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Original Article

9-cis retinoic acid antagonizes the stimulatory effect of 1,25 dihydroxyvitamin D_3 on chondrogenesis of chick limb bud mesenchymal cells: interactions of their receptors

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ABSTRACT Retinoids or vitamin D have been found to profoundly affect pattern formation and chondrogenesis in the developing limb. These substances mediate their actions through their nuclear receptors. In the present investigation, we present data showing that 9-cis RA, the ligand for RXR can stimulate chondrogenesis of chick limb bud mesenchymal cells, however, in combination, it antagonizes the stimulatory effect of vitamin D in the same system. The receptors for 9-cis RA (RXR) and vitamin D (VDR) were also shown to be present in the mesenchymal cells and to form heterodimers. These results implicate these receptors in cartilage differentiation during limb development.

KEY WORDS: *vitamin D, retinoic acid, chondrogenesis, steroid receptors, RXR-VDR formation*

Introduction

Retinoids have been shown to cause profound effects on limb morphogenesis and regeneration. All-trans retinoic acid (RA) affects the positional information of cells in the developing chick limb bud. The effect of retinoic acid is similar to transplanting the zone of polarizing activity (ZPA) in the chick limb bud. The ZPA, located on the posterior side of the chick limb bud, is believed to control positional signaling in the developing limb. When RA is administered or the ZPA is transplanted to the anterior side of a normal chick limb bud, a mirror image duplication in the anterior-posterior axis is obtained (Tickle *et al.*, 1985, 1982; Eichele, 1989; Thaller and Eichele, 1990; Duboule, 1991). It has also been proven that other retinoids such as 3,4 didehydroretinoic acid and 9-cis RA have similar effects in the chick limb bud (Thaller and Eichele, 1990; Thaller *et al.*, 1993; Tsonis *et al.*, 1994). All-trans RA has also been shown to affect the positional information within the blastema of the regenerating limb on salamanders. When all-trans RA is administered to the blastema it results in a dose dependent proximalization of the regenerate (Maden, 1982). In other words, when a limb is amputated at the wrist and treated with all-trans RA, an additional humerus, ulna and radius can be formed.

In both the regenerating and developing limb, 9-cis RA has been shown to be more potent than all-trans RA. Proximalization results when 9-cis RA is administered to axolotls *(Ambystoma mexicanum)* undergoing limb regeneration (Tsonis *et al.*, 1994). In fact, the dose of 9-cis RA needed to cause such an effect is lower than that needed for all-trans RA. In the developing chick limb bud, 9-cis RA administration to the limb induces pattern duplications similar to all-trans RA, however 9-cis RA was found to be approximately 25 times more potent (Thaller *et al.*, 1993).

This action of vitamin A metabolites suggests the existence of morphogens. The finding that retinoids are endogenously present in higher concentration in the posterior than in the anterior region of the chick limb suggests that retinoids might be the best candidates for the role of morphogens (Thaller and Eichele, 1987). Similar gradients have been observed in the regenerating limb as well (Scadding and Maden, 1994). While the effects of all-trans RA on limb patterning can be explained by its graded presence, other studies have shown that anterior chick limb bud cells can be converted into cells with posterior identity by the treatment with all-trans RA (Wanek *et al.*, 1991; Hayamizu and Bryant, 1992). This means that perhaps diffusible morphogens are not released, but rather cell-cell interactions have a role in limb patterning. This of course does not exclude the possibility that other diffusible factors may act as morphogens.

Skeletal specification and chondrogenesis in the developing limb bud begins with differentiation of mesenchymal cells. At the early limb bud stage (4 days of development), the mesenchymal cells condense, and these prechondrogenic condensations differentiate to cartilage. Study of such phenomenon has been facilitated enormously by the fact that the chick limb bud offers a reliable and efficient system for *in vitro* chondrogenesis studies. When limb bud mesenchymal cells from stage 20-24 are plated at high cell density ($1x10^6$ cells/1.6 cm plate) or in micromass culture ($2x10^5$

Abbreviations used in this paper: RA, All-trans retinoic acid; RAR, retinoic acid receptor; RXR, retinoic x receptor; VDR, vitamin D receptor.

