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Regulation of Growth, Protein Synthesis, and Maturation of Fetal Bovine Epiphyseal Chondrocytes Grown in High-Density Culture in the Presence of Ascorbic Acid, Retinoic Acid, and Dihydroxycholesterol B

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Abstract Phenotypic expression of chondrocytes can be modulated *in vitro* by changing the culture technique and by agents such as vitamins and growth factors. We studied the effects of ascorbic acid, retinoic acid (0.5 and 10 μM), and dihydroxycholesterol B (3, 10, 20 μM DHCB), separately or in combination (ascorbic acid + retinoic acid or ascorbic acid + DHCB), on the induction of maturation of fetal bovine epiphyseal chondrocytes grown for up to 4 weeks at high density in medium containing 10% fetal calf serum and the various agents. In the absence of any agent or with retinoic acid or DHCB alone, the metabolic activity of the cells remained very low after day 6, with no induction of type I or X collagen synthesis nor increase in alkaline phosphatase activity. Chondrocytes treated with fresh ascorbic acid showed active protein synthesis associated with expression of types I and X after 6 and 13 days, respectively. This maturation was not accompanied by obvious hypertrophy of the cells or high alkaline phosphatase activity. Addition of retinoic acid to the ascorbic acid-treated cultures decreased the level of type II collagen synthesis and delayed the induction of types I and X collagen, which were present only after 30 days. A striking increase in alkaline phosphatase activity (15–20-fold) was observed in the presence of both ascorbic acid and the highest dose of retinoic acid (10 μM). DHCB was also a potent inhibitor of the maturation induced by treatment with ascorbic acid, as the chondrocytes maintained their rounded shape and synthesized type II collagen without induction of type I or X collagen. The pattern of protein secretion was compared under all culture conditions by two-dimensional gel electrophoresis. The different regulations of chondrocyte differentiation by ascorbic acid, retinoic acid, and DHCB were confirmed by the important qualitative and quantitative changes in the pattern of secreted proteins observed by two-dimensional gel electrophoresis along the study.

Key words: chondrocyte maturation; collagen types I, II, and X; ascorbic acid; retinoic acid; dihydroxycholesterol B

During endochondral ossification, chondrocytes undergo maturation from resting and proliferating cells to hypertrophic cells [Brighton, 1978; Hunziker, 1994]. This maturation process is controlled by programmed events, with changes in cell morphology and qualitative and quantitative variations in the synthesis of matrix macromolecules. Culture conditions and the presence of agents such as vitamins and

growth or differentiation factors can modulate the phenotypic expression of the chondrocytes [Solursh, 1989; Sandberg, 1991; Adolphe and Benya, 1992; Cancedda et al., 1995; Labourdette et al., 1996; Hickok et al., 1998]. After release from their cartilagenous matrix, rabbit differentiated articular chondrocytes grown in a monolayer on plastic surfaces rapidly lose their initial phenotype [Benya et al., 1978]. Under culture conditions that limit their attachment and proliferation, in liquid suspension [Horwitz and Dorfman, 1970; Pacifici and Oettinger, 1985; Tacchetti et al., 1987] or in a three-dimensional matrix such as agarose [Benya and Shaffer, 1982; Aydelotte et al., 1986], alginate beads [Guo et al., 1989; Häuselmann et al., 1992], or collagen gels [Gibson et al.,

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1982; Kimura et al., 1984], chondrocytes from various sources maintain their rounded configuration and a cartilage phenotype. Inhibition of chondrocyte spread by a high initial cell seeding density on plastic or by using the cell pellet technique has been shown by several authors to allow simple culture conditions for analysis of changes in chondrocyte gene expression during maturation in vitro. Under these conditions, fetal bovine epiphyseal or adolescent bovine articular cells [Harmand et al., 1982; Kuettner et al., 1982; Daniel et al., 1984; Ruggiero et al., 1993; Hering et al., 1994; Freyria et al., 1995a,b; Ronziere et al., 1997], chicken growth plate cells [Farquharson and Whitehead, 1995] rabbit growth plate cells [Kato et al., 1988; Iwamoto et al., 1989], or rat epiphyseal cells [Ballock et al., 1993] rapidly synthesize their own extracellular matrix, form multicellular layers, and can be maintained in culture for longer than 1 month.

Ascorbic acid and retinoic acid, factors known to modulate the phenotype of the chondrocytes, were frequently added in the different culture systems described to study the chondrocyte maturation. Most of these studies were conducted in chicken embryo utilizing either vertebra [Oettinger and Pacifici, 1990; Gerstenfeld and Landis, 1991; Adams et al., 1991] or sterna (caudal as immature chondrocyte zone and cephalic as mature chondrocyte region) [Yasui et al., 1986; Horton et al., 1987; Bruckner et al., 1989; Leboy et al., 1989; Dietz et al., 1993; Iwamoto et al., 1993a,b, 1994; Sullivan et al., 1994; Chen et al., 1995; Leboy et al., 1997; Venezian et al., 1998]. The chicken growth plate was also frequently used [Castagnola et al., 1986; Takishita et al., 1990; Wu et al., 1997; Nie et al., 1998]. These studies and studies with chondrocytes from other species such as quail [Sanchez et al., 1991], rat [Ballock et al., 1993], rabbit [Kato et al., 1988; Iwamoto et al., 1989], bovine [Hering et al., 1994; Koyano et al., 1996], and human [Kirsch et al., 1992; Stephens et al., 1992] and from different tissues showed that all of the cell types analyzed could undergo a sequence of changes up to the final maturation with type X collagen synthesis and increase in alkaline phosphatase activity, depending on the culture conditions and on the presence of appropriate activating agents. Thus, cells from the reserve (resting) zone and the permanent articular region can undergo hypertrophy, although

at a slower rate than cells from the growth plate.

In previous studies, we determined the composition and organization of the collagen network produced by fetal bovine epiphyseal chondrocytes in long-term culture at high density [Ruggiero et al., 1993] and the effect on these parameters of retinoic acid [Freyria et al., 1995a,b] and ascorbic acid [Ronziere et al., 1997]. In this paper, we report a study conducted to examine phenotypic modulation of the cells in the same culture model in the presence of ascorbic acid, retinoic acid (0.5 or 10 μM) and dihydrocytochalasin B (DHCB, 3, 10, and 20 μM), added separately or in combination (ascorbic acid + retinoic acid or ascorbic acid + DHCB). Cell morphology and proliferation, synthesis of total protein and collagen types I, II, and X in the cell layer and the medium and alkaline phosphatase activity were characterized under various culture conditions and various lengths of culture. The pattern of secreted proteins was determined by two-dimensional electrophoresis in order to elucidate the qualitative and quantitative modifications induced by these factors in fetal bovine epiphyseal chondrocytes.

MATERIALS AND METHODS

Cell Culture

Chondrocytes were isolated by enzymatic digestion from the reserve zone of the distal femoral epiphyses of 4-month-old bovine fetuses (five different animals were used in this study) after careful removal of the articular surface and the growth plate. They are noted here "fetal bovine epiphyseal chondrocytes" according to Koyano et al. [1996]. Chondrocytes were cultured at high density (0.8×10^6 cells/cm²) in RPMI/NCTC medium containing 10% fetal calf serum and antibiotic supplements, in the absence or presence of freshly prepared ascorbic acid (25 $\mu\text{g}/\text{ml}$) throughout the culture. The medium was changed every three days, as previously described [Ruggiero et al., 1993; Freyria et al., 1995a; Ronziere et al., 1997]. All-*trans* retinoic acid or DHCB was dissolved in 95% ethanol and added to the culture media at a volume of 0.2% after 2 days of culture and when the treated cultures were fed. Cells cultured with retinoic acid received 0.5 or 10 μM for 4, 11, 21, or 28 days, whereas cells cultured with DHCB received 3, 10, or 20 μM for 11 or 21 days; the control cultures received the same amounts of

ethanol. The cultures were analyzed after 6, 13, 23, and 30 days of culture.

