The role of cell adhesion in retinoic acid-induced modulation of chondrocyte phenotype

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Retinoic acid (RA) treatment of a suspension of quail chondrocytes inhibits the expression of cartilage collagens and induces cell adhesion along with fibronectin expression. We asked whether the RA-induced modulation of the chondrocyte phenotype was dependent on cell adhesion. Prevention of cell adhesion blocks cell growth and many of the effects associated with RA, such as collagen II inhibition, collagen I activation and fibronectin induction. The activity of the bone/tendon promoter of the $\alpha 2(I)$ collagen gene was determined by measuring the transient expression of COL1A2-CAT, a chimaeric gene bearing 3500 bp from upstream of the transcription start site of the human $\alpha 2(I)$

gene fused to the chloramphenicol acetyltransferase (CAT) gene. This promoter is activated only in permissive conditions for cell adhesion. The attachment activities of chondrocytes on protein substrates was studied by an *in vitro* cell adhesion assay. Untreated cells or cells maintained in suspension while undergoing RA treatment do not attach when replated on protein substrates. Chondrocytes treated with RA in permissive conditions for cell adhesion rapidly attach and spread instead on collagen-coated wells. Altogether the results suggest that cell adhesion plays a major role in RA-induced modulation of the chondrocyte phenotype.

INTRODUCTION

All-*trans* retinoic acid (RA) and other vitamin A derivatives are known to have profound effects on cell proliferation, differentiation and pattern formation [1]. The action of RA is mediated through a family of nuclear receptors homologous to steroid and thyroid hormone receptors [2–4]. In addition to the RA receptors (RARs) [5,6], RA is also recognized with lower affinity by cytoplasmic binding proteins and by 9-*cis* RARs, the nuclear retinoid X receptors [7]. The retinoid X receptors can also form heterodimers with the RARs and other members of the steroid superfamily [8–10]. RA has been shown to modulate the collagen expression pattern of chondrocytes *in vitro* at doses of 0.01–10 μ M [11–17]. Exposure of quail embryo chondrocytes to 0.5 μ M RA for 3 days causes them to lose the differentiated phenotype [18]. After RA addition, the previously floating chondrocytes adhere to the dish, assume a flattened dedifferentiated shape and synthesize fibronectin. RA-treated chondrocytes can re-express collagen II if they are cultured in the absence of RA for an additional 15 days. We have reported [19] that, during the recovery to the chondrocyte phenotype, $\alpha 2(I)$ collagen gene expression is induced and the transcription activation of this gene results from the utilization of the bone/tendon promoter [20].

Fibronectin and cell adhesion are irreversibly induced by RA [18]. Once withdrawn from RA medium, cells continue to synthesize fibronectin and keep growing as adherent cells, assuming a polygonal morphology like that of typical chondrocytes growing in monolayer.

In the present report we consider whether the changes in gene expression of matrix proteins might be subsequent to changes occurring upon cell adhesion or be a direct effect of RA and its nuclear receptors on the regulation of these genes. Therefore we prevented RA-induced cell adhesion by culturing chondrocytes on agarose-coated dishes, and analysed cell growth and ex-

pression patterns of extracellular matrix proteins in treated chondrocytes. We studied, at protein and mRNA levels, the expression of collagens I, II and IX along with fibronectin and $\beta 1$ integrin subunit genes in treated chondrocytes cultured in ‘suspension’ (on agarose-coated dishes) or in ‘monolayer’ (on tissue-culture dishes, i.e. in permissive conditions for RA-induced cell adhesion). Moreover, we developed an *in vitro* cell adhesion assay to study the attachment activities of chondrocytes treated with RA in permissive and non-permissive conditions for cell adhesion.

Altogether the data presented support the hypothesis that alterations of cell–matrix interactions occurring upon RA-induced cell adhesion play a major role in RA-induced modulation of chondrocyte phenotype.