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cells spotted with 20 μl of media), they spontaneously differentiate into chondrocytes (Caplan, 1970; Ahrens *et al.*, 1977; Gay and Kosher, 1984). This high cell density requirement is reminiscent of the *in vivo* cell condensation. However, when these cells are plated at low density ($1x10^5$ cells/1.6 cm), chondrogenesis does not occur unless treated with all-trans RA or 1,25 dihydroxyvitamin D_3. All-trans RA promotes cellular proliferation and nodule formation in distal mesodermal cells (stages 20-24) of the developing limb bud (Ide and Aono, 1988). 1,25 dihydroxyvitamin D_3 also stimulates chondrogenesis in cultures of mesenchymal cells from stage 24 chick limb buds but does not promote proliferation of mesenchymal cells (Tsonis, 1991).

Actions of retinoids or 1,25 dihydroxyvitamin D_3 are mediated by ligand dependent nuclear, DNA binding receptors that belong to the same steroid receptor superfamily (Beato, 1989). All-trans RA binds to the retinoic acid receptor (RAR) (Giguere *et al.*, 1987; Petkovich *et al.*, 1987; Ragsdale and Brockes, 1991; Laudet and Stehelin, 1992), 9-cis RA binds to the retinoid X receptor (RXR) as well as RAR (Mangelsdorf *et al.*, 1990; Heyman *et al.*, 1992; Levin *et al.*, 1992), and 1,25 dihydroxyvitamin D_3 binds to the vitamin D receptor (VDR) (Minghetti and Norman, 1988; Liao *et al.*, 1990). These steroid receptors bind to DNA sequences that are similar, yet specific (Beato, 1989; Umesono *et al.*, 1991). DNA binding of these receptors is through homodimer formation. However, studies have also shown that RXR can heterodimerize with the thyroid hormone receptor (TR), VDR, or RAR and increase the binding affinity of the receptors to the DNA (Umesono *et al.*, 1991; Bugge *et al.*, 1992; Kliewer *et al.*, 1992; Laudet and Stehelin, 1992; Leid *et al.*, 1992). This ability of the steroid receptors can explain results showing that all-trans RA and 1,25 dihydroxyvitamin D_3 act synergistically, or cooperatively, to inhibit cellular proliferation in human breast cancer cells, possibly through heterodimerization of the receptors RAR and VDR (Koga and Sutherland, 1991). Cooperation or antagonism among the ligands for steroid receptors has been shown during gene expression in rat liver and kidney (Shull *et al.*, 1995) and in photoreceptor cell differentiation (Kelly *et al.*, 1995). Similarly, evidence for the cooperation of T_4 and RA (Vincinti and Crawford, 1993), or RA and vitamin D during morphogenesis of the regenerating limb has been suggested (Washabaugh and Tsonis, 1995). Other studies demonstrate that 9-cis RA greatly inhibits 1,25 dihydroxyvitamin D_3-dependent gene expression, but 1,25 dihydroxyvitamin D_3 induces the heterodimerization of VDR and unliganded RXR (MacDonald *et al.*, 1993) and exerts its transcriptional effects through RXR-VDR heterodimer formation (Sasaki *et al.*, 1995).

Due to the profound effects of retinoids and vitamin D on limb development and chondrogenesis, the implications of RXR in limb pattern formation, and the potential molecular interactions between the nuclear receptors, RXR and VDR, we decided to study the effects of 9-cis RA, alone or combined with vitamin D metabolites, on chondrogenesis in the chick limb bud in order to establish a molecular framework that could lead us to the identification of important genes governing this phenomenon.