Electron Microscopy

For transmission electron microscopy, cultures were fixed in 2% glutaraldehyde in phosphate-buffered saline for 30 min at room temperature and post-fixed in 2% osmium tetroxide in phosphate-buffered saline for 1 h. After dehydration in successive dilutions of ethanol, the material was embedded in Epon. Thin sections were mounted on copper grids, stained with uranyl acetate, and lead citrate and observed on a Jeol 1200 EX electron microscope at a voltage of 80 kV.

For scanning electron microscopy, cultures were fixed in a 4% glutaraldehyde cacodylate-buffered solution for 1 h at room temperature then washed for 1 h with 0.4 M sodium cacodylate, pH 7.4, and 360 mOsmol/L. The samples were dehydrated by acetone diffusion, critical point-dried, coated with gold palladium, and viewed under a S800 Hitachi microscope at 15 kV. All of these experiments were performed at the Centre for Electron Microscopy Applied to Biology and Geology, Université Claude Bernard, Lyon.

Analytical Procedures

Protein synthesis after 12 and 22 days of culture of DHCB-treated cells was measured by labelling duplicate cultures with 8 μ Ci/ml of 14 C-proline (290 mCi/mmol, NEN) for 24 h in fresh medium. For the retinoic acid-treated chondrocytes, labelling was performed after 5, 12, 22, or 29 days of culture with 20 μ Ci/ml of 35 S-methionine (1,000 Ci/mmol, SJ204 Amersham Pharmacia Biotech) for 14 h in fresh RPMI lacking methionine and in the absence of fetal calf serum, as previously reported [Freyria et al., 1995a]. After labelling, the media and cell layers were collected and treated separately, as previously described [Freyria et al., 1995a; Ronzière et al., 1997].

The amounts and types of collagens were monitored in both the medium and the cell layer fractions after salt precipitation and pepsin digestion [Freyria et al., 1995a]. Pepsin-digested samples were fractionated under reducing and non-reducing conditions on a 7% sodium dodecylsulfate-polyacrylamide gel (SDS) [Laemmli, 1970]. Aliquots containing a known number of dpm were loaded onto the gels. The bands corresponding to the various types of

collagens were visualized by fluorography, and the relative proportions of collagen types I and II were quantified by scanning densitometry with a Personal Densitometer (Molecular Dynamics). As the samples did not represent the same percentage of the corresponding matrix and culture medium and as they were not extracted from the same number of cells, these variations were integrated in the quantitative analysis.

The presence of type X collagen was detected by immunoblot analysis with a mouse anti-deer antibody (a generous gift from G. Gibson [Gibson et al., 1996]). Pepsin-digested samples containing aliquots equivalent to 150 μ l of the original cell layer extract were applied to each lane, reduced with dithiothreitol and electroblotted onto polyvinylidene difluoride membranes after electrophoresis on 10% polyacrylamide gels. After the membrane had been blocked with a 1% bovine serum albumin solution, the blotted proteins were immunostained with the antibody at a 1:200 dilution, a biotinylated second antibody, biotin-avidin-horseradish peroxidase complex and 4-chloro-1-naphthol as a color substrate.

The cellular DNA content was determined using bis-benzimidazole (Hoechst 33258) after extraction of the DNA according to Lipman [1989]. DNA calf thymus was used as standard. Cell number was calculated by using a conversion factor of 8 pg DNA per chondrocyte (personal data). Alkaline phosphatase activity was determined with *para*-nitrophenylphosphate as the substrate [Freyria et al., 1991]. Aliquots (50 μ l) of the cell layer lysate (0.1% Triton \times 100 in 0.1 M Tris-HCl pH 8.1; 600 μ l for a 25-cm² flask) were incubated after sonication with 250 μ l of substrate. Hydrolysis of the substrate was followed for 5–10 mn at 405 nm and 37°C. Proteins in cell lysates were measured with the Pierce microbicinchoninic acid assay kit and bovine serum albumin as the standard. Student's *t*-test was performed and significant differences were taken as $P < 0.02$.

The secreted proteins were analyzed by two-dimensional electrophoresis with Immobiline dry strips pH 3–10.5, pre-cast ExcelGel XL SDS, 12–14% acrylamide and ExcelGel SDS buffer strips (Amersham Pharmacia Biotech), as previously reported [Freyria et al., 1995a,b]. The culture media were labelled as for the collagen analyses with 50 μ Ci/ml of 35 S-methionine (1,000 Ci/mmol, SJ 204 Amersham). After

dialysis and freeze-drying, they were solubilized in lysis solution (9.8 M urea, 2% Nonidet P-40, 2% carrier ampholytes [1.7% pH 5–7 (Serva) and 0.3% pH 3–10 (Bio Rad SA)] and 100 μ M dithiothreitol. The same number of dpm were loaded for each sample at the cathodic side of the strip and focused at 15°C for 22 kVh. The Bio Rad two-dimensional polyacrylamide gel electrophoresis (PAGE) marker kit was used for determining molecular mass and pI. After SDS-PAGE, the gels were stained with Coomassie Blue, dried, and autoradiographed on reflection autoradiography film.

RESULTS

Cell Proliferation

When fetal bovine epiphyseal chondrocytes were grown for 30 days in high-density culture, the DNA content increased slightly throughout the culture in the absence of ascorbic acid, with $24 \pm 2.3 \times 10^6$ cells per flask at day 6 and $40 \pm 4.6 \times 10^6$ cells at day 30. The presence of ascorbic acid induced a three-fold increase in the DNA content at day 30 ($70.2 \pm 1 \times 10^6$ cells) as compared with the level in untreated cultures. The DNA content did not vary over time in the presence of 10 μ M retinoic acid ($21 \pm 3 \times 10^6$ cells at day 30), whereas a small increase was measured at day 30 ($39 \pm 1 \times 10^6$ cells) in cultures treated with ascorbic acid + retinoic acid as compared with the three-fold increase with ascorbic acid alone. The protein content of the cultures followed the changes in cell number.

When the cultures were treated with DHCB alone or with DHCB + ascorbic acid, a rapid decrease in the DNA content was measured, as cells detached themselves from the culture flask. At day 13, a mean 30% decrease was seen with each dose, which reached 50% at day 23.

