels of RA, especially if exposed continuously. Even when exposed for only 3 days (days 7–10), there was a significant decrease in PG content. When exposed transiently from days 7–14, there was some recovery of PG synthesis by day 21 in serum-free HL-1 cultures; this recovery was only seen at the lowest level of RA (10 nM) on day 21 in DATP cultures.

Stimulation of Mineral Deposition by RA

While there was some variability from culture to culture in the time at which mineralization was first detected, generally day 14 was the earliest date. When preconfluent cultures (days 7–14) were exposed transiently to RA at levels of 10–250 nM, a significant increase (138–189%) was observed in calcium deposition between days 14–21 (Fig. 3A). When the cells were treated continuously with RA, an increase in calcium content of the cultures was also observed (110–200%), depending on the RA concentration (Fig. 3B). A similar pattern of increase in Pi content was also obtained. In general, Ca/P ratios of 1.56 $\pm$ 0.17 were measured for the mineral deposits; this value is comparable to the Ca/P ratio of biological apatite seen in vivo.

To verify that enhanced mineral deposition had indeed occurred in the cell-matrix layer after treatment with RA, separate cultures were stained with alizarin red S which specifically binds to the Ca^{2+} deposits. It is evident that increased staining with alizarin red S was seen after treatment with RA in either serum-containing DATP5 (Fig. 3C) or serum-free HL-1 (Fig. 3D) medium.

Morphology of RA-Treated Cells

Figure 4B,D shows the morphology of cells treated continuously with 50 nM RA from day 7 onward, in either HL-1 (Fig. 4, panel) or DATP5 (left panel) medium. Note the progressive change from the polygonal (Fig. 4A) and round (Fig. 4C) shape of the controls, to the osteoblast-like spindle shape with increasing time of exposure to RA. On the other hand, cells treated transiently with 50 nM RA from days 7–14 when examined on day 21 (Fig. 4E) recovered their typical morphology in HL-1 medium (Fig. 4, right); whereas when treated in DATP5 medium they failed to fully recover (Fig. 4, left). Thus, chondrocytes treated only transiently with RA in serum-free medium recovered normal morphology after removal of RA.

Release of Type II and X Collagen into Culture Medium

The effects of RA on the secretion of collagens and fibronectin into the culture medium was investigated. Figure 5 shows the effect of RA on the amount of collagen precipitated from the serum-containing DATP5 culture medium with 30% saturation ammonium sulfate. This represents steady-state production of collagen. The intensity of the type X collagen doublet bands migrating near the 66-kDa bovine serum albumin (BSA) standard was decreased with increasing RA concentration in DATP5 medium. This effect was seen regardless of whether the cells were treated transiently or continuously. By contrast, a new band corresponding to the α2-chain of type I collagen appeared just below the α1-type II collagen band on the SDS–PAGE gels. The identity of type I collagen was confirmed with Western blots using type I-specific polyclonal antibodies (see below). By contrast, the control cells grown in the absence of RA, when examined at the same time periods, showed no evidence of the type I collagen band. The pattern of type II and X collagen in culture media from untreated chondrocytes was previously documented by Western blotting [Wu et al., 1995]. The decrease in type X and increase in α2-type I collagen with RA was consistently seen by SDS–PAGE, regardless of whether the cells were treated from days 7–14, 7–28, or 14–21, and examined periodically from 3 to 7 days after termination of RA treatment. It was also evident that fibronectin (FN) was also induced by low dosages of RA (50 nM). This 250-kDa FN band had been seen previously by Western blot analysis [Wu et al., 1992] and was confirmed in this study.

This RA-dependent change in collagen secretion was also investigated using serum-free HL-1 medium. [^{3}H]-Proline labeling studies showed that during RA treatment in serum-free medium from days 6–10, proline incorpora-

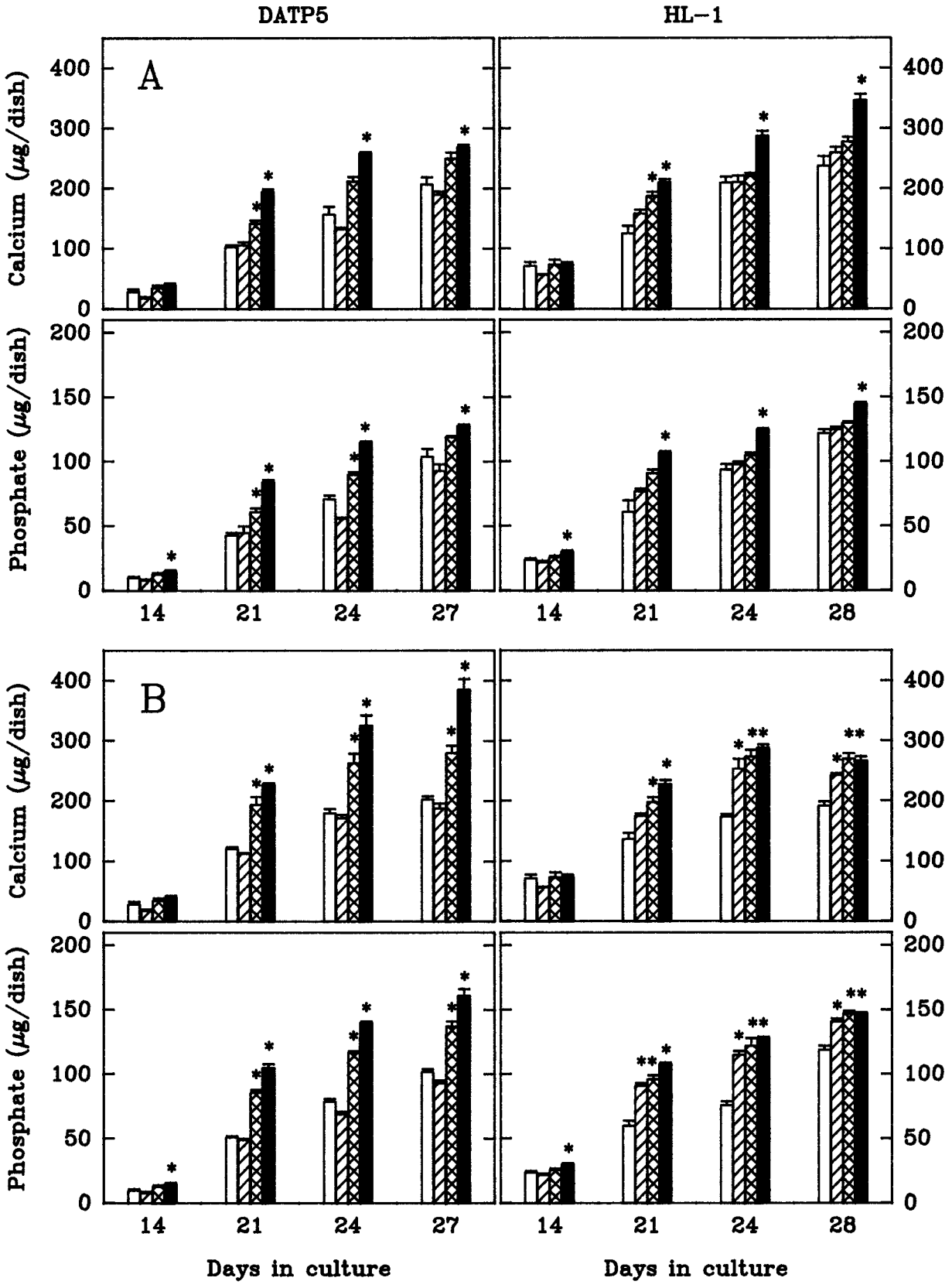

DATP5
HL-1
A
B
Calcium (μg/dish)
Phosphate (μg/dish)
0
50
100
150
200
300
400
14
21
24
27
28
Days in culture

Figure 3.