TABLE 1

PROLIFERATION STUDY OF CHICK LIMB BUD MESENCHYMAL CELLS

Treatment	Day 1 (cpm)	Day 2 (cpm)
Control	4302.5*	15970#
10^{-8} all trans RA	2201.5*	14924.5
10^{-10} all trans RA	3013.2	10856.5*
10^{-12} all trans RA	3621	14990
10^{-8} 9-cis RA	5138	6960.8*#
10^{-9} 9-cis RA	4826	12282.8
10^{-10} 9-cis RA	6390.8	17698.5*
10^{-11} 9-cis RA	6107.3*	16010.5
10^{-13} 9-cis RA	6969.8	16037*
10^{-8} 1060	5820.3	13780
10^{-10} 1060	6112.5	13933.5*
10^{-12} 1060	6898.7	11502.5*
10^{-8} 1,25	6529.3	2571*
10^{-10} 1,25	8116.2	6919.3
10^{-12} 1,25	7300	5440.3

Average of triplicates. *Average of duplicates. #$p<0.05$

Results and Discussion

Effects of vitamin A and vitamin D metabolites on chondrogenesis

The micromass cultures were maintained for 2.5 days. By the end of that time, chondrogenesis had occurred in the control cultures. Usually, cartilage nodules begin to form on the second day. In our experiments, nodule formation was observed after 24 h predominantly in the cultures treated with the vitamin D analog, KH 1060, and 1,25 dihydroxyvitamin D_3 (10^{-8} M and 10^{-10} M) although cellular proliferation had not increased over the other cultures (Table 1). No obvious antagonistic effects of combined treatments on proliferation were observed (data not shown). No nodules were observed in the other cultures at that time. Sixty hours post-plating, nodules were prominent in the KH 1060 and 1,25 dihydroxyvitamin D_3 treated cultures while other cultures showed nodule formation of varying degrees (Fig. 1).

The amount of chondrogenesis caused by the vitamin D analog KH 1060 was much higher than the untreated cells (Table 2). In fact, the vitamin D metabolite KH 1060 stimulated chondrogenesis the most, compared to the other treatments (Table 2, Fig. 2). Even at lower concentrations (10^{-12} M), the KH 1060 metabolite stimulated chondrogenesis to levels higher than in the control cultures. However, the stimulatory effect was not due to an increase in cellular proliferation as demonstrated by [^{3}H] thymidine incorporation (Table 1). The mechanism used by this analog has not been elucidated, but binding to VDR, the only known mode of action for 1,25 dihydroxyvitamin D_3, has been shown (Binderup *et al.*, 1991).

As expected, 1,25 dihydroxyvitamin D_3 at concentrations of 10^{-8} M and 10^{-10} M also showed higher core proteoglycan mRNA expression and degree of chondrogenesis in comparison to the untreated control (Table 2, Fig. 2). Proliferation studies by [^{3}H] thymidine incorporation performed on these treated cultures showed that the stimulatory effects of this metabolite are not due to proliferation rate, which was even lower than the rate of the control cultures (Table 1). These data agree with previous studies in which 1,25 dihydroxyvitamin D_3 did not promote cellular proliferation (Tsonis, 1991).