Cell Morphology

Within 4 weeks in culture under the various conditions, the cells changed in shape and size and in the abundance of extracellular matrix. In the absence of ascorbic acid, the cells had a characteristic round or polygonal morphology with a surrounding matrix after 23 days (Fig. 1a), but after 30 days they appeared to have lost their round shape (Fig. 1e). In the presence of ascorbic acid, the cells showed extensive accumulation of extracellular matrix (Fig. 1c,g). In the presence of 10 μ M retinoic acid, the cells

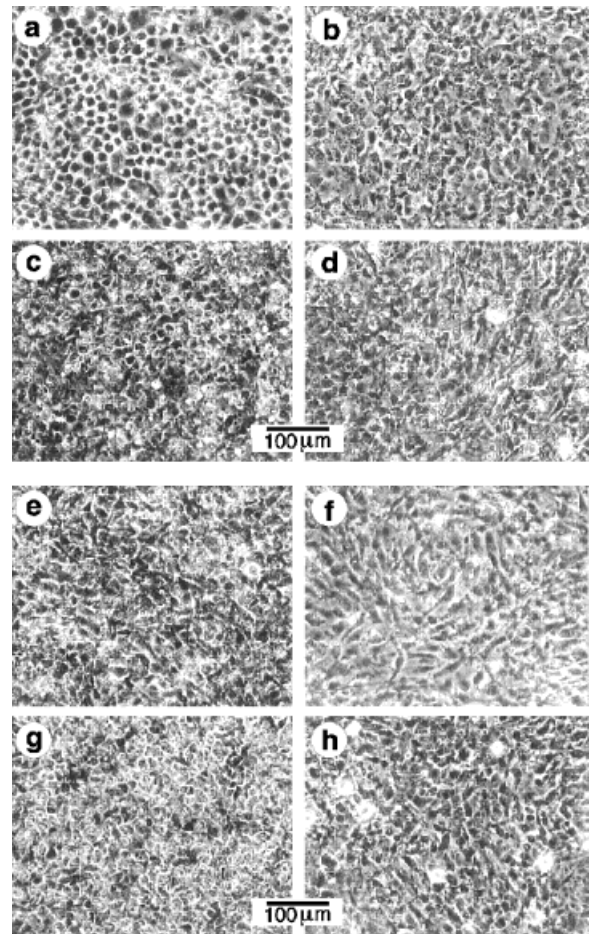


Fig. 1. Phase-contrast photomicrographs of fetal bovine epiphyseal chondrocytes treated with and without retinoic acid. Cultures were observed after 23 days (a–d) and 30 days (e–h) of culture: control (a,e), ascorbic acid-treated (c,g), 10 μ M retinoic acid-treated (b,f) and ascorbic acid + 10 μ M retinoic acid-treated chondrocytes (d,h); $\times 110$.

lost these characteristic traits and became longer and even smaller than in the control culture (Fig. 1b,f) as more and more were entrapped in the extracellular matrix. The ascorbic acid + retinoic acid-treated cells were shorter and were surrounded by an abundant matrix (Fig. 1d,h). The average cell size did not increase with time in culture, regardless of whether ascorbic acid was present.

The addition of DHCB was followed by a change in cell shape: after 24 h of treatment with either dose of DHCB, the polygonal cells became rounded and kept this morphology with time of culture (Fig. 2). In contrast to the other culture conditions, in presence of DHCB was observed in only slight deposition of extracellular matrix (Fig. 2e).

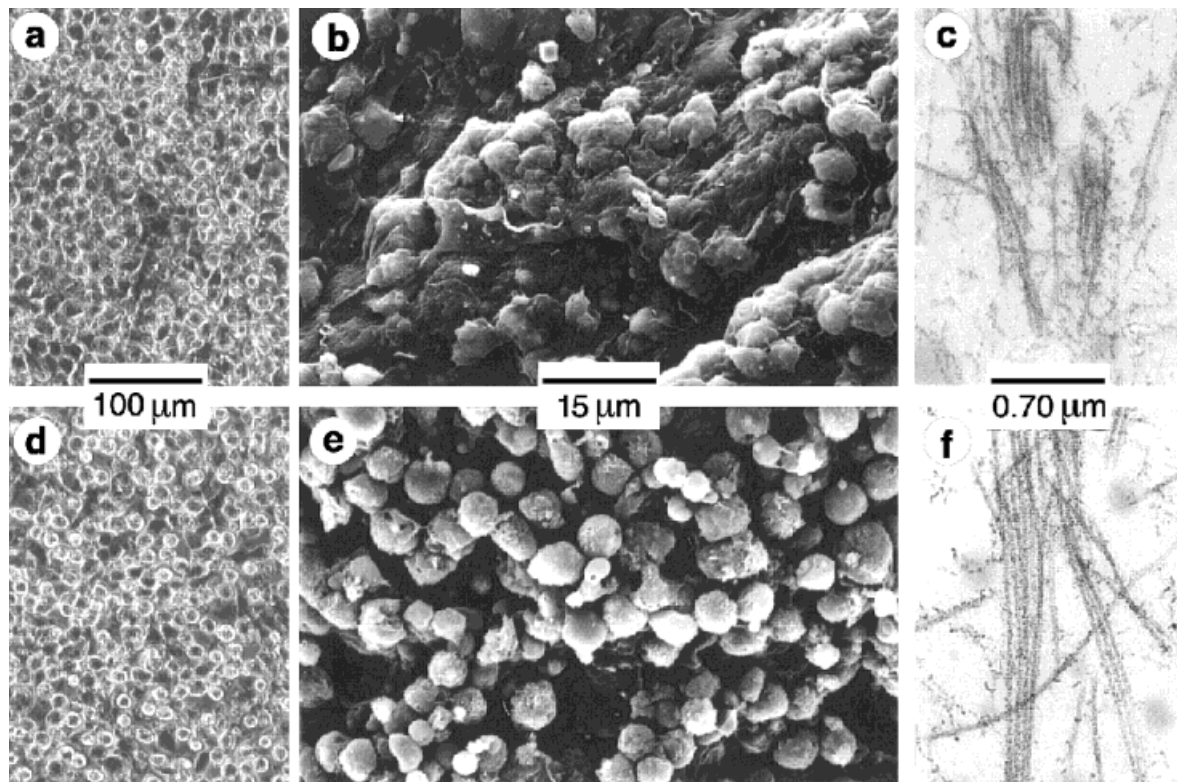


Fig. 2. Phase-contrast and scanning and transmission electron micrographs of fetal bovine epiphyseal chondrocytes treated with and without 10 μM dihydrocytochalasin B (DHCB) after 13 days of culture. No fresh ascorbic acid was added during this period. Chondrocytes in control culture are shown in **a**, **b**, **c** and in the DHCB-treated culture in **d**, **e**, **f**. Note the change in morphology and abundance of the pericellular matrix when comparing the controls **a** and **b** with DHCB-treated **d** and **e**. $\times 142$ (**a**,**d**) and $\times 10,000$ (**b**,**e**). Chondrocytes are surrounded by fine and striated fibrils (**c**,**f**); $\times 21,500$.

Deposition and Composition of the Extracellular Matrix

As seen by transmission electronic microscopy, cells in the control cultures grew close together throughout the period of culture and were embedded in a loose matrix composed mainly of collagen fibrils of about 13–18 nm in width (Fig. 3a,e), which were either isolated, irregularly spaced, or organized in bundles. In the presence of ascorbic acid, the cells were widely spread at day 23 (Fig. 3c), and at this time many thin filaments were observed that interconnected the fibrils (Fig. 3g) and resembled a proteoglycan structure. During the 3 weeks of treatment with 10 μM retinoic acid, the cells appeared to grow tightly together, with many contacts (Fig. 3b). The extracellular matrix, deposited as a thin network at the boundaries of the cells, was composed mainly of collagen fibrils with a mean thickness of 15 nm (Fig. 3f). In the culture with the two vitamins (Fig. 3d), the organization and composition of

the pericellular matrix (Fig. 3h) resembled that with retinoic acid alone. Chondrocytes grown with 10 μM DHCB were rounded and isolated (Fig. 2d), and there was a less abundant pericellular matrix containing fewer collagen fibrils (Fig. 2e); when they were present (Fig. 2f), they were of the same width as in the control culture (Fig. 2c).