MATERIALS AND METHODS

Cell culture

Chondrocytes were isolated from 10-day-old quail embryo tibiae as previously described [21]. Floating cells were resuspended in Coon’s modified F12 medium [22] supplemented with 10% (v/v) foetal calf serum (FCS); after addition of RA, cells were plated at 1×10^5 cells/ml. All-*trans*-RA (Hoffmann–La Roche) was dissolved in 95% ethanol and stored at -80°C in the dark. This solution was diluted with growth medium on day 0, and control cultures received an equivalent amount of ethanol. Chondrocytes were treated with RA for 72 h and subsequently cultured in normal medium for about 7 days. The experiments reported were performed on chondrocytes exposed to RA at 0.5 μ M; no toxic effect was ever detected at this concentration in treated cultures. All the experiments described in this report were carried out in ordinary FCS. Parallel experiments in which delipidized serum was used did not show significant differences in the expression

Abbreviations used: RA, retinoic acid; 1,25(OH) $_2$ D $_3$, 1,25-dihydroxyvitamin D $_3$; FCS, fetal calf serum; COL1A2, $\alpha 2(I)$ collagen; tK, thymidine kinase; RAR, retinoic acid receptor; RARE, RAR-responsive element; CAT, chloramphenicol acetyltransferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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patterns of extracellular matrix genes. To prevent cell adhesion promoted by RA treatment, culture dishes were coated with a film of agarose.

Preparation of extracellular-matrix-protein substrates

Tissue-culture plates (96-well plate; Falcon) were coated with bovine plasma fibronectin (Sigma) by overnight incubation at 4 °C. For collagen substrates, collagen I from calf skin (Sigma) or collagen II from chick embryo sterna was solubilized in acetic acid and then dialysed against culture medium. Purity of collagens was more than 95 % as assessed by SDS/PAGE. Wells were coated with 100 μ l of collagen solution (100 μ g/ml), excess solution was removed, and the plates were dried at room temperature. All plates were washed twice with PBS before use.

Cell adhesion assay

After trypsinization, cells were resuspended in medium containing 0.3 % FCS, plated on to coated wells (70 000 cells/well) and allowed to attach for 45 min or overnight. Fibroblasts were allowed to attach for 10 min and 3 h respectively. All assays were performed in octuplicate and the experiments repeated several times. After incubation, wells were washed three times with PBS to remove the unattached cells. Attached cells were then fixed and stained with 0.5 % Crystal Violet in 20 % methanol. After extensive washings with distilled water, plates were allowed to dry and colour yields were measured with a Bio-Rad ELISA Reader using a 570 nm filter. In this assay, colour yields are proportional to the number of attached cells. Colour yields read after short incubations were normalized to the highest yield observed after extended incubations. To evaluate the percentage of attached cells corresponding to the highest yield, parallel experiments were performed with protein-coated dishes. After 16 h, cells were detached with collagenase/trypsin and counted. We evaluated that, when dealing with chondrocytes pretreated with RA in permissive conditions for cell adhesion and replated on collagen II, more than 95 % of total cells initially added attach to the protein-coated dishes after overnight incubation.

Protein labelling and PAGE

After incubation in proline-free F12 medium, cultures were labelled for 2 h with 50 μ Ci/ml of [2,3-³H]proline (Amersham; 49 Ci/mmol). Samples were digested with collagenase (Advanced Biofactures) and then electrophoresed in SDS/7 % polyacrylamide gels as described previously [23]. Scanning of autoradiograms was performed using a discovery system from Pharmacia Biotech served by software from PDI RFL-PRINT.

DNA probes

The pro α 1(II) collagen probe used was pYN 2142, harbouring a 1200 bp cDNA insert coding for the C-propeptide of chicken type II collagen [24]. pYN 535 was a 760 bp cDNA fragment coding for the C-propeptide of chicken α 2(I) collagen [25]. p1E1, which was kindly provided by Dr. U. Dietz (Erlangen, Germany), carries a 180 bp cDNA fragment encoding the first exon of the chicken α 1(I) collagen gene; since this exon is α 1(I) specific, this probe does not give cross-hybridization with α 1(II) [26]. pc FN-V is a 214 bp fibronectin cDNA clone encoding part of the alternative spliced V region and part of the adjacent 5'-unspliced exon [27]. pc INT β is a 620 bp cDNA of the chicken integrin β 1 subunit [28]. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was a full-length cDNA clone encoding rat GAPDH [29]. The inserts of these plasmids were used as probes.

High-specific-activity random-primed probes were prepared with the Amersham kit as specified by the supplier.

RNA extraction and Northern blot hybridization

Total cellular RNA was extracted by following the procedure described previously [30]. For each sample, equivalent amounts of total RNA (15 μ g/lane) were denatured and fractionated on formaldehyde/agarose gels [31]. RNA was then transferred to Amersham Hybond N nylon membranes.