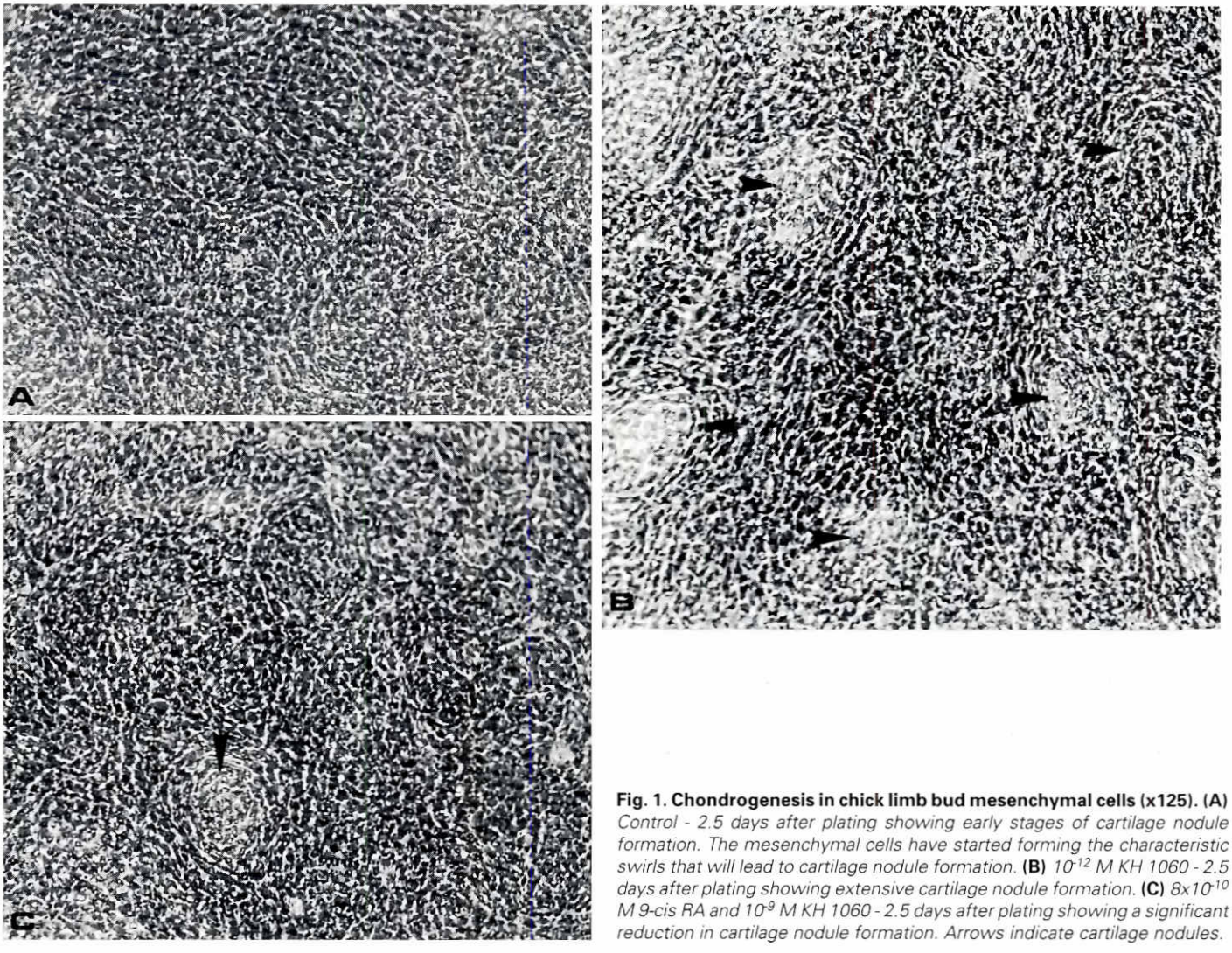

Fig. 1. Chondrogenesis in chick limb bud mesenchymal cells (x125). (A) *Control - 2.5 days after plating showing early stages of cartilage nodule formation. The mesenchymal cells have started forming the characteristic swirls that will lead to cartilage nodule formation.* **(B)** *10^{-12} M KH 1060 - 2.5 days after plating showing extensive cartilage nodule formation.* **(C)** *$8x10^{-10}$ M 9-cis RA and 10^{-9} M KH 1060 - 2.5 days after plating showing a significant reduction in cartilage nodule formation. Arrows indicate cartilage nodules.*

At the highest concentrations used (10^{-8} M), all-trans retinoic acid treated cultures showed a degree of chondrogenesis slightly above the untreated cells. At the lower doses (10^{-10} M and 10^{-12} M) of all-trans RA, the amount of chondrogenesis increased over the control cells (Table 2). It has been previously shown that high doses of all-trans RA inhibit chondrogenesis in chick limb bud mesenchymal cells, and chondrogenesis increases with lower doses of all-trans RA (Ide and Aono, 1988). The cellular proliferation in these cultures at all the concentrations was similar to that in the untreated cultures (Table 1).