Rates of Protein Synthesis and Collagen Typing

Changes in the total amount of protein, collagen synthesis, and typing in the presence of retinoic acid are presented on Figure 4. In control cultures with and without ascorbic acid, most total and pepsin-resistant protein synthesis occurred in the cell layer, with a net maximum at day 6. Addition of 0.5 μM retinoic acid suppressed this maximum, with an 80% decrease in type II collagen (Fig. 4c) and complete inhibition of the synthesis of type I collagen seen with ascorbic acid (Fig. 4d). On day 13, 10 μM retinoic acid were necessary to obtain simi-

Bovine Epiphyseal Chondrocyte Phenotype Modulation

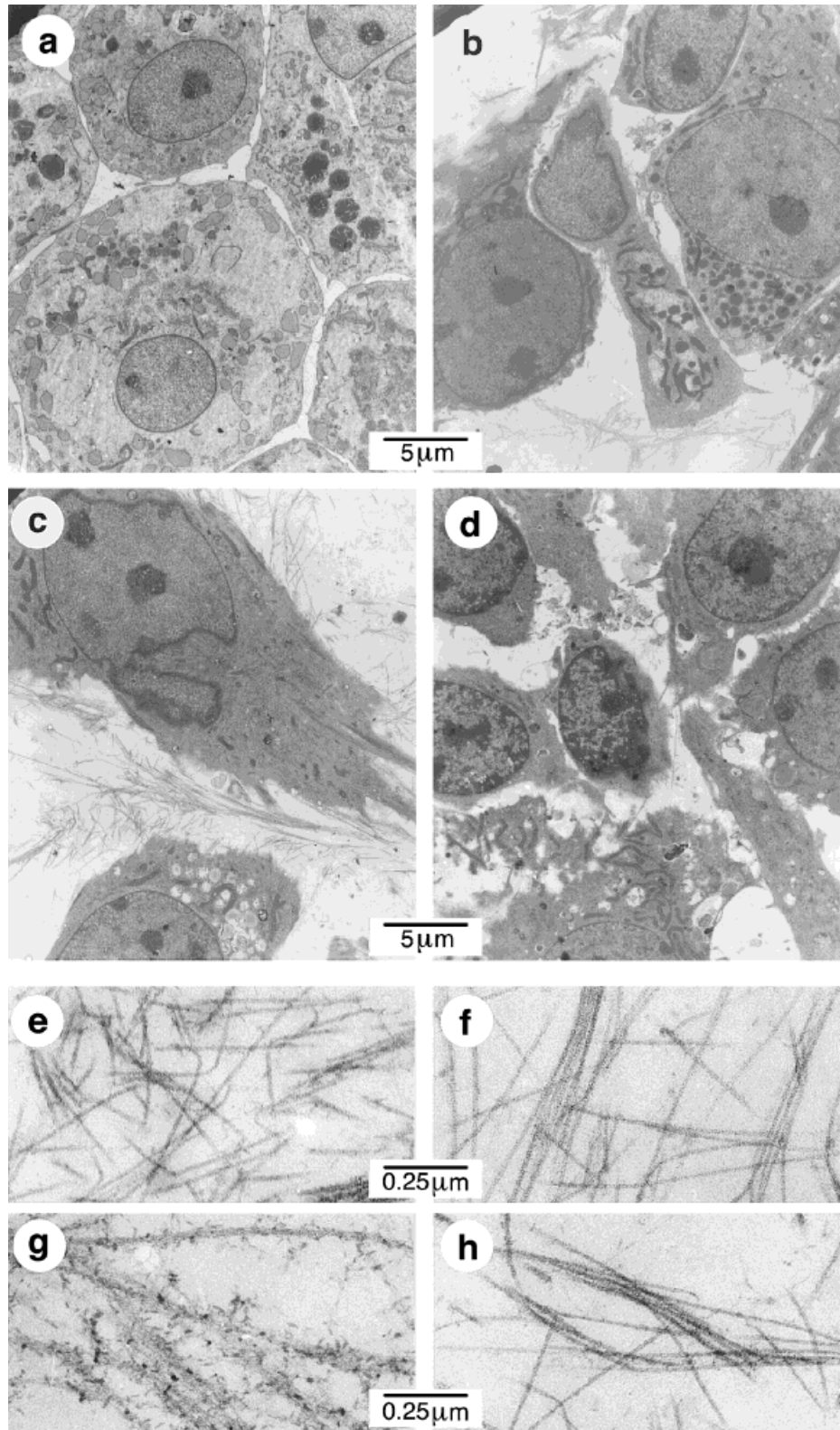


Fig. 3. Transmission electron micrographs of fetal bovine epiphyseal chondrocytes treated with 10 μ M retinoic acid for 23 days. Cells are shown in **a–d** and the extracellular matrix in **e–h**; control cultures are shown in **a** and **e**, retinoic acid-treated cultures in **b** and **f**, ascorbic acid-treated culture in **c** and **g** and ascorbic acid + retinoic acid-treated chondrocytes in **d** and **h**. $\times 2500$. Chondrocytes in the various culture conditions are surrounded by fine and striated fibrils (**e–h**); $\times 49,200$.