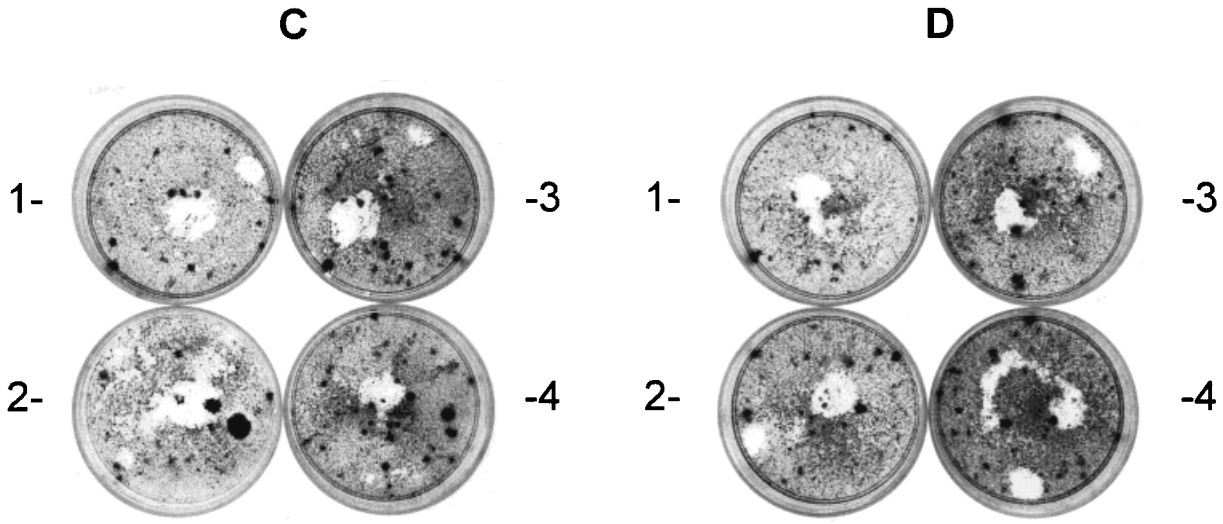


Figure 3. (Continued)

tion into the collagen fraction was decreased by ~40% (data not shown). The appearance of a new band migrating below the α1-type II collagen band was also observed on SDS–PAGE gels (Fig. 6). Western blots confirmed that this band was the α2-type I collagen band. When the cells were treated with RA from days 7–14, days 14–21, or continuously from days 7–28, a dramatic decrease in type X collagen was observed on days 14 and 28. In HL-1 medium, FN levels were initially stimulated by RA treatment, but were suppressed when treatment with RA was continuous from days 7–28 (data not shown).

Densitometric analysis of SDS–PAGE gels of RA-treated cultures in the region of the type II and type I bands revealed that the ratio of the top band, representing α1(I), and α1(II) over the bottom band, representing α2(I), was ~1.4–2.3 (Table II). In the non-RA-treated control cultures, these ratios were within the range of 3.2–8.4. This indicates that most of the collagen secreted by the cultures after RA treatment was, in fact, type I collagen. This calculation was based on the assumption that the ratio of α1/α2 in type I collagen is 2. We conclude that there was an increase in type I and a decrease in type II collagen secretion into the medium of the RA-treated chondrocytes.

Induction of Matrix Metalloproteinase by RA

In order to detect the presence of any matrix metalloproteinase (MMP) in the culture medium and to examine the effect of RA on MMP production, gelatin zymography was performed. Based on migration in comparison to MW standards, we observed a lucent band of gelatinase activity at 72,000 Da M_r (Fig. 7) and confirmed by N-terminal sequencing (Nie et al., unpublished data) that this enzyme was MMP-2 [Wilhelm et al., 1993; Woessner, 1991]. Treating cells with RA in HL-1 serum-free medium (left gel) from day 7–14 (set A), or from day 14–21 (set B), caused a marked increase in the activity of the 72-kDa MMP; levels of MMP

Fig. 3. Effect of all-trans retinoic acid (RA) on mineral deposition. Preconfluent primary cultures of avian growth plate chondrocytes were treated either transiently (**A:** days 7–14) or continuously (**B:** from day 7 onward) with varying levels of RA in either serum-containing (DATP5, *left*), or serum-free (HL-1, *right*) media. The cell-matrix pellet was incubated in 0.1 N HCl to dissolve the mineral and the Ca^{2+} and Pi content was analyzed colorimetrically (see Methods). Values represent the mean ±SEM of four samples each. Open bars, control; hatched bars, RA 10 nM; cross-hatched bars, RA 50 nM; solid bars, RA 250 nM. Calcium levels, *top;* phosphate levels, *bottom.* Note that phosphate deposition was similar to that of calcium deposition; mean Ca/Pi ratio was 1.56 ± 0.17. Asterisks (*) indicate values significantly different from the control; $P < 0.01$. To verify that RA had increased mineral deposition, calcium deposits were stained with 0.5% alizarin red S solution and the culture dishes photographed. RA was supplied to the cultures from day 7 until the dishes were harvested and stained on day 15. **C:** Cells cultured in serum-containing DATP5 medium. **D:** Cells cultured in serum-free HL-1 medium. **C,D:** 1–control, 2–10 nM, 3–50 nM, and 4–100 nM RA.

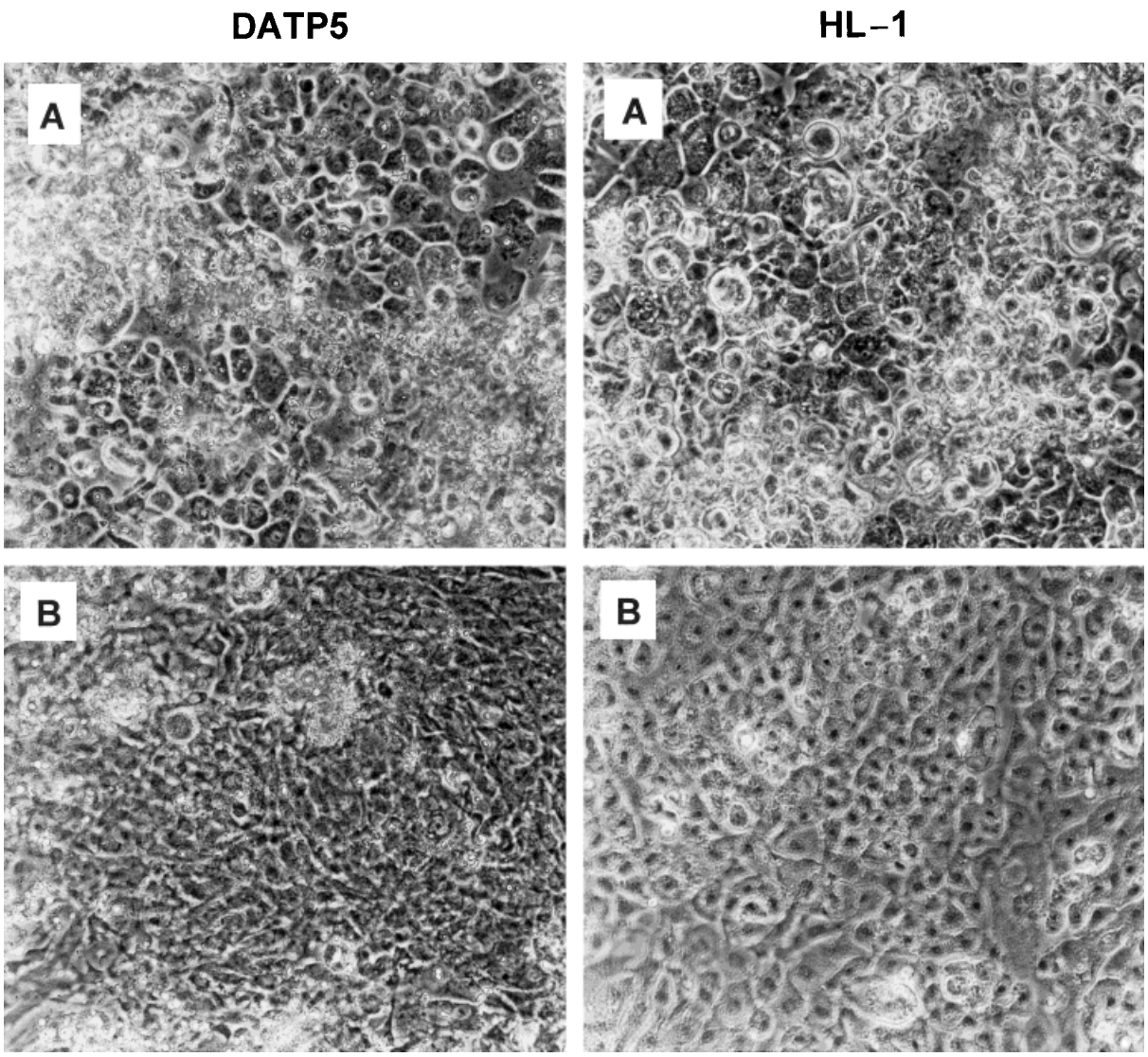


Fig. 4. Effect of all-trans retinoic acid (RA) on cell morphology. Preconfluent primary cultures of avian growth plate chondrocytes were treated with 50 nM RA in either serum-containing (DATP5, *left*), or serum-free (HL-1, *right*) media. Cells were treated either continuously from days 7–15 and examined on day 15 (**B**), or from days 7–21 and examined on day 21 (**D**), or transiently from days 7–14 and examined on day 21 (**E**). Untreated controls for day 15 (**A**) and for day 21 (**C**) are shown. Note the change in morphology when comparing the control **A** with RA-treated **B** (day 15), or control **C** with RA-treated **D** or **E** (day 21).

activity were almost 3-fold higher in RA-treated than in control cells.