9-cis RA (10^{-8} M) affected chondrogenesis in a similar fashion to all-trans RA. However, lower doses of 9-cis RA resulted in a higher degree of chondrogenesis than all-trans RA (Table 2, Fig. 2). Proliferation studies of these cultures showed a significant decrease in [^{3}H] thymidine incorporation in the 10^{-8} M 9-cis RA cultures, therefore, the lower degree of chondrogenesis in these cultures could be attributed to the inhibition of cellular proliferation. However, at the lower concentrations of 10^{-9} M-10^{-13} M, proliferation was not inhibited (Table 1). Our results also show that 20-30 fold less 9-cis RA than all-trans RA is needed to cause the same amount of chondrogenesis (compare 10^{-10} M all-trans RA with 10^{-11} M 9-cis RA in Table 2). This is reminiscent of the potency of 9-cis RA in pattern duplication in the developing chick and the axolotl regenerating limb (Thaller *et al.*, 1993; Tsonis *et al.*, 1994).

Taken together, the results of the various treatments indicate that the vitamin D analog KH 1060 is the most potent in stimulating chondrogenesis at all concentrations. 1,25 dihydroxyvitamin D_3 is more potent than 9-cis RA at higher concentrations, but 9-cis RA is more potent at lower concentrations. All-trans RA is obviously the least potent of the compounds tested (Fig. 2).

The unique ability of RXR to exert effects by heterodimerizing with RAR or VDR and the possible interactions of vitamin D and retinoids in other systems prompted us to study the effects of 9-cis RA in combination with vitamin D compounds. 9-cis RA, at the concentrations of 10^{-9} M to 10^{-13} M, showed a stimulation of chondrogenesis without affecting cellular proliferation. When 9-cis RA ($8x10^{-10}$ M) was combined with 1,25 dihydroxyvitamin D_3 (at concentrations of 10^{-8} M or 10^{-9} M) or with the analog KH 1060 (at concentrations of 10^{-8} M, 10^{-9} M or 10^{-10} M), which alone promoted chondrogenesis considerably, the stimulating effect was blocked (Table 2). The blocking effect varied depending on the treatment. In general, the combined treatment lowered the chondrogenesis

level to that induced by 9-cis RA alone (compare 10^{-9} M 9-cis RA, 10^{-8} M KH 1060 and the combined treatment of 9-cis RA with 10^{-8} M KH 1060) (Table 2).

Expression and implication of RXR and VDR dimers

The aforementioned effects of 9-cis RA and vitamin D on chondrogenesis strongly imply that they are mediated by the differential use of their receptors RXR and VDR. Presence of RXR and VDR has been shown before in the chick limb bud (Rowe *et al.*, 1991; Tsonis, 1991), but the mode of dimerization is not known. Such possibility prompted us to examine the presence of these receptors and their mode of dimerization in our system. For this purpose we performed CAT assays with CAT plasmids having responsive elements in their 5' regulatory regions specific to either RXR-RXR or RXR-VDR. Our results show considerable CAT activity in cells transfected with the plasmids containing responsive elements for RXR-VDR and RXR-RXR as compared with the negative control, the untransfected cultures (Table 3). Our results show that CAT activity obtained through RXR-VDR elements is nearly four times higher than the one received from the CRBPII elements, and double than the one received using the original