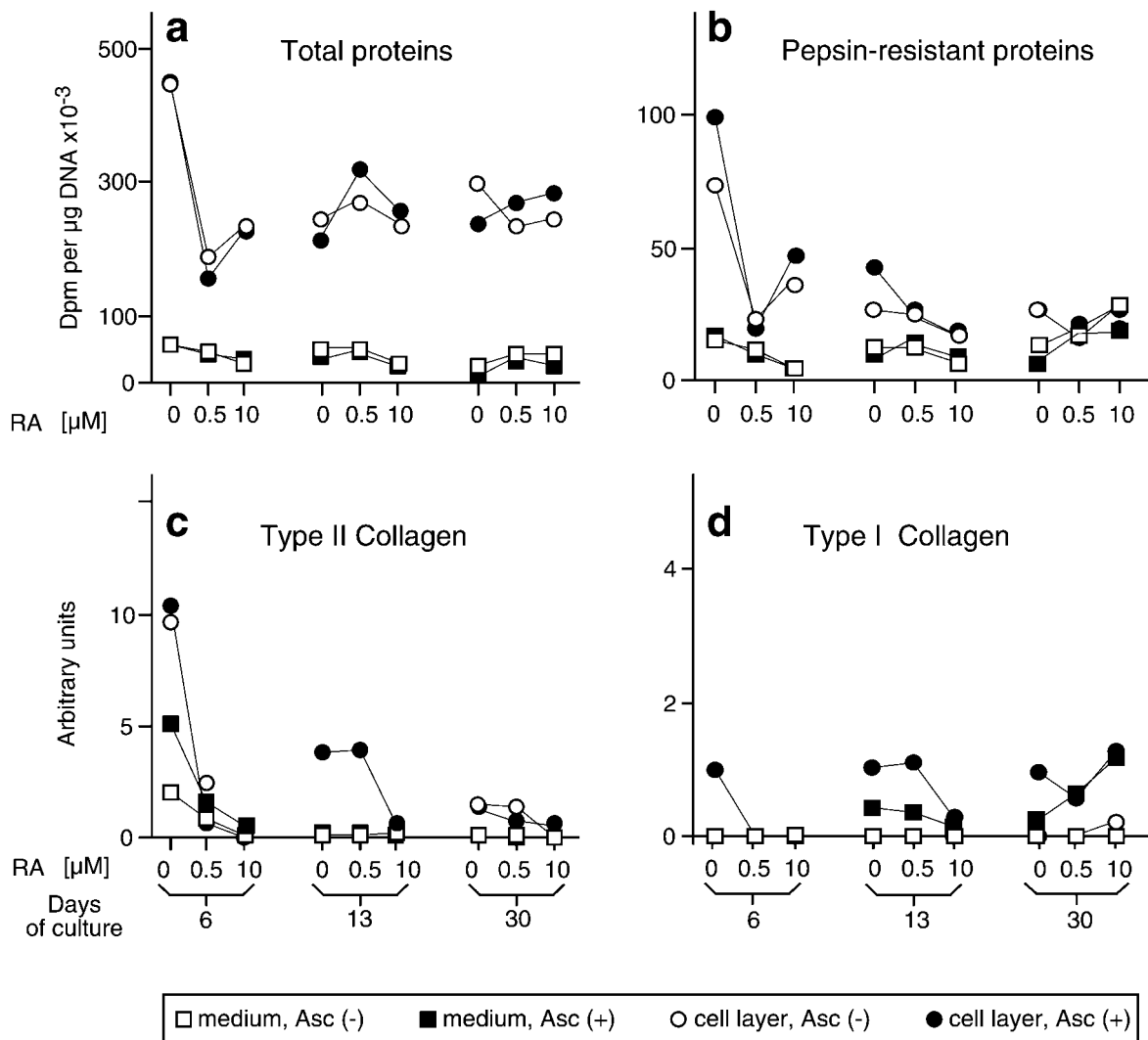


Fig. 4. Effects of retinoic acid (RA) alone and with ascorbic acid (Asc) on the uptake of ^{35}S -methionine into newly synthesized total (a) and pepsin-resistant proteins (b) and on the levels of types II (c) and I (d) collagen. Fetal bovine epiphyseal chondrocytes were grown in high-density culture in the presence or absence of 0.5 or 10 μM RA and/or Asc. Media and cell layers were collected on days 6, 13, and 30 after labelling for the last 14 h of each time and submitted to limited pepsin digestion. Samples were precipitated with alcohol, and aliquots equivalent to the same volume of extract were counted in

duplicate. The values in $\text{dpm}/\mu\text{g DNA} \times 10^{-3}$ are the means of counts for three to five different cultures. Cell layer and media samples containing the same number of dpm were run on a 7% sodium dodecyl sulfate-polyacrylamide gel to separate the collagens. The relative amounts of types II and I collagen were measured on the fluorographs by densitometry and expressed in arbitrary units as absorbance per microgram of DNA for the total amount in each samples. Values represent the data from one representative single experiment. Comparable results were obtained with chondrocytes isolated from two other fetuses.

lar decreases in types I and II collagen. By day 30, retinoic acid was much less effective: the synthesis level was very low, and type I collagen synthesis was still occurring in cultures with 10 μM retinoic acid + ascorbic acid, in both the medium and the cell layer (Fig. 4d).

In the study with DHCB, the rates of total and pepsin-resistant protein synthesis in the medium and the cell layer were comparable in

control cultures on days 13 and 23, and a two- to three-fold increase in protein was seen with ascorbic acid in the cell layer (Fig. 5). With 3 μM DHCB, a mean 65% increase in total and pepsin-resistant proteins was seen, independently of the presence of ascorbic acid (Fig. 5a,b). A slight increase in protein synthesis was seen with 10 μM DHCB, whereas in the presence of 20 μM the level of total protein synthe-

Bovine Epiphyseal Chondrocyte Phenotype Modulation

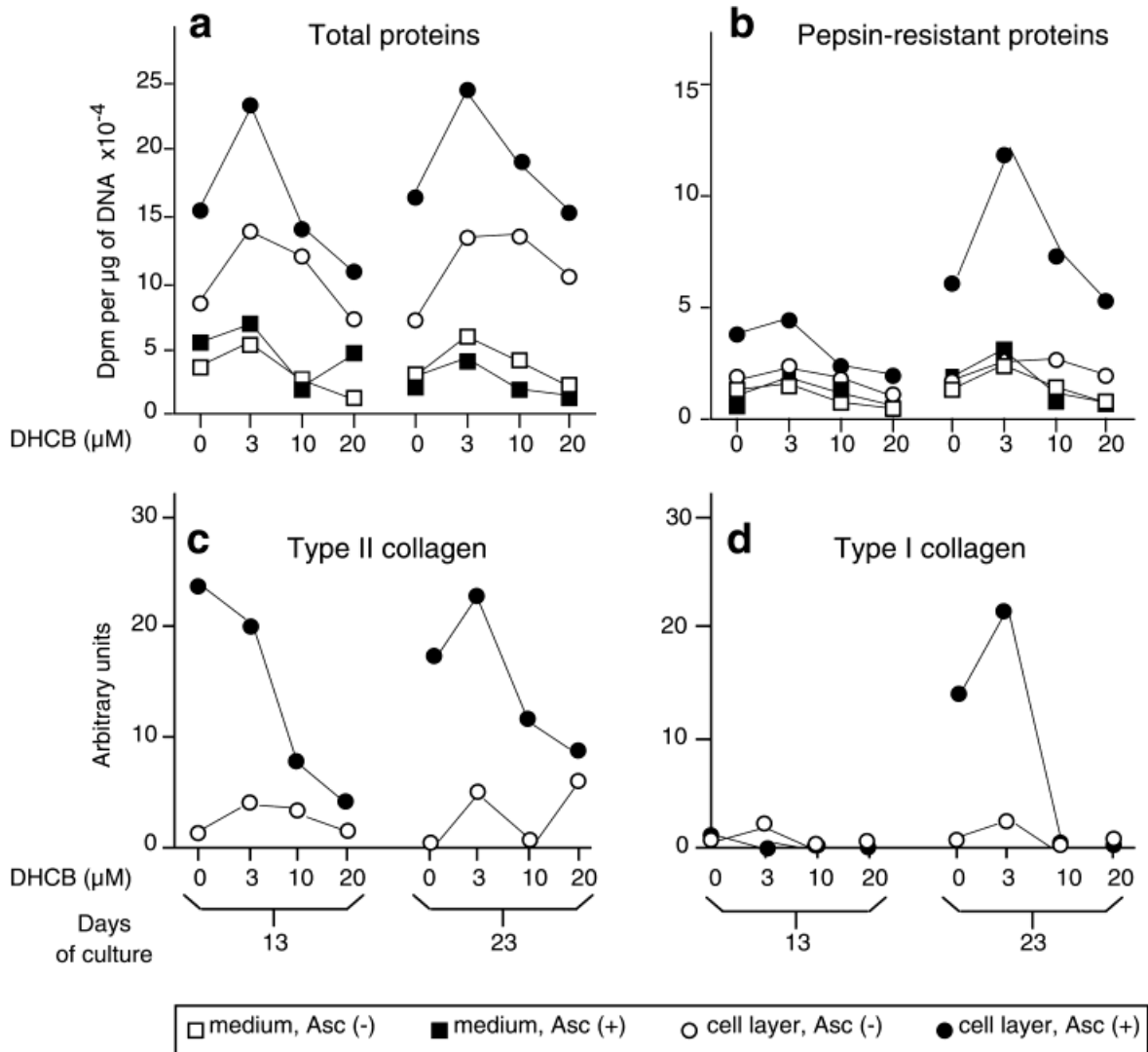


Fig. 5. Effects of dihydrocytochalasin B (DHCB) alone and in combination with ascorbic acid (Asc) on the uptake of ^{14}C -proline into newly synthesized total (a) and pepsin-resistant proteins (b) and on the levels of types II (c) and I (d) collagen. Fetal bovine epiphyseal chondrocytes were grown in high-density culture in the presence or absence of 3, 10, or 20 μM