Prehybridization and hybridization were performed in 500 mM NaH₂PO₄ (pH 7.2)/7 % SDS/1 mM EDTA for 30 min and 16 h respectively; the hybridization temperature was 65 °C. Filters were washed three times at 65 °C in 50 mM NaH₂PO₄ (pH 7.2)/1 % SDS. Washed filters were dried and exposed to Fuji films with intensifying screens at -80 °C.

Chimaeric DNA constructs

COL1A2-CAT (formerly pMS-3.5/CAT) contains the COL1A2 proximal promoter fused to the chloramphenicol acetyltransferase (CAT) gene and was derived from a 3.5 kb *EcoRI/SphI* genomic subclone that spans from position -3500 to +58 of the 5'-flanking sequences of human α 2(I) collagen gene [32].

RARE β -tK-CAT carries the promoter of herpes simplex thymidine kinase (tK) gene fused to the CAT gene. A synthetic 25-mer oligonucleotide, representative of the RAR-responsive element (RARE) from the RAR β promoter [33,34] was inserted upstream of the tK promoter [35].

Transfection experiments

Cells were transfected by the liposome technique according to the manufacturer's directions (Boehringer, DOTAP transfection reagent). Transfection was performed on 1×10^6 suspension cells. Test DNA was 5 μ g. Parallel cultures were transfected with 5 μ g of RSV-CAT as a reference plasmid [36]. Less DNA gives no appreciable transfection efficiency with the DOTAP reagent. When appropriate, fresh medium with RA was added after transfection. Cells were analysed for CAT activity after 48–72 h, and cell extracts were assayed for conversion of ¹⁴C-labelled chloramphenicol into acetylated chloramphenicol by TLC coupled with fluorography as described previously [37]. Multiple DNA preparations were used for each experiment, and care was taken to ensure that all assays were carried out in the linear phase. Parallel transfections with the reference plasmid were performed in all experiments.

RESULTS

RA-induced modulation of chondrocyte phenotype

Cultured quail embryo chondrocytes can grow either in a monolayer, displaying a polygonal morphology, or in suspension as floating cells [21]. Floating chondrocytes poorly convert into attached cells and do not express fibronectin. Addition of RA to floating quail chondrocytes causes them to attach to the plastic dish and induces a dedifferentiated morphology. Following removal of RA some of them spread further and form focal contacts. Thereafter cells gradually recover the polygonal morphology characteristic of chondrocytes growing in a monolayer.

As previously reported [18], RA treatment of floating quail chondrocytes results in the inhibition of cartilage collagens and induction of cell adhesion along with fibronectin expression. By metabolic labelling with L-[2,3-³H]proline and SDS/PAGE, we confirmed that the synthesis of collagen II and IX is inhibited by

Figure 1 Effect of cell adhesion on the chondrocyte (QEC) response to RA-induced inhibition of cell growth

(a) Monolayer, i.e. cultures in permissive conditions for cell adhesion. (b) Suspension (cultures on agarose-coated culture dishes), i.e. in non-permissive conditions for cell adhesion. Arrows refer to withdrawal of cultures from RA medium. Cell number per dish at each indicated time was determined with a haemocytometer and represents the mean value of duplicate cultures. C, control untreated QEC.

RA treatment while $\alpha 1(I)$ chain synthesis is stimulated. Culture in the absence of RA leads to gradual re-expression of collagen II and a reduced rate of synthesis of pro $\alpha 1(I)$ chain (results not shown). The fibronectin gene is turned on shortly after RA addition (within the first 24 h), and its expression increases as chondrocytes attach to the dish, as detected by Northern-blot analysis of total RNAs (results not shown). Once withdrawn from RA medium, chondrocytes continue to synthesize fibronectin and keep growing as attached cells [18].

To understand whether cell adhesion is an essential step in the mechanisms by which RA inhibits the chondrocyte phenotype, we blocked cell adhesion by culturing chondrocytes on agarose-coated dishes. During this study we will refer to chondrocytes kept in suspension on agarose-coated dishes as 'suspension' cultures and to chondrocytes cultured in permissive conditions for cell adhesion as 'monolayer' cultures.