A similar induction of the 72-kDa MMP was seen in serum-containing DATP5 medium after treating the cells with RA (Fig. 7, right gel). A slight increase in the activity of the 72-kDa MMP was observed even at 10 nM RA during the day 7–14 treatment (set C); a 2-fold or more increase was seen with day 14–21 treatment (set D) at RA levels of 50 and 250 nM. The lower band under the 72-kDa MMP band (set A) is probably a truncated, active form of the enzyme.

Effect of RA on Collagen Synthesis in the Cell-Matrix Layer

We examined the effects of RA on the deposition of the various collagens into the extracellular matrix because this is a vital parameter of cellular function. Two ways of examining the effect of RA on synthesis of collagen into the cell-matrix layer were used. First, the cell-

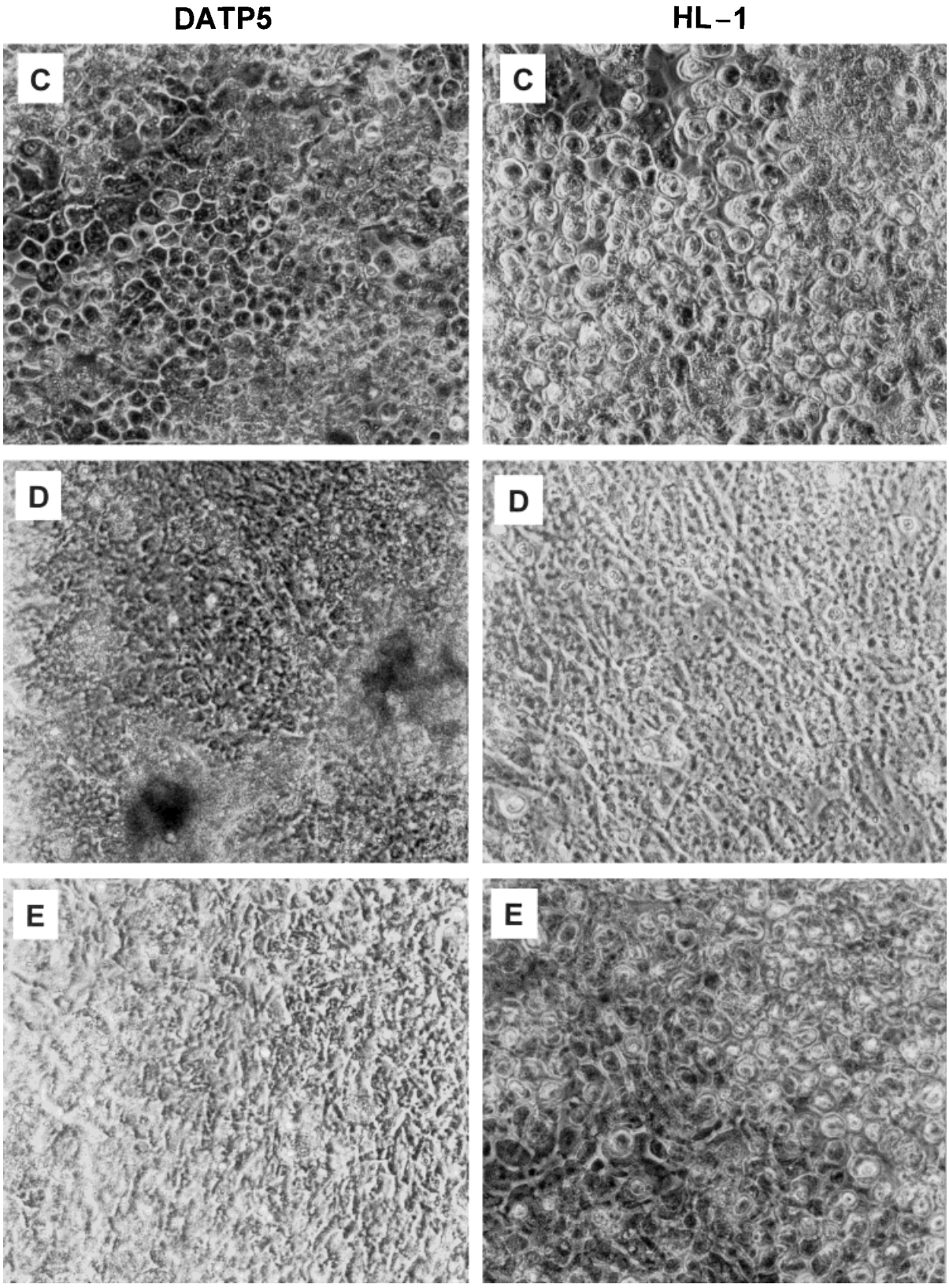


Figure 4. (Continued)

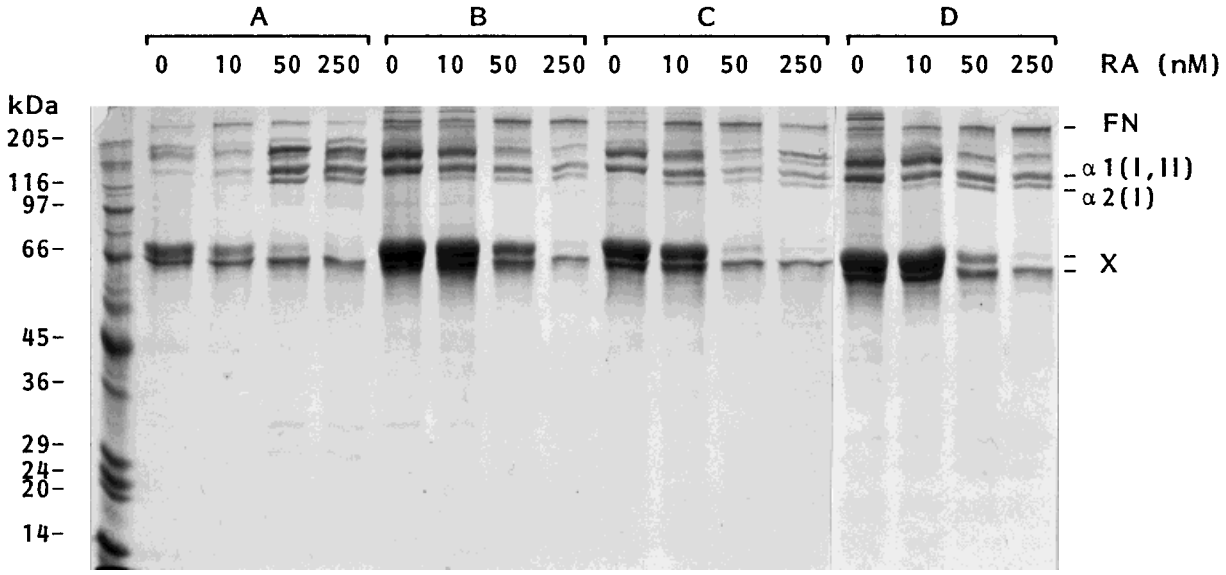


Fig. 5. Effect of all-trans retinoic acid (RA) on the type of collagen synthesized into serum-containing DATP5 culture medium. Preconfluent primary cultures of avian growth plate chondrocytes were treated with varying levels of RA either transiently from days 7–14 and the media collected on day 14 (set A), or on day 28 (set B) and analyzed. Alternatively, the cells were either treated continuously from days 7–28 (set C), or treated transiently from days 14–21 (set D), and the media collected on day 28 and analyzed. Collagen in the culture medium, collected on either day 14 or 28, was precipitated with 30% saturation ammonium sulfate, and the collagen pellet analyzed by SDS–PAGE. Protein bands on the gels were visualized using Coomassie Blue stain. *Lanes 1–4* (set A), control, RA 10 nM, 50 nM, and 250 nM; *lanes 5–8* (set B), control, RA 10 nM, 50 nM, and 250 nM; *lanes 9–12* (set C), control, RA 10 nM, 50 nM, and 250 nM; *lanes 13–16* (set D): control, RA 10 nM, 50 nM, and 250 nM. FN, fibronectin; α1(I, II), α1 chain of type I and II collagen; α2(I), α2 chain of type I collagen; X, type X collagen.