DHCB and/or Asc. Media and cell layers were collected on days 13 and 23 after labelling for the last 24 h of each time and treated as specified in Figure 4. Values represent the data from one representative single experiment. Comparable results were obtained with chondrocytes isolated from one other fetus.

sis was close to or below that of the control culture (Fig. 5a). With all three doses, most of the total proteins (70%) and pepsin-resistant material (60%) was found in the cell layer. No change in type I or II collagen synthesis was observed in the cell layer at days 13 and 23 after treatment with 3 μM DHCB + ascorbic acid (Fig. 5). With 10 and 20 μM DHCB + ascorbic acid, a large decrease in type II collagen synthesis was observed on days 13 and 23, with complete inhibition of type I collagen syn-

thesis; the contents of types I and II collagen in the medium were too low to be measured.

Chondrocyte Differentiation Markers

Synthesis of type X collagen was analyzed in both the culture medium and the cell layer after treatment with retinoic acid (Fig. 6). Type X collagen was absent from all culture media throughout culture and from the cell layer in the absence of ascorbic acid with or without retinoic acid. Type X collagen was observed in

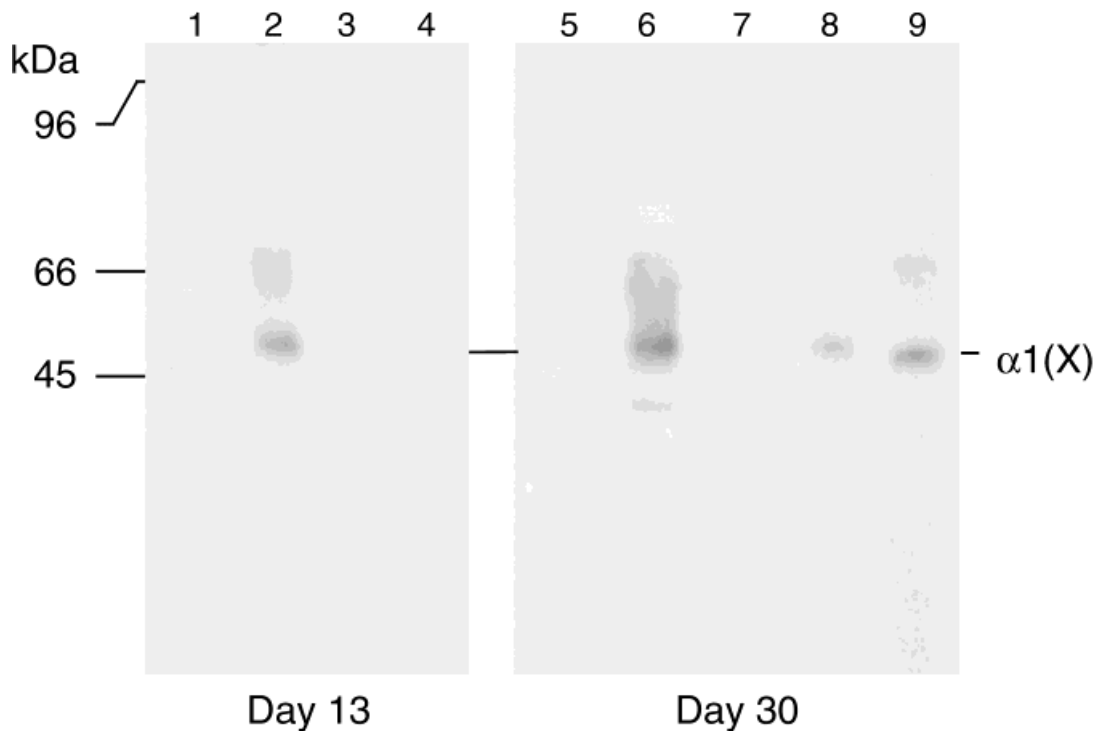


Fig. 6. Immunoblot analysis of the effects of ascorbic acid (Asc) and retinoic acid (RA) on type X collagen deposition in the cell layers of chondrocytes. Fetal bovine epiphyseal chondrocytes were grown in high-density culture in the presence or absence of 0.5 or 10 μ M RA and/or Asc. Cell layers were collected on days 13 and 30. The pepsin-digested collagens were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride mem-

brane and probed with antibodies to type X collagen. Untreated cultures (**lanes 1 and 5**), and Asc+ (**lanes 2 and 6**) and RA-treated cultures (**lanes 3 and 7**) are represented on day 13 and 30, respectively. The combined effects of RA + Asc are represented on day 13 with 10 μ M RA (**lane 4**) and on day 30 (**lanes 8 and 9**) with 0.5 and 10 μ M RA. Molecular mass markers are shown on the left axis.

TABLE I. Effects of Ascorbic Acid (Asc) and Retinoic Acid (RA) on Alkaline Phosphatase Activity (mU/10⁶ cells) in Fetal Bovine Epiphyseal Chondrocytes During 30 Days in Culture^a

Days of treatment	RA 0 μ M		RA 0.5 μ M		RA 10 μ M	
	Asc (-)	Asc (+)	Asc (-)	Asc (+)	Asc (-)	Asc (+)
13	135 \pm 24	167 \pm 33	187 \pm 47	531 \pm 357	378 \pm 113	1308 \pm 232
23	261 \pm 30	200 \pm 81	111 \pm 13	519 \pm 112	354 \pm 170	3148 \pm 292*
30	126 \pm 23	55 \pm 17	155 \pm 8	214 \pm 27	656 \pm 182	3565 \pm 89*

^aChondrocytes were grown in high-density culture in the presence or absence of 0.5 and 10 μ M RA and/or Asc. Cell layers were collected on days 13, 23, and 30 and alkaline phosphatase activity was measured. Values expressed in milliunit per million of cells (mU/10⁶ cells) represent the average levels \pm SD for three to five independent experiments.

* $P < 0.02$ when untreated cultures were compared with treated cultures.

the cell layer of ascorbic acid-treated cultures at days 13 and 30 of culture and in ascorbic acid + retinoic acid-treated cultures at day 30 (Fig. 6).

Table I shows the alkaline phosphatase activity in the cell layer during culture. A low mean level of 135.5 \pm 21 mU per 10⁶ cells was measured on days 13, 23, and 30 in the control culture, and addition of ascorbic acid did not change this level at any time. In the presence of

0.5 μ M retinoic acid, a two- to three-fold increase was observed only in the presence of ascorbic acid. With 10 μ M retinoic acid and in the absence of ascorbic acid, the increase was only three-fold on day 30. The presence of both ascorbic acid and 10 μ M retinoic acid strongly stimulated alkaline phosphatase activity in a time-dependent manner, increasing it statistically by as much as 12-fold by day 23 ($P < 0.02$) and 23–26-fold by day 30 ($P < 0.02$). No change

in activity could be detected when the cells were grown in the presence of DHCB.