Effect of cell adhesion on the chondrocyte response to RA-induced inhibition of cell growth

Cell proliferation was examined (Figure 1). After an initial inhibition of cell growth occurring during RA treatment, withdrawal of RA medium restored cell growth in monolayer cultures, that is in cultures where RA-induced cell adhesion has not been prevented. However, in suspension cultures, cell proliferation was inhibited even after withdrawal of RA medium. Even though cell growth was blocked in suspension cultures, most of the cells (90 %) retained the ability to exclude Trypan Blue and remained metabolically active. In some experiments they appeared to be organized in loosely packaged small aggregates.

Effect of cell adhesion on the chondrocyte response to the RA-induced modulation of extracellular-matrix-gene expression

Total RNAs from control and RA-treated chondrocytes cultured either in suspension or in monolayer were analysed by Northern-blot hybridization (Figure 2). As a control, RNA from quail fibroblasts was also examined. A strong accumulation of steady-state levels of fibronectin and pro $\alpha 1(I)$ collagen mRNAs was

Figure 2 RA-induced modulation of chondrocyte phenotype and cell adhesion

(a) Steady-state levels of pro $\alpha 1(I)$ collagen, fibronectin and $\beta 1$ integrin subunit mRNAs in quail embryo chondrocytes (QEC) treated with RA and allowed or not allowed to attach to the culture dish (monolayer and suspension respectively). Lanes 1 and 5, control chondrocytes; lanes 2 and 6, chondrocytes undergoing a 3 day treatment; lanes 3 and 7, chondrocytes withdrawn from RA medium and allowed to grow in RA-free medium for an additional 6 days; lanes 4 and 8, quail embryo fibroblasts (QEF). (b) Steady-state levels of pro $\alpha 1(II)$ collagen and GAPDH mRNAs in quail embryo chondrocytes (QEC) treated with RA and allowed or not allowed to attach (monolayer and suspension respectively). Lanes 1 and 4, control chondrocytes; lanes 2 and 5, chondrocytes undergoing a 3 day treatment; lanes 3 and 6, chondrocytes withdrawn from RA medium and allowed to grow in RA-free medium for 6 days; lane 7, quail embryo fibroblasts (QEF). In the experiment shown in (b) only 5 μ g/sample of total RNA was loaded on the gel.

observed after a 3 day treatment in permissive conditions for cell adhesion (Figure 2a, lanes 1 and 2). By 6 days after RA removal, pro $\alpha 1(I)$ collagen mRNA levels fell to the background levels of control untreated chondrocytes, while fibronectin mRNA was still present, although at lower extents than in cells undergoing RA treatment (Figure 2a, lane 3). Prevention of cell adhesion resulted in a lack of accumulation of fibronectin and $\alpha 1(I)$ collagen mRNAs (Figure 2a, lanes 5–7). We examined the steady-state levels of $\alpha 1(II)$ collagen in monolayer and suspension cultures (Figure 2b). As previously described [18,19], pro $\alpha 1(II)$ mRNA levels were severely reduced in cells undergoing RA treatment in permissive conditions for cell adhesion (Figure 2b, lanes 1 and 2); on day 6 of culture without RA, cells re-expressed this collagen, although at lower extents than control cells did (Figure 2b, lane 3). Prevention of cell adhesion significantly decreased RA-induced inhibition of collagen II mRNA levels (Figure 2b, lanes 4–6). Hybridization with pc INT β , a chicken

Figure 3 RA-induced regulation of pro $\alpha 2(I)$ and pro $\alpha 1(II)$ collagen genes and cell adhesion

Steady-state levels of pro $\alpha 2(I)$, pro $\alpha 1(II)$ collagen and GAPDH mRNAs in RA-treated quail embryo chondrocytes (QEC) cultured in monolayer on plastic dishes or in suspension on agarose-coated dishes. Lanes 1 and 4, control chondrocytes; lanes 2 and 5, chondrocytes undergoing a 3 day treatment; lanes 3 and 6, chondrocytes withdrawn from RA medium and allowed to grow in RA-free medium for 6 days; lane 7, quail embryo fibroblasts (QEF).