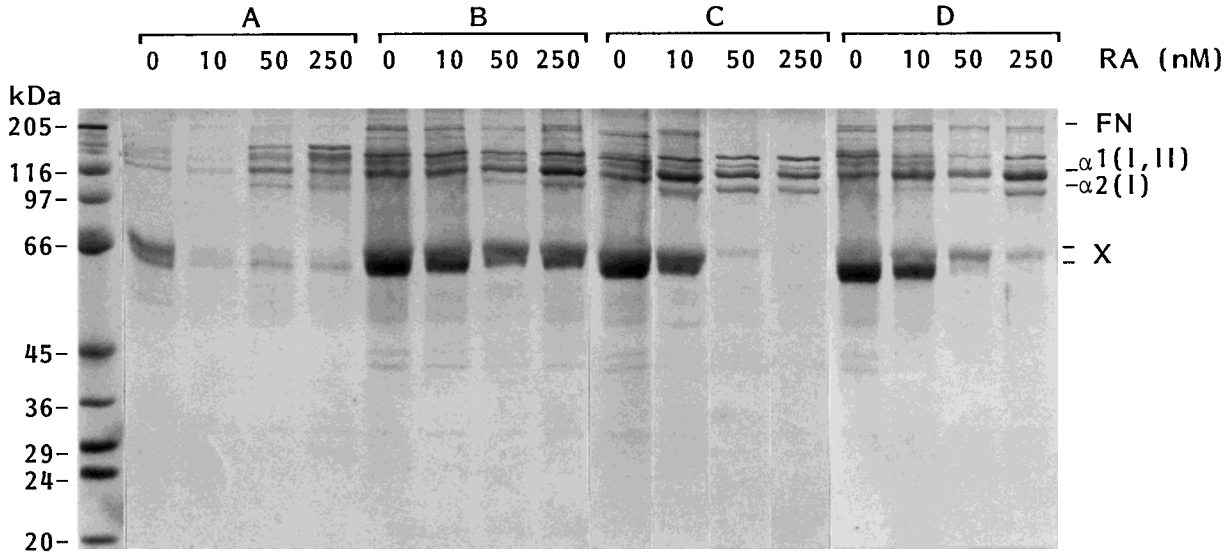


Fig. 6. Effect of all-trans retinoic acid (RA) on the type of collagen synthesized into serum-free HL-1 culture medium. Preconfluent primary cultures of avian growth plate chondrocytes were treated with varying levels of RA either transiently from days 7–14 and the media collected on day 14 (set A), or on day 28 (set B) and analyzed. Alternatively, the cells were either treated continuously from days 7–28 (set C), or treated transiently from days 14–21 (set D) and the media collected on day 28 and analyzed. Collagen in the culture medium, collected on either day 14 or 28, was precipitated with 30% saturation ammonium sulfate, and the collagen pellet analyzed by SDS–PAGE. Protein bands on the gels were visualized using the Coomassie Blue stain. *Lanes 1–4* (set A), control, RA 10 nM, 50 nM, and 250 nM; *lanes 5–8* (set B), control, RA 10 nM, 50 nM, and 250 nM; *lanes 9–12* (set C), control, RA 10 nM, 50 nM, and 250 nM; *lanes 13–16* (set D), control, RA 10 nM, 50 nM, and 250 nM. FN, fibronectin; α1(I, II), α1 chain of type I and II collagen; α2(I), α2 chain of type I collagen; X, type X collagen.

TABLE II. Densitometric Image Analyses of the Effect of Retinoic Acid on Synthesis of Type I and II Collagen into the Medium by Primary Cultures of Chicken Growth Plate Chondrocytes

RA level (nM)	Integrated density value[a]		Ratio of α1(I) + α1(II)/α2(I)
	α1(I) + α1(II)	α2(I)	
Cells grown on DATP5 (RA administered from days 7–14, analyzed day 14)			
0	13,479	4,173	3.23
10	41,649	21,538	1.93
50	205,679	150,343	1.43
250	215,594	144,222	1.49
Cells grown on DATP5 (RA administered from days 7–28, analyzed day 28)			
0	165,928	19,828	8.37
10	166,588	88,754	1.88
50	91,700	47,187	1.94
250	104,729	51,195	2.04
Cells grown on HL-1 (RA administered from days 7–14, analyzed day 14)			
0	33,668	4,736	7.11
10	23,701	8,552	2.77
50	77,512	45,401	1.71
250	92,629	65,229	1.42
Cells grown on HL-1 (RA administered from days 7–28, analyzed day 28)			
0	76,334	10,639	7.17
10	125,447	53,648	2.33
50	102,825	68,951	1.49
250	84,815	42,557	1.99

[a]Integrated density value is the intensity of the respective collagen bands on the SDS–PAGE gels after Coomassie Blue staining (taken from Figs. 5, 6). The top band contained both α1(I) and α1(II) collagens; the lower band contained only α2(I) collagen. As there are two α1(I) subunits for each α2(I) subunit in type I collagen, whereas there are three α1(II) subunits in type II collagen, the ratio of the two bands provides a measure of the synthesis of the two collagen types. When the ratios are ≤2.0, this indicates that the cells were synthesizing predominantly type I collagen.

matrix layer was harvested in TMS and sonicated in TMT buffer as described above. This provided a measure of the whole spectrum of proteins present in the cell-matrix layer. Second, the cell-matrix layer was treated with pepsin-acetic acid to yield a soluble extract. This allowed us to focus specifically on the collagens, since they are resistant to pepsin. While pepsin treatment does not degrade the collagen triple helix and can release collagen from the cell-matrix layer, cross-linking and interaction with proteoglycans can make the collagens resistant to pepsin extraction [Wardale and Duance, 1993]. Thus, the pepsin-insoluble fraction from the cell/matrix layer was also analyzed (see later). All fractions were analyzed by SDS-PAGE.

When the proteins present in the TMT sonicate of the cell-matrix layer from cells grown in serum-containing DATP5 medium were analyzed (Fig. 8A), a new protein band appeared under the type II (α1) band after RA treatment. This new band was identified (see later) as α2-type I collagen. The increase in α2-type I was prominent when cells were treated with RA during days 7–14, 7–17, and 7–21 and analyzed on days 14, 17, and 21. FN levels in the cell-matrix layer also appeared to be increased by the RA treatment. However, the Western blot shown in Figure 8B reveals that the levels of type X collagen were markedly decreased after RA treatment. These changes in the cell-matrix layer represent cumulative effects of RA treatment.

The proteins present in the TMT sonicate of the cell-matrix layer from cells grown in serum-free HL-1 medium in the presence or absence of RA were also analyzed. Native type X collagen was decreased, and the shift to the production of type I collagen, as indicated by the appearance of the α2 band, was pronounced in the presence of RA (data not shown). FN was also increased by RA on days 17 and 21. The effect of RA on collagen mRNA levels is currently being analyzed to explore more precisely the on–off relationship between type I, II, and X collagens.

Identification of Type I Collagen in RA-Treated Cultures of Growth Plate Chondrocytes

To verify the effect of RA on the type of collagen production, portions of the medium were precipitated with 30% ammonium sulfate and further digested with pepsin to remove noncollagenous proteins. Figure 9 (top) shows that pepsinized type X collagen was greatly reduced after treating the cells with RA. On the contrary, synthesis of α2 type I collagen was switched on by RA in both the DATP5 and HL-1 media. That the newly synthesized collagen was indeed type I, was confirmed by Western blot analysis (Fig. 9, bottom) of proteins on a gel essentially identical to that seen in the Coomassie Blue-stained gel (Fig. 9, top panel) after