Analysis of Secreted Protein by Two-Dimensional Polyacrylamide Gel Electrophoresis

In order to examine the extent of the changes in protein synthesis induced by the various culture conditions, the media were subjected to two-dimensional electrophoresis. We selected three spots on the autoradiographs for comparison of the proteins expressed on the gels. The synthesis of proteins with a molecular mass of less than 29 kDa was down-regulated between days 6 and 13 in the absence of ascorbic acid (Fig. 7a vs. b), whereas acidic proteins with a mass of 45–95 kDa were expressed. The presence of ascorbic acid maintained a high level of expression of the low-molecular-mass proteins after 12 days (Fig. 7e,f). The subsets of proteins 1, 2, and 3 were also up-regulated in comparison with the untreated culture. Cells grown in the presence of 10 μ M DHCB (Fig. 7c) ex-

pressed nearly the same pattern of secreted proteins as untreated cells in the absence of ascorbic acid (Fig. 7b), but DHCB inhibited the large increase in protein synthesis due to ascorbic acid alone (Fig. 7g). When 10 μ M retinoic acid were added to the culture, alone (Fig. 7d) or in combination with ascorbic acid (Fig. 7h), the cells had a different pattern of secreted protein from untreated cells (Fig. 7b,f): the low-molecular-mass proteins were mainly down-regulated as early as day 3 of treatment (not shown). Subsets 2 and 3 were no longer detected.

DISCUSSION

This study provides additional information [Ruggiero et al., 1993; Freyria et al., 1995a,b; Ronzière et al., 1997] about modulation of the phenotype of fetal bovine epiphyseal chondrocytes cultured for up to 30 days in high density in RPMI/NCTC medium containing 10% fetal calf serum, with or without fresh ascorbic acid

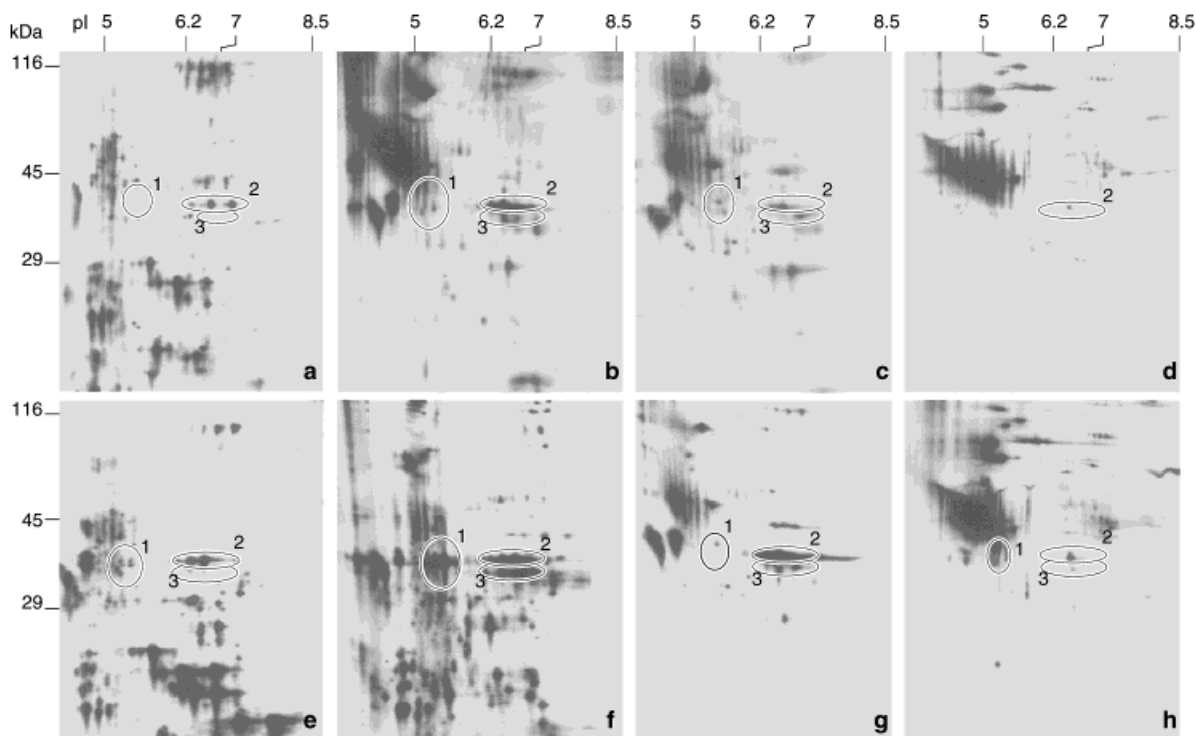


Fig. 7. Two-dimensional polyacrylamide gel electrophoresis autoradiographs of secreted proteins of chondrocytes collected on days 6 and 13 and labelled with 35 S-methionine for the last 14 h of culture. Samples containing the same number of counts per minute were separated in the first dimension on Immobiline dry strips pH 3–10.5 and in the second dimension on 12–14% acrylamide SDS ExcelGel XL. Control chondrocytes grown for 6 (a) or 12 days (b). Ascorbic acid-treated chondrocytes grown for

6 (e) or 13 days (f). Chondrocytes treated with 10 μ M dihydroxytolchalin B for the last 10 days in culture without (c) and with ascorbic acid (g); chondrocytes treated with 10 μ M retinoic acid for the last 10 days in culture without (d) and with ascorbic acid (h). The apparent molecular masses of known proteins are indicated on the left side. Gels present the data from one representative single experiment. Comparable results were obtained with chondrocytes isolated from one other fetus.

and in the presence of various concentrations of retinoic acid or DHCB. In the absence of ascorbic acid, chondrocytes have a low proliferation rate ($\times 1.5$ after 1 month), with a rapid decrease in total and pepsin-resistant protein synthesis after six days. The rate of type II collagen synthesis was very low at 13 and 30 days, and no type I collagen was synthesized. No deposition of type X collagen could be detected in the cell layer, and the alkaline phosphatase activity in the medium and cell layer remained very low. Thus, under these conditions, epiphyseal chondrocytes have a stable phenotype with low metabolic activity, as observed *in vivo*.