integrin $\beta 1$ subunit cDNA, showed a moderate increase of $\beta 1$ integrin mRNA levels in monolayer cultures; no significant changes could be detected in suspension cultures (Figure 2a). Although equivalent amounts of total RNA were loaded, GAPDH mRNA levels were reduced in chondrocytes undergoing RA treatment in permissive conditions for cell adhesion. Since we have often observed that RA treatment of monolayer cultures induces inhibition of GAPDH expression, it is probable that this gene is also regulated by RA. By metabolic labelling with L-[2,3- 3H]proline and SDS/PAGE, we confirmed that in non-permissive conditions for cell adhesion, RA-induced inhibition of collagen II synthesis is significantly decreased and fibronectin synthesis is not detectable (results not shown). The pattern of secreted proteins after $(NH_4)_2SO_4$ precipitation confirms that RA treatment does not induce fibronectin synthesis if cells are kept in suspension (results not shown).

We next analysed by Northern-blot hybridization the steady-state levels of pro $\alpha 2(I)$ collagen mRNAs from fibroblasts and RA-treated chondrocytes (Figure 3). Equal amounts of total RNA were loaded per lane; as a control, pro $\alpha 1(II)$ collagen mRNA levels were examined. In this experiment, pro $\alpha 1(II)$ mRNA levels decreased sharply upon RA-induced cell adhesion; on day 6 of culture without RA, cells accumulated pro $\alpha 2(I)$ collagen mRNA and re-expressed $\alpha 1(II)$ collagen mRNA, although in lower amounts than did untreated control cells (Figure 3, lanes 1–3). By this time, however, the rate of synthesis of the pro $\alpha 1(II)$ collagen chain was almost completely recovered compared with the rate of synthesis observed in control cells (results not shown). Prevention of cell adhesion resulted in the failure of the inhibition of pro $\alpha 1(II)$ expression; accumulation of pro $\alpha 2(I)$ collagen mRNA was not noticeable in suspension cultures (Figure 3, lanes 4–6).

The expression of the CAT gene driven by the bone/tendon promoter from the human $\alpha 2(I)$ collagen gene was evaluated and compared with the expression of RARE β -tK-CAT, a RAR β -responsive CAT reporter plasmid (Figure 4). As to RAR expression in chondrocytes, Iwamoto et al. [17] reported that RAR β mRNA is strongly accumulated upon RA treatment. We also observed by Northern-blot analysis a strong accumulation

Figure 4 Transcription activity of COL1A2-CAT and RARE β -tK-CAT in RA-treated chondrocytes cultured in monolayer (a) or kept in suspension (b)

Upper panels: at each time point, cells were transfected in duplicate with three different fusion genes, namely RSV-CAT, COL1A2-CAT and RARE β -tK-CAT. To normalize, collagen-driven CAT activity and RARE β -tK-driven CAT activity were expressed as the relative chloramphenicol conversion with respect to the conversion obtained with RSV-CAT reference plasmid (control). The values obtained are reported as histograms. Lower panels: each autoradiogram depicts, from left to right, duplicate CAT assays from RARE β -tK-CAT transfected cells (lanes 1, 2, 5, 6, 9 and 10) and COL1A2-CAT transfected cells (lanes 3, 4, 7, 8, 11, and 12). QEC C, control untreated chondrocytes. QEC RA (+3d), chondrocytes undergoing a 3 day treatment. QEC RA (-6d), treated chondrocytes withdrawn from RA medium and then cultured in RA-free medium for an additional 6 days. Autoradiograms report, in order of increasing mobility, chloramphenicol, 1-acetate chloramphenicol and 3-acetate chloramphenicol.

of RAR β mRNA occurring within a few hours after RA addition (results not shown). As previously reported [19], collagen-driven CAT activity was low in cells undergoing RA treatment, and it was strongly induced after withdrawal of RA medium when dealing with monolayer cultures. Conversely, collagen-driven CAT activity remained at the background levels throughout the entire experiment in cells kept in suspension on agarose-coated dishes. Therefore, prevention of RA-induced cell adhesion results in the failure of transcriptional activation of bone/tendon promoter from $\alpha 2(I)$ collagen gene. As expected, RARE β -tK-CAT was readily expressed in cells undergoing RA treatment, its expression being even higher in suspension than in monolayer cultures. Following RA removal, the activity of the RARE β -tK promoter declines in both suspension and monolayer cultures.