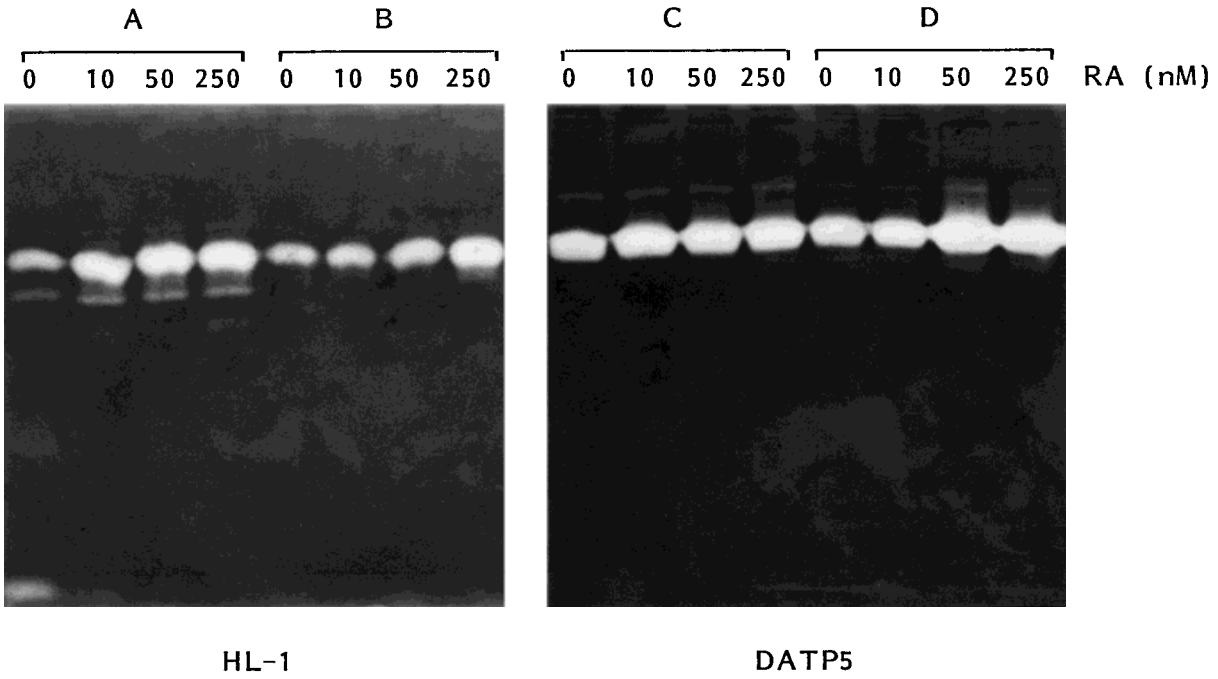


Fig. 7. Effect of all-trans retinoic acid (RA) on the synthesis of matrix metalloproteinase 2 (MMP-2, gelatinase) into the culture medium. Preconfluent primary cultures of avian growth plate chondrocytes in serum-free HL-1 were treated from days 7–14 (set A), or from days 14–21 (set B), and the media collected on days 14 and 21, respectively, and analyzed. In serum-containing DATP5, the cells were treated with varying levels of RA from days 7–14 (set C) or from days 14–21 (set D), and the media collected on days 14 and 21, respectively, and analyzed. MMP-2 activity in the culture medium was assayed by zymography using 0.2% gelatin in SDS–PAGE (see Methods). *Lanes 1–4* (set A), control, RA 10 nM, 50 nM, and 250 nM; *lanes 5–8* (set B), control, RA 10 nM, 50 nM, and 250 nM; *lanes 9–12* (set C), control, RA 10 nM, 50 nM, and 250 nM; *lanes 13–16* (set D), control, RA 10 nM, 50 nM, and 250 nM.

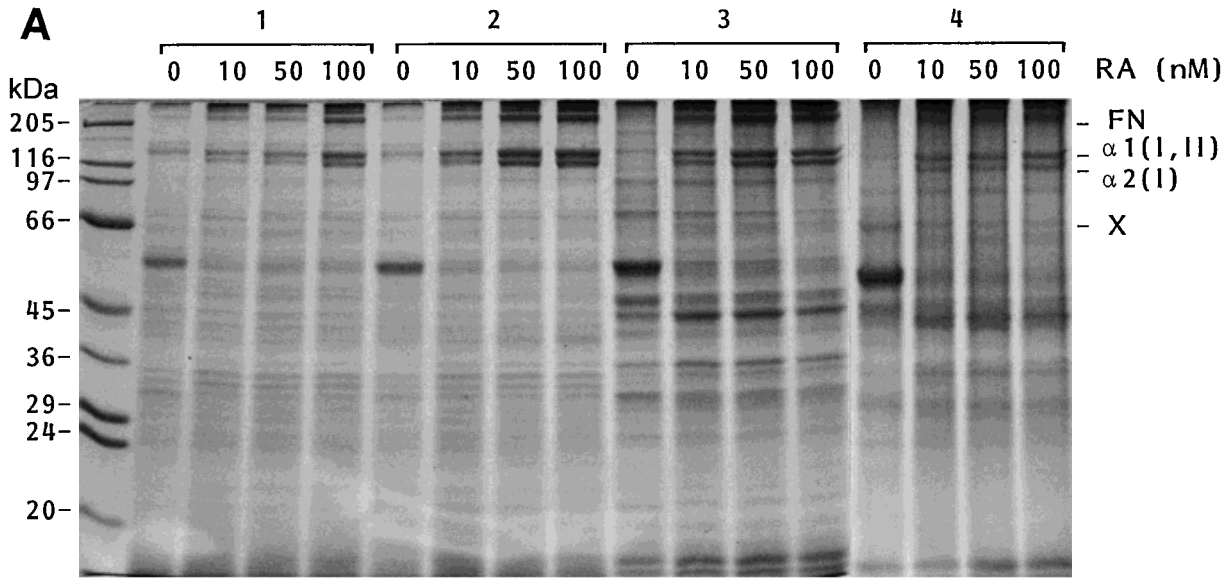
transfer to nitrocellulose paper. The commercially available anti-type I collagen antibody was tested for specificity and was found to bind only to type I collagen.

To confirm that RA also increased type I collagen levels in the cell-matrix layer, TMT sonicates, or pepsin digests, of the cell-matrix layer were subjected to Western blot analysis using anti-type I collagen antibodies. The Coomassie Blue-stained SDS–PAGE (Fig. 10, top) of the pepsin-soluble (left) and pepsin-insoluble pellet fractions (right) show that while both type II and X collagen were present, type I collagen was absent in the control cultures.

Fig. 8. Effect of all-trans retinoic acid (RA) on the type of collagen synthesized into the cell-matrix layer. Preconfluent primary cultures of avian growth plate chondrocytes grown in serum-containing DATP5 medium, were treated with varying levels of RA either from days 7–14 and analyzed on day 14 (set 1), from days 7–17 and analyzed on day 17 (set 2), from days 7–21 and analyzed on day 21 (set 3), or from days 7–14 and analyzed on day 21 (set 4). **A:** Proteins in the cell-matrix layer were extracted with TMT-buffer with sonication and an aliquot was analyzed by SDS–PAGE to show the cumulative effect of RA treatment. *Lanes 1–4* (set A), control, RA 10 nM, 50 nM, and 100 nM; *lanes 5–8* (set B), control, RA 10 nM, 50 nM, and 100 nM; *lanes 9–12* (set C), control, RA 10 nM, 50 nM, and 100 nM; *lanes 13–16* (set D), control, RA 10 nM, 50 nM, and 100 nM. FN, fibronectin; α1(I, II), α1 chain of type I and II collagen; α2(I), α2 chain of type I collagen; X, type X collagen. Note that a 55-kDa protein present in the cell-matrix layer was decreased with RA treatment. This band migrates to the position of pepsinized type X collagen (see Figs. 9, 10) but does not react with the 6F6 monoclonal antibody to type X collagen (**B**). Pepsinized type X collagen is not immunoreactive with the 6F6 monoclonal antibody [Wu et al., 1989]. **B:** Immunoblot of protein transferred from SDS–PAGE gels onto nitrocellulose paper and reacted with anti-type X collagen antibodies (see Methods). *Lane 1,* type X collagen standard, isolated from culture medium showed two bands of MW 70 kDa and 60 kDa (globular standards) corresponding to 59 kDa and 50 kDa type X; *lane 2,* prestained MW standards (123, 89, 58, 49, and 34 kDa); *lanes 3–6,* TMT sonicate of the DATP5 cell layer on day 21 (0, 10, 50, and 100 nM RA), treated from days 7–14; *lanes 7–10,* TMT sonicate of the HL-1 cell layer on day 21 (0, 10, 50, and 100 nm RA), treated from days 7–21. From many studies, we found that the continuous presence of RA in serum-free HL-1 medium was required to maintain decreased type X collagen synthesis into the cell/matrix layer.

With increasing levels of RA, it is evident that type I collagen was present in increasing amounts in both fractions. The Western blot (bottom), using antibodies to type I collagen, confirms this finding.