TABLE 2

QUANTIFICATION OF CHONDROGENESIS IN MICROMASS CULTURES OF CHICK LIMB BUD MESENCHYMAL CELLS 60 h AFTER PLATING

Treatment	Absorbance (core protein expression scanning)	% Increase over control
Control	0.63	–
10^{-8} all trans RA	0.72	14
10^{-10} all trans RA	0.87	38
10^{-12} all trans RA	0.84	33
10^{-8} 9-cis RA	0.77	22
10^{-9} 9-cis RA	1.01	60
10^{-10} 9-cis RA	1.20	90
10^{-11} 9-cis RA	1.13	79
10^{-13} 9-cis RA	0.99	57
10^{-8} 1,25	1.08	71+
10^{-10} 1,25	1.15	82+
10^{-12} 1,25	0.77	22
10^{-8} 1060	1.16	84+
10^{-10} 1060	1.25	98
10^{-12} 1060	1.33	111
8×10^{-10} 9-cis + 10^{-8} 1060	1.03	63+
8×10^{-10} 9-cis + 10^{-9} 1060	0.99	57+
8×10^{-10} 9-cis + 10^{-10} 1060	1.20	90
8×10^{-10} 9-cis + 10^{-8} 1,25	1.04	65+
8×10^{-10} 9-cis + 10^{-9} 1,25	1.04	65+

+$p < 0.05$

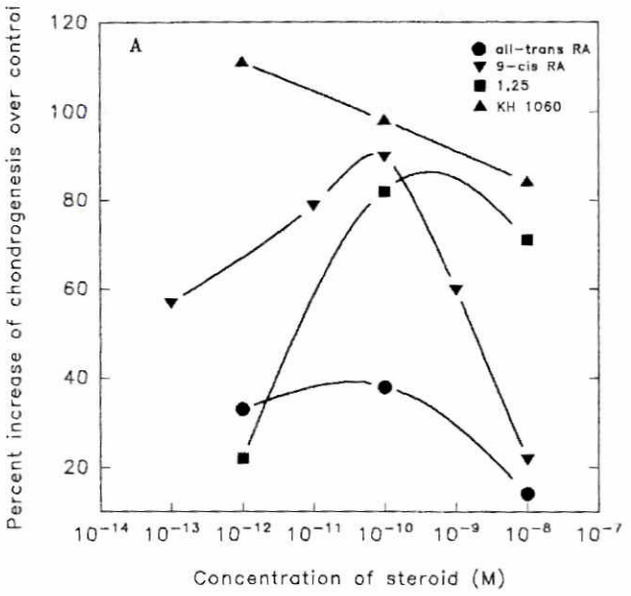

Fig. 2. Representation of degrees of chondrogenesis with the various treatments. *Degree of chondrogenesis is depicted as percent increase over control from Table 2.*

parental plasmid δSVCAT. This suggests that the RXR-VDR heterodimers must be the most predominant in the mesenchymal cells. Alternatively, RXR-RXR could be a stronger suppressor in this system. This is indeed the case for RAR which is known to repress RXR-mediated activation through CRBPII elements (Kliewer *et al.*, 1992). Another intriguing observation was the fact that treatment did not increase the levels of CAT expression. Instead, the treatments, especially 9-cis or 9-cis+1,25 reduced the levels, 15% in the presence of RXR-VDR and 30% in the presence of RXR-RXR elements. The most likely explanation for such an effect is that these treatments promote the RXR-RXR dimerization which results then in more suppression. The reader, however, should bear in mind here that our cultures are primary and all these molecules and their ligands are probably present. Usually this kind of experiments are done with cell lines that do not have endogenous receptors or ligands. In such a case clear effects on gene regulation can be studied upon ligand addition. In our case of primary culture endogenous receptors and ligands might create "noise" and render the results on transcription unclear. However, even such primary cultures as the ones used in the present study cannot undermine the conclusion that the dimers are present, which was the purpose of this experiment. The fact that there is regulation of CAT expression from the different responsive elements strongly indicates that RXR-VDR and RXR-RXR dimers do exist in our system.