Effect of cell adhesion on the modulation of extracellular-matrix-gene expression of untreated control chondrocytes

In order to investigate the role of cell adhesion in initiating the modulation of the chondrocyte phenotype in the absence of RA, we studied the effect of plating untreated control chondrocytes on to specific and non-specific adhesive substrates. We therefore plated, in complete medium, untrypsinized floating chondrocytes on to uncoated culture dishes or dishes previously coated with collagens I and II, fibronectin or poly-L-lysine. After prolonged incubations they attached only to poly-L-lysine- or collagen-coated dishes. Attachment to collagen substrates was not associated with cell spreading. Fibronectin did not support cell adhesion of untreated control chondrocytes, not even after prolonged incubations, suggesting that the fibronectin receptor is

Figure 5 Adhesion of control and RA-treated chondrocytes to different substrates

Trypsinized cells were resuspended in F12 medium containing 0.3% FCS and plated at 7×10^4 cells/well in 96-well plates coated with collagen II (Coll. II), collagen I (Coll. I), fibronectin (FN), poly-L-lysine (PLL) or plastic (Plas). After a 45 min incubation (a) or a 16 h incubation (b), the cultures were washed with PBS to remove unattached cells; the attached cells were fixed and stained with a Crystal Violet solution. Colour yields measured with a Bio-Rad ELISA Reader were normalized against the highest colour yield observed after overnight incubation, which corresponds to chondrocytes pretreated with RA in permissive conditions for cell adhesion and replated on to collagen II-coated wells. QEC, control untreated chondrocytes. QEC (RA) ML, chondrocytes pretreated with RA in permissive conditions for cell adhesion. QEC (RA) SSP, chondrocytes pretreated with RA in non-permissive conditions for cell adhesion.

either missing or inactive. Four days after plating on to these substrates, cells were labelled with [2,3- ^3H]proline, and the proteins were analysed by SDS/PAGE (results not shown). No major differences could be detected in the collagen expression patterns, suggesting that cell adhesion to specific or unspecific substrates is not sufficient to initiate the modulation of chondrocyte phenotype.

Attachment of control and RA-treated chondrocytes to extracellular-matrix proteins

In order to investigate the mechanisms responsible for the different responses to RA of chondrocytes maintained in suspension or allowed to attach, a microtitre attachment assay was developed. Untreated chondrocytes or chondrocytes treated with RA for 3 days in permissive or non-permissive conditions for cell adhesion were trypsinized, resuspended in medium containing 0.3% FCS and plated in microtitre wells previously coated with collagen I, collagen II, fibronectin or poly-L-lysine (Figure 5). As an internal control, the attachment of quail fibroblasts to the same substrates was also studied. After a 45 min incubation, cells were washed and then stained (Figure 5a). The colour yields obtained were normalized to the highest colour yield observed after longer incubation (16 h), the highest yield being associated with chondrocytes allowed to adhere during RA treatment and replated on to collagen II-coated wells (Figure 5b). Untreated control chondrocytes did not attach to any of the protein substrates in a short-term assay; after an overnight incubation they attached to collagen II-coated wells. Chondrocytes treated

with RA in permissive conditions for cell adhesion rapidly attached and spread on collagen-coated wells. Like untreated control cells, cells pretreated with RA in non-permissive conditions for cell adhesion did not attach to any of the protein substrates in a short-term assay. Prolonging the attachment assay to 16 h allowed these cells to attach and spread on to collagen II, collagen I and fibronectin; again the adhesion to collagen II appeared to be higher.

DISCUSSION

Cell adhesion and RA-induced modulation of chondrocyte phenotype

This study aimed to investigate the role of cell adhesion in RA-induced regulation of cell growth and extracellular-matrix-gene expression in quail chondrocytes. Primary cultures of quail embryo chondrocytes synthesize the specialized products characteristic of differentiated chondrocytes, including collagens II and IX. This phenotype is very stable, as chondrocytes do not dedifferentiate when they are maintained in culture for prolonged periods. The differentiated phenotype of chondrocytes from different species is generally associated with a round or a polygonal morphology and can be maintained in high-density cultures. Transition from round or polygonal morphology to a spread morphology occurs, for instance, when chondrocytes are maintained in low-density cultures. During this transition, loss of differentiated phenotype is observed [38].