The effects just described suggest that RA has the capability to transform chondrocytes into cells that behave like osteoblasts. At this point we asked whether these in vitro effects of RA have any counterpart in vivo, or more explic-



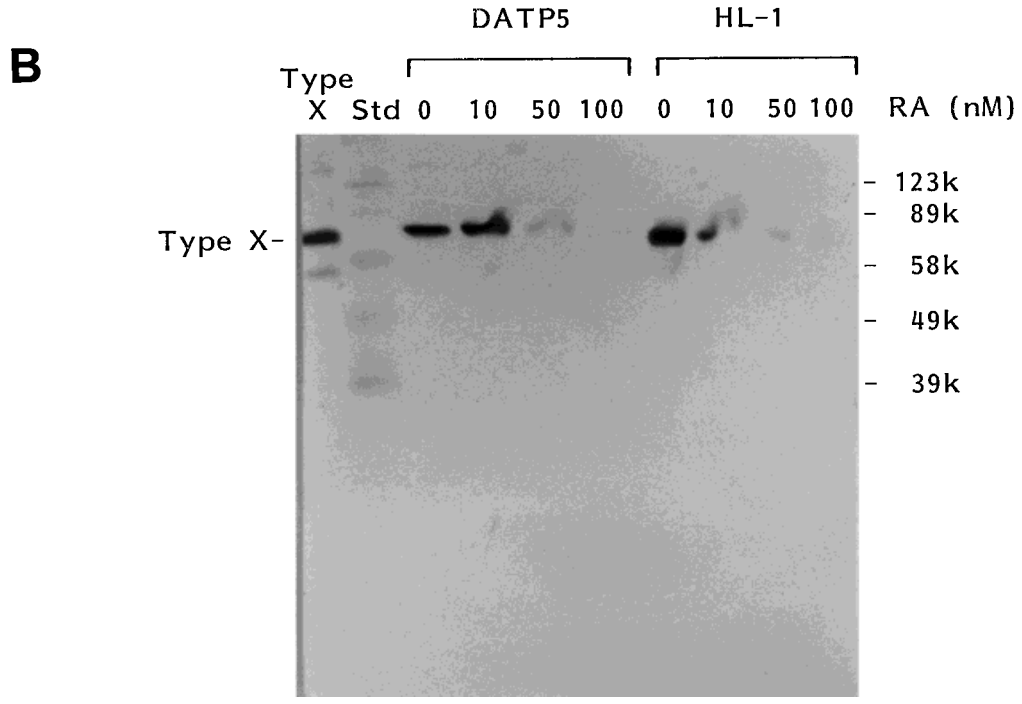


Figure 8.

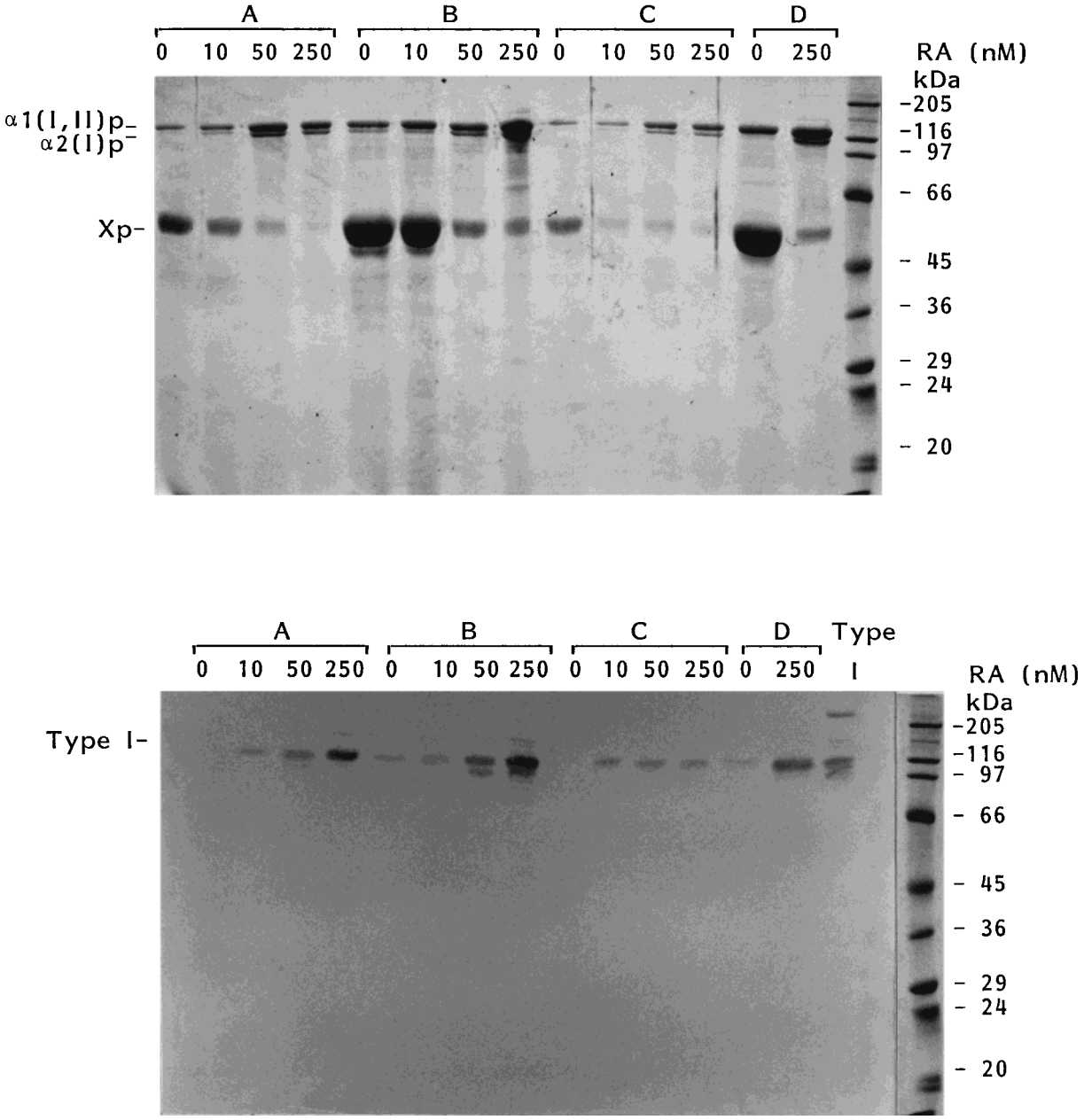


Fig. 9. SDS–PAGE and immunoblot analysis of the effect of all-trans retinoic acid (RA) on type I collagen synthesis into the culture medium. Collagen secreted into the culture medium was purified by precipitation with 30% saturation ammonium sulfate and subsequent treatment of the precipitate with pepsin digestion in 0.5 M acetic acid (see Methods). Preconfluent primary cultures of avian growth plate chondrocytes in either serum-containing DATP5 (*lanes 1–8*) or serum-free HL-1 (*lanes 9–14*) medium were treated with varying levels of RA from days 7–14 and analyzed on day 14 (set A, *lanes 1–4*), from days 14–21 and analyzed on day 21 (set B, *lanes 5–8*), from days 7–14 and analyzed on day 14 (set C, *lanes 9–12*), or from days 14–21 and analyzed on day 21 (set D, *lanes 13,14*). In sets A–C, the first lane is the control; the next three lanes correspond to treatment from 10, 50, and 250 nM RA, respectively. In set D, the first lane is the control; the second lane is 250 nM RA. *Top:* SDS–PAGE; *bottom:* Immunoblot of protein transferred from the SDS–PAGE gel onto nitrocellulose paper and reacted with anti-type I collagen antibodies (see Methods).

itly, whether the growth plate chondrocytes can switch phenotype and begin to act like osteoblasts. To answer this question, sections of avian growth plate were examined with the van Gieson stain (Fig. 11), a stain that has long been known to be specific for fibrillar, type I collagen [Fullmer, 1979]. It is important to note in the avian growth plate that capillary invasion does not occur in a uniform front but leaves islands of hypertrophic chondrocytes in areas well re-