Addition of fresh ascorbic acid (25 $\mu\text{g/ml}$) induced extensive changes in chondrocyte growth and metabolism and in the formation of the extracellular matrix, leading to maturation of the cells: cell proliferation was induced ($\times 3$ after 30 days), and type II collagen was still being synthesized after 13 days; type I collagen synthesis was seen after 6 days (in the cell layer), and type X collagen was present after 13 days. After 30 days, both types I and X collagen were present in the cell layer, whereas the rate of synthesis of type II collagen and the alkaline phosphatase activity were very low. The presence of type X collagen was not associated with cell hypertrophy, in accordance with results obtained *in vivo* in the chick growth plate [Oshima et al., 1989] and *in vitro* with immature sternal chondrocytes [Pacifci et al., 1991a,b] or with cells isolated from the mandibular ectomesenchyme [Ekayanake and Hall, 1994]. Similar data on proliferation, the expression of types I and II collagen genes and the synthesis of these types were observed [Daniel et al., 1984; Hering et al., 1994] with adolescent bovine articular chondrocytes plated at high density and grown in the presence of ascorbic acid. Several studies with chicken chondrocytes (epiphyseal, growth plate, or sternal cells) have shown that ascorbic acid stimulates type X collagen synthesis and matrix calcification [Taccetti et al., 1987; Leboy et al., 1989; Wu et al., 1989; Gerstenfeld and Landis, 1991; Shapiro et al., 1991; Sullivan et al., 1994; Leboy et al., 1997; Farquharson et al., 1998]. Ballock et al. [1993] showed that chondrocytes isolated from the reserve zone of the epiphyses of neonatal rats and cultured as a three-dimensional cell pellet with fresh ascorbic acid acquired a hypertrophic phenotype by day 28 with expression of type X collagen and inhibition of type II colla-

gen. This terminal differentiation was inhibited by TGF beta-1. In contrast, Kirsch et al. [1992] have shown that in the absence of ascorbic acid fetal human chondrocytes in long-term culture in suspension over agarose differentiate into so-called 'post-hypertrophic' chondrocytes, which synthesize types I and X collagen. Under these culture conditions, addition of ascorbic acid during the initial phase inhibited the passage to hypertrophic cells, perhaps due to production of radicals to which human chondrocytes may be more sensitive than bovine, rat, or chicken chondrocytes [Tschan et al., 1990].

Retinoic acid is one of the more biologically active derivatives of retinol (vitamin A). It functions as an important regulatory signalling molecule for cell growth and differentiation both during embryogenesis and in the adult animal [Gudas, 1994]. It regulates the expression of genes directly through interactions with DNA-binding retinoic acid receptors, the gamma subtype of which is found predominantly in cartilage [Kastner et al., 1990]. We have shown here that addition of high doses of retinoic acid (0.5 and 10 μM) to a culture medium in the absence of fresh ascorbic acid does not modify the metabolic activity or the phenotype of fetal bovine epiphyseal chondrocytes in high-density culture. Addition of retinoic acid in combination with ascorbic acid decreased the cell proliferation induced by ascorbic acid and suppressed the high level of total protein and collagen II and I synthesis observed at day 6. The synthesis of types I and X collagen was delayed but these two collagens were present at day 30. The presence of type X collagen, a marker of maturation, was again not associated with hypertrophy of the cells, as already shown with ascorbic acid without retinoic acid. Interestingly, with 10 μM retinoic acid + ascorbic acid, we observed a striking increase in alkaline phosphatase activity in comparison with that seen with each vitamin alone (15–25-fold increase at days 23 and 30). At day 30, no mineralization of the matrix was found, probably because beta-glycerophosphate, a well-known activator of calcification, was not added to the culture medium [Chung et al., 1992; Coe et al., 1992].

Previous studies have also shown that retinoic acid inhibits the expression of the genes coding for cartilage matrix proteins such as type II collagen [Benya and Padilla, 1986; Horton et al., 1987]. It has also been shown that retinoic acid induces maturation and matrix

mineralization specifically in pre-hypertrophic cells, isolated from the cephalic portion of the chicken sternum or from growth plate cartilage [Oettinger and Pacifici, 1990; Pacifici et al., 1991a,b; Iwamoto et al., 1993a, 1994; Wu et al., 1997]; however, alkaline phosphatase and mineralization were not induced in immature chondrocytes isolated from the caudal region of the chick embryo sternum in the presence of 35 or 100 nM retinoic acid, with or without ascorbic acid [Pacifici et al., 1991b; Iwamoto et al., 1993a, 1994]. Thus, our results demonstrate for the first time that combined treatment with ascorbic acid and high doses of retinoic acid is necessary to induce both maturation of fetal epiphyseal chondrocytes and stimulation of alkaline phosphatase activity.

Regulation of the gene expression of alkaline phosphatase by retinoic acid (1 μ M) has been demonstrated at both the transcriptional and post-transcriptional level [Zhou et al., 1994]. Other mechanisms of action of retinoids in chondrocytes include modulation of their integrin-mediated attachment to extracellular matrix proteins [Loeser, 1994; Sanchez et al., 1996] and up-regulation of metalloprotease genes [Balloch et al., 1994; Nie et al., 1998]. The importance of retinoic acid in regulating protein synthesis by fetal epiphyseal chondrocytes is well demonstrated by the numerous modifications induced in the pattern of secreted proteins, at both the quantitative and qualitative levels. Two-dimensional electrophoresis is a powerful technique for analysing complex mixtures of proteins, and specific differences in map composition were revealed when extracellular proteins derived from fibroblastic and osteoblastic cells were compared [Hankey et al., 1992]. This technique was used by Mathieu et al. [1994] to identify novel proteins as markers of osteoblast differentiation. In our laboratory, changes in the chondrocyte phenotype were recorded in two-dimensional patterns after induction by retinoic acid as a decrease in type II collagen synthesis and expression of the small proteoglycan, decorin [Freyria et al., 1995b]. In the present study, major changes occurred on day 13, when type I collagen was strongly expressed in culture with ascorbic acid and suppressed by retinoic acid. This inducer further counteracted the effect of ascorbic acid on the expression of various proteins in the extracellular compartments. In the culture grown without ascorbic acid, in which retinoic acid delayed maturation

of the chondrocytes, little variation was seen in the level of secreted proteins, suggesting that the mechanisms for this effect involve few extracellular proteins. Another important inhibitor of the maturation of fetal bovine epiphyseal chondrocytes induced by ascorbic acid in our culture conditions is DHCB. After addition of 10 or 20 μ M DHCB to the culture medium, with or without fresh ascorbic acid, chondrocytes maintained their round morphology and showed a dramatic decrease in total and collagen protein synthesis, leading to a low deposition of extracellular matrix and partial detachment of the cells. Synthesis of types I and X collagen and alkaline phosphatase activity were completely inhibited, demonstrating that a cartilage phenotype with a low level of type II collagen synthesis and a pattern of secreted proteins is maintained after 13 days. This is very similar to the pattern obtained without ascorbic acid. It was reported previously that DHCB, a microfilament-modifying agent, enhances the expression of chondroblastic markers by rat mesenchymal cells cultured with cartilage-inducing factor [Rosen et al., 1986] or can cause restoration of type II collagen synthesis by dedifferentiated chondrocytes [Benya et al., 1988; Brown and Benya, 1988; Benya and Padilla, 1993] indicating a potential role of the cytoskeleton in the maintenance of the phenotype. The disruption of the cytoskeleton microfilament by DHCB has been also reported to inhibit beta-1 integrin increased-expression by TGF-beta-1 in articular chondrocytes [Loeser et al., 1995]. Cytochalasin B was reported to inhibit the transport of dehydroascorbic acid in human neutrophils through the inhibition of the hexose transport system [Vera et al., 1993]. Similar mechanisms may be involved, in the bovine epiphyseal chondrocytes, regarding the inhibition of ascorbic acid effects by DHCB.

In conclusion, the phenotype of fetal bovine epiphyseal chondrocytes appears to be modified readily in high-density culture by the addition of inducers such as ascorbic acid, retinoic acid and DHCB. Depending on the culture conditions, cells showed a maintained, a partially dedifferentiated or a terminally differentiated phenotype with expression of type X collagen, with or without high alkaline phosphatase activity. Our two-dimensional PAGE analyses have also shown that the changes in the chondrocyte phenotype are accompanied by extensive changes in the pattern of excreted proteins.

Complete identification of these proteins would be useful for defining the mechanisms involved more precisely.

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