RA treatment of suspension quail chondrocytes inhibits the expression of the differentiated phenotype and induces a spread morphology [18,19]. In order to dissect primary responses to RA versus the secondary responses related to the alteration of cell morphology, we prevented chondrocyte attachment by culturing them on agarose-coated dishes throughout the entire experiment. Prevention of RA-induced cell adhesion resulted in an irreversible block of cell proliferation and in a lack of modulation of chondrocyte phenotype. In these conditions, the RA-induced inhibition of collagen II is significantly decreased, the expression of fibronectin and $\alpha 1(\text{I})$ collagen is not modulated, and transcription activation of $\alpha 2(\text{I})$ collagen is not detectable.

The differentiated phenotype of chondrocytes is subject to extensive modulation in cell culture, and the mechanisms regulating differential gene expression are largely unknown. Culture conditions that induce cell spreading and the formation of focal contacts result in alteration of collagen expression patterns from types II, IX and XI to collagen types I, III and V [39], cessation of aggrecan synthesis [40] and re-arrangement of the actin cytoskeleton [41]. Chondrocytes could be considered shape-responsive cells in that their regulatory mechanisms are responsive to changes in cell shape. Horton and Hassel [12] reported that treatment with RA ($10 \mu\text{M}$) of chicken sternal chondrocytes suspended in methyl cellulose results in an irreversible loss of differentiated phenotype. They suggested that RA directly affects chondrocyte phenotype in the absence of cell shape change. Here we report instead that RA treatment does not significantly affect the expression of extracellular-matrix genes in quail chondrocytes if the initial morphological alteration from spherical floating to adherent chondrocytes is blocked by culturing them on agarose-coated dishes. Moreover, Gerstenfeld et al. [42] observed that the block of cell adhesion performed by culturing chick sternal chondrocytes on bacteriological Petri dishes abolishes the modulation of extracellular-matrix genes promoted by $1,25\text{-(OH)}_2\text{D}_3$. RA-dedifferentiated rabbit chondrocytes can recover the differentiated phenotype in the absence of detectable cell rounding if treated with the microfilament-disruptive drug dihydrocytochalasin B [15,43]. These results,

together with another report [44], suggest that actin organization rather than changes in cell shape could function as a signal in the modulation of chondrocyte phenotype.

Chondrocyte adhesion to extracellular-matrix proteins

Activation of cellular signalling events mediated by extracellular-matrix receptors could play a role in regulating the chondrocyte differentiation programme. We observed that induction of cell adhesion to collagen substrates in the absence of RA is not associated with cell spreading and does not promote the modulation of the chondrocyte phenotype. Chondrocytes pretreated with RA in permissive conditions for cell adhesion rapidly attach and spread on to collagens I and II. Chondrocytes pretreated with RA in non-permissive conditions for cell adhesion are not able to attach to any substrate in a short-term assay; longer incubations are needed to observe their attachment and spreading to protein substrates. The molecular basis of these differential adhesive activities must be further investigated. The interaction between RA and the differentiation program of chondrocytes appears to be a sequence of temporally distinct steps through which the cells progress under the effect of RA and its receptors. Prevention of cell adhesion could block the progression of the cells towards a different stage characterized by a different pattern of integrin receptors and a high adhesiveness on collagen substrates. Alterations of cell-matrix interactions and cytoskeletal reorganization resulting from changes in function and/or expression of integrins could in turn influence cell proliferation and differentiation pathways. In our experiments, we could not detect significant changes in $\beta 1$ integrin mRNA levels in cells maintained in suspension, while a moderate increase of these levels is observed after RA-induced cell adhesion. A moderate increase in the rate of $\beta 1$ integrin synthesis was observed by Ross et al. [45] in RA-treated F9 cells. Enomoto et al. [46] studied integrin-receptor distribution in chick sternal chondrocytes and demonstrated that the $\beta 1$ integrin subunit mediates their adhesion to collagen I, collagen II and fibronectin. They proposed $\alpha 2\beta 1$ as the most likely candidate for the collagen receptor and $\alpha 5\beta 1$ as the major fibronectin receptor in chick chondrocytes. In agreement, Durr et al. [47] and Tuckwell et al. [48] reported that $\alpha 2\beta 1$ can be involved in cell-collagen interactions of human chondrocytes. Defining the α subunits expressed in our experimental system could help to explore the receptor-ligand binding events responsible for adhesion and cell-cycle transitions occurring upon RA treatment.

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