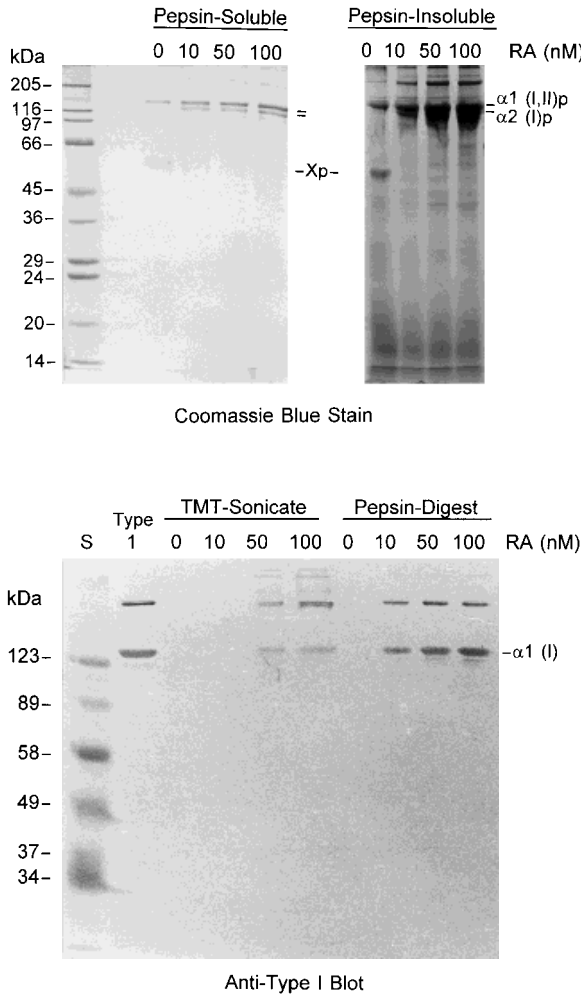


Fig. 10. SDS–PAGE and immunoblot analysis of the effect of all-trans retinoic acid (RA) on type I collagen synthesis into the cell-matrix layer. Preconfluent primary cultures of avian growth plate chondrocytes in serum-containing DATP5 medium were treated with 0, 10, 50, and 100 nM RA from days 7–21 and harvested on day 21. The TMS cell layer pellet was digested with pepsin in 0.5 M acetic acid. After centrifugation, the pepsin-digested collagen was analyzed by SDS–PAGE and stained with Coomassie Blue. *Top:* pepsin-soluble (*left*) and pepsin-insoluble (*right*) (see Methods). For verification of type I collagen induction by RA, TMT sonicates, or the entire pepsin digest, of the DATP5 cell-matrix layer was analyzed by SDS–PAGE, transferred to nitrocellulose, and probed with type I collagen antibodies. Bottom Panel: Prestained MW markers are shown in *lane 1;* type I collagen standard in *lane 2.* The next four lanes (*lanes 3–6*) represent TMT sonicates of the cell layer of cultures treated with the indicated levels of RA between days 7–14 and harvested on day 21. The last four lanes (*lanes 7–10*) represent samples obtained after pepsin digestion of the TMS pellet of the cell layer from cultures treated with the indicated levels of RA between days 7–21 and harvested on day 21 (see Methods).

moved from the site of capillary penetration. As is evident from Figure 11, the lacunae of numerous hypertrophic chondrocytes in this distal region of the growth plate reveal the presence of intense van Gieson stain, whereas the lacunae of other chondrocytes in the more proximal regions were van Gieson negative. The van Gieson-positive lacunae were well removed (several microns) from sites of capillary penetration with their accompanying osteoblastic progenitor cells. Further, the surrounding matrix was van Gieson negative. Thus the van Gieson-positive material must have been synthesized by the resident cells.

DISCUSSION

The response of cells to retinoids depends on their type and developmental stage; it can be either positive or negative [Iwamoto et al., 1993b]. The present study describes the effect of exogenous RA on growth, differentiation, and synthesis of specific matrix-related proteins by chicken growth plate chondrocytes in primary cell cultures containing ascorbic acid. The lower concentrations of RA used (10–50 nM) were

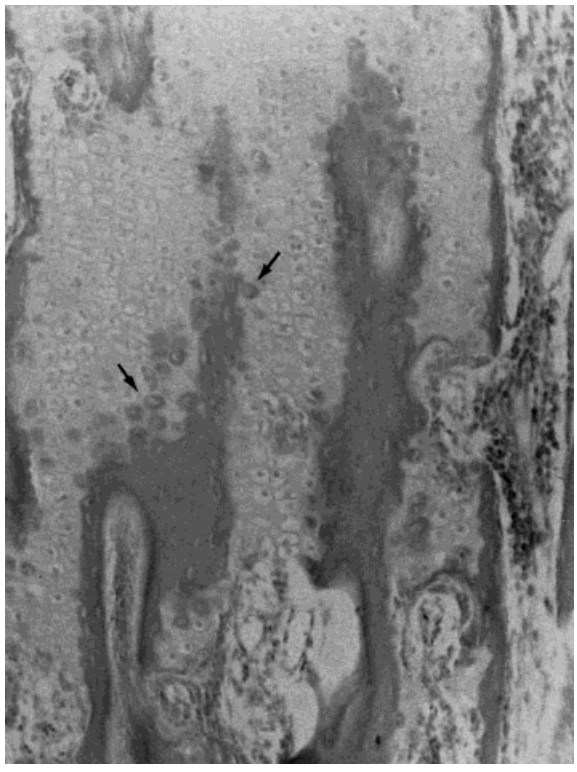


Fig. 11. Histological analysis of normal growth plate cartilage from 2-week-old chickens with van Gieson stain for type I collagen fibrils. The region shown is the distal growth plate at the site at which capillary penetration occurs in the region of calcified cartilage. Van Gieson-positive areas appear dark gray; negative areas are very light gray or white. Note that the matrix around the chondrocytes is generally negative, except near the sites of vascular penetration, where strongly positive stain can be seen around many of the hypertrophic chondrocytes (*arrows*). The matrix of the cancellous bone (type I collagen) is strongly positive to van Gieson stain.

physiological, similar to those observed in the posterior chick limb bud [Thaller and Eichele, 1987] and in serum [Satre and Kochhar, 1989]. We also used higher levels (100 and 250 nM) of RA to discern the RA-induced changes more rapidly. We found that RA typically caused a 2- to 5-fold increase in AP activity, depending on the dosage and duration of treatment. Administered either transiently or continuously, RA increased AP activity in both serum-containing and serum-free media. This observation of AP induction by RA is similar to that reported on chick embryo sternal chondrocytes [Iwamoto et al., 1993a; Chen et al., 1995].

It should be noted that our culture systems induce calcium phosphate mineral deposition, even in the absence of RA [Wu et al., 1989, 1995]. Based on analyses of Ca^{2+} and Pi in the cell-matrix layer, RA increased mineral formation in both serum-free and serum-containing cell cultures by 110–200% of the control level. Cells treated continuously with RA showed greater mineral deposition than those treated only transiently. Ca^{2+} deposition in RA-treated cultures has been reported previously, but in that case, the control cultures did not mineralize [Iwamoto et al., 1993a]. From those studies, it was concluded that RA directly stimulated endochondral calcification.

In our cultures, the effect of RA on the synthesis of cellular protein was more evident in serum-containing (DATP5) than in serum-free (HL-1) media. RA stimulated proliferation of chondrocytes (increased levels of DNA and total cellular protein) in serum-containing DATP5 medium. In serum-free HL-1 medium, transient exposure to RA also stimulated protein synthesis, but continuous exposure to RA, especially at higher concentrations, caused inhibition. These differing effects may result from the presence of serum albumin which would bind RA and buffer the amount accessible to the cells. Further, depending on the timing, duration, and level of RA administration, there may be a shift in the balance between proliferation, cell growth, differentiation, and programmed cell death [Herrlich and Ponta, 1994]. The effects of RA we observed on the growth plate chondrocytes included increased proliferation in the early phases (days 7–14) and inhibition of (HL-1) or no change in (DATP5) growth during the later phases (days 14–21) of development.

PG content of the cultures, both in the cell/matrix layer and in the medium, was greatly reduced by treatment with RA at levels $\geq$10 nM (Fig. 2A,B), indicating that at these levels, RA reduced PG synthesis. This decrease in PG is also consistent with earlier findings that indicate that RA stimulates resorption of the cartilage matrix. For example, in cultured rat epiphyseal chondrocytes, inhibition by RA of the expression of genes for type II collagen and PG core protein has been shown to be correlated with the upregulation of metalloprotease genes through the AP-1 transcription factors, c-*fos* and c-*jun* [Ballock et al., 1994]. In the current studies we show that RA increases the activity of a 72-kDa MMP in our primary cultures of avian growth plate chondrocytes in both serum-free and serum-containing media. As early as 16 h after treatment with 10 nM RA, MMP activity was enhanced (data not shown); this preceded the reduction in PG seen after 3 days of RA treatment. MMP are recognized as important enzymes for both normal connective tissue remodeling [Dean et al., 1989, 1992] and appear to be involved in the excessive matrix breakdown seen in rheumatoid arthritis [Woessner, 1991; Matresian, 1990]. Recent studies have shown that complete degradation of type X collagen requires the combined action of interstitial collagenases such as the 72-kDa gelatinase and osteoblast-derived cathepsin B provided by the invading osteoblasts [Sires et al., 1995]. We now have evidence that the 72-kDa MMP is present and is induced by RA in our growth plate primary cultures. It is possible that the increased expression of this protease may have contributed to the breakdown of type X collagen and the decreased levels seen after RA treatment.

In fact, in Figure 5 it should be noted that type X collagen secreted into the medium appears as a doublet, both forms of which react with the type X monoclonal antibody. The lower band (smaller form), which appears to be a proteolyzed version of type X collagen distinct from that produced by pepsin digestion, predominated when the cells were treated with the higher concentrations of RA in serum-containing media. We previously noted [Wu et al., 1989] that two forms of type X collagen are present in the culture medium. However, the lower band should not be confused with ovotransferrin observed by Cancedda et al. [1992], since that protein is not precipitated under the same salt conditions, and would have been hydrolyzed by pepsin. Thus, our findings support the possibility that increased metalloprotease activity could be partially responsible for the

decreased levels of type X collagen seen after RA treatment.

It is currently unclear from published data whether type X collagen is transiently induced by or decreased by RA, and whether type I collagen is switched on by RA. Thus, we examined the effect of RA on synthesis of type II, X, and I collagens in our ascorbate-containing media which allow assembly of properly hydroxylated collagens, and which undergo mineralization. It is apparent that in ascorbate-free cultures where type X collagen is minimally produced, RA (35 nM) can transiently increase either the mRNA level (36–48 h) or protein level (72–96 h) [Iwamoto et al., 1993b; Oettinger and Pacifici, 1990]. Transient induction of type X mRNA has also been described by Cockshutt et al. [1993] in an ascorbate-free medium in which the control cells did not show any type X mRNA expression. Both studies indicate that type X collagen expression must reach a critical setpoint before chondrocyte differentiation can progress forward, supporting the notion that RA promotes chondrocyte hypertrophy. Ultimately, however, the outcome is that type X collagen expression becomes downregulated after 72-h exposure to RA, and remains low thereafter [Oettinger and Pacifici, 1990]. We found that type X collagen levels in the culture media and cell layer are drastically reduced after 7-day exposure to RA at levels as low as 10 nM. The continuing presence of RA in both serum-containing and serum-free media potently suppressed type X collagen levels (Figs. 5, 6, sets B and C). The lack of a transient increase in type X collagen can probably be attributed to the abundance of type X collagen already present in our ascorbate-containing culture systems.

The decrease in type X and II collagens and the initiation of the synthesis of type I collagen suggest that growth plate chondrocytes become reprogrammed for an osteoblast-like function when exposed to appropriate levels of RA. Chondrocytes do not normally produce type I collagen; however, type I collagen has been observed to be deposited at the eroded surface of the cartilage matrix in the "transition zone" in embryonic chick bone development [von der Mark and von der Mark, 1977; von der Mark et al., 1976]. Further evidence of a transition from hypertrophic chondrocytic to osteoblastic cellular phenotype has been demonstrated recently in vivo at the chondro-osseus junction of long bones where synthesis of type I collagen has been observed in cells that still retain the chondrocyte surface antigen [Galotto et al., 1994].

In our cultures during continuous RA treatment, type X collagen synthesis was seen only transiently and then ceased, whereas synthesis of type I collagen was induced. Under these conditions, there was also a significant increase in mineral formation by the cultures, as well as a change to an osteoblastic-like morphology. Type X collagen is developmentally associated and is exclusively and transiently present at sites where calcification is occurring in hypertrophic chondrocytes. Transcription of type X collagen appears to occur before onset of mineralization [LuValle et al., 1992]. Nevertheless, the function of type X collagen remains a mystery. Complete knockout of type X collagen in mice causes no growth plate abnormality [Rosati et al., 1994]; however expression of an abnormal type X collagen causes serious disruption of growth plate development [Olsen, 1995]. In endochondral bone formation, growth plate chondrocytes pass through a series of stages of differentiation before they cease to divide, mature, become hypertrophic (synthesize type X collagen), and finally mineralize. The repression of type II and X, and the emergence of type I collagen synthesis, plus the enhancement of MMP-2 activity, indicate that in our cultures RA induced chondrocytic differentiation beyond the hypertrophic stage. This switch may represent another key transition point where chondrogenesis expands to osteogenesis.

In fact, it has been suggested by several investigators that hypertrophic chondrocytes can undergo further osteogenic differentiation, depending on their microenvironment [Cancedda, 1992; Lian et al., 1993; Iwamoto et al., 1994; Roach et al., 1995]. In Cancedda's studies, the addition of RA to the hypertrophic chondrocyte cultures accelerated maturation of the cells to produce type I collagen and ovotransferrin, and to acquire an osteoblast-like phenotype. The concept that chondrocytes progress towards an osteoblastic phenotype was also shown by Lian et al. [1993], demonstrating that bone-specific proteins (osteocalcin and osteopontin) are expressed by embryonic chondrocytes when they become hypertrophic. Similarly, Iwamoto et al. [1994] showed that genes for the bone-related proteins, osteonectin and osteopontin, were increased severalfold after treatment with 10–100 nM RA, indicating that RA can rapidly induce expression of late maturation genes related to mineralization. Roach et al. [1995] dem-

onstrated that the osteogenic differentiation of hypertrophic chondrocytes involves asymmetric cell divisions and apoptosis. Finally, a recent review by Gerstenfeld et al. [1996, and references therein] discusses various hypotheses concerning the sequence whereby growth cartilage differentiates to an osteoblastic phenotype and exhibits such gene products as alkaline phosphatase, osteopontin, osteonectin, osteocalcin, and bone sialoprotein. Thus, how hypertrophic chondrocytes progress to an osteoblastic phenotype has remained unsettled.

A possible lineage relationship between chondroprogenitors and osteoprogenitors for differentiation into mature cartilage and bone phenotypes has been previously proposed [Rosen et al., 1994]. It was speculated that cartilage retains its phenotype unless it is subjected to another inductive signal such as the bone morphogeneic proteins (BMPs) or others yet to be identified. Francis et al. [1994] showed that RA induces BMP-2 expression in the developing chick limb bud in vivo, and very recently RA has been found to greatly increase BMP-7 expression in maturing growth plate chondrocytes [Grimsrud et al., 1996]. The mirror image duplications of bones in the developing limb by RA appear to be mediated through the induction of sonic hedgehog [Wanek et al., 1991]. It is possible that RA induces the expression of select BMPs, which in turn affect further differentiation of hypertrophic chondrocytes into osteoblasts.

Finally, the effects of RA seen here on GP chondrocytes in culture appear to have direct counterparts in vivo. Penetration of vascular buds into the terminal zone of the growth plate would deliver Ca^{2+} and protein-bound RA to the growth plate chondrocytes, induce their differentiation to the hypertrophic state and their transformation to osteoblast-like cells, and thus contribute to formation of metaphyseal bone. An early manifestation of vitamin A deficiency in the growth plate is an alteration in bone growth [Moore et al., 1935]. In addition, histological examination of the terminal region of chicken growth plate cartilage with the van Gieson stain (Fig. 11) provides evidence of in vivo type I collagen synthesis by hypertrophic chondrocytes. This van Gieson-positive material is indistinguishable from that present in the matrix of cancellous bone, and the stain has been shown to be specific for type I collagen by Silbermann et al. [1983] in studies of the transformation of mandibular condyle cartilage progenitor cells to osteoblasts. Further, the close association between hypertrophic chondrocytes and osteoblasts (occupying the same lacunae) in avian growth plate has been shown earlier by Boyde and Shapiro [1987] and this concept has been extended recently by the elegant studies of Roach et al. [1995]. At this point, it is appropriate to mention that there are significant differences between mammalians and avians in the pattern of vascular penetration of the growth plate. In avians there are typically regions of calcified cartilage between the penetrating vascular channels. Thus, the survival of the hypertrophic cells and their expression of an osteoblastic phenotype may be a more prominent feature in avians than in mammals.

Thus in summary, we have shown that RA affects chondrocyte proliferation and the ongoing processes of hypertrophy and mineralization. In addition, RA decreased synthesis of the cartilage-specific proteins, and caused a transition from the synthesis of predominantly type II and X collagen to primarily type I, linking the regulatory events of growth plate differentiation to subsequent bone formation. However, the precise role of RA in transforming hypertrophic to osteoblast-like phenotype in the terminal cells of the avian growth plate remains to be established.

ACKNOWLEDGMENTS

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