These results and ideas can provide a molecular framework whereby they can be explained. It seems that vitamin D can induce chondrogenesis by promoting the formation of RXR-VDR heterodimers which subsequently activate responsive genes. Other treatments, such as 9-cis or all-trans can promote the formation of different dimers and can interfere with chondrogenesis possibly via different pathways depending on RXR-RXR levels. Such hypothetical scheme is presented in Figure 3. In fact, such a scenario is supported by the results obtained in other systems. It has been

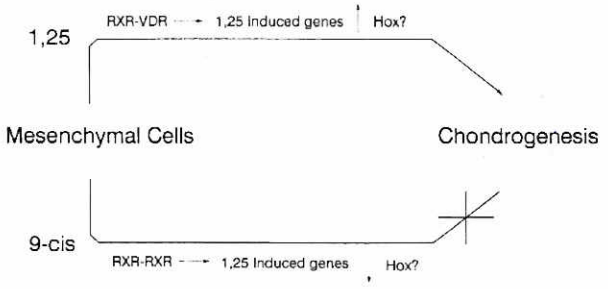

Fig. 3. Hypothetical illustration of the two possible pathways by which chondrogenesis of the chick limb bud mesenchymal cells can occur by treatment of 1,25 dihydroxyvitamin D_3 or 9-cis RA.

recently shown in HeLa and the osteoblastic-like Ros 17/2.8 cells that vitamin D promoted the heterodimerization of RXR-VDR and induced expression of the osteocalcin gene (MacDonald *et al.*, 1993). In the same system, it was also demonstrated that 9-cis RA treatment promoted RXR-RXR homodimer formation and inhibited vitamin D-induced expression of the osteocalcin gene. This antagonistic action of 9-cis RA could in fact provide a molecular framework whereby our results can be explained. In our system, the action of vitamin D was inhibited by 9-cis RA; combinations of vitamin D and 9-cis RA significantly decreased the stimulation of chondrogenesis. In other words, two distinct pathways of differentiation can be caused by the action of vitamin D or 9-cis RA. One through the action of vitamin D, which presumably induces RXR-VDR heterodimerization, and the other through 9-cis RA, presumably by RXR-RXR homodimerization (Fig. 3).

The RXR-RXR homodimers or the RXR-VDR heterodimers regulate distinct sets of genes that govern different pathways. RA and vitamin D have been shown to induce the expression of Hox genes, which are also key regulatory molecules that govern cell differentiation and pattern formation (Tsonis and Adamson, 1986; Stornaiuolo *et al.*, 1990; Morriss-Kay *et al.*, 1991; Simeone *et al.*, 1991; Hodgkinson *et al.*, 1993). The role of Hox genes in limb pattern formation has been established (Duboule, 1991). However, their role in the differentiation of mesenchymal cells to chondrocytes *in vitro*, has not been proven. It is very possible that aggregates of mesenchymal cells and subsequent differentiation to chondrocytes are regulated by Hox genes. In this respect, it is possible that Hox genes might be involved in various differentiation pathways in our system.

We have identified pathways that regulate chondrogenesis of the chick limb bud mesenchymal cells by treatment of 9-cis RA or vitamin D. Our results share similarities with other systems and by analogy strongly implicate the nuclear receptors for these compounds in the differentiation process. Such regulation might provide the molecular framework from which to identify other genes involved in chondrogenesis and help us fathom the mechanisms behind the generation of skeletal elements and pattern formation of the developing limb.

TABLE 3

CAT ACTIVITY (cpm) IN TRANSFECTED LIMB BUD MESENCHYMAL CELLS

	Plasmids		
Treatment	δSV-ost-VDR*	δSV-CRBPII*	δSVCAT*
1,25	39,454	12,259+	
9-cis	37,929	9,910+	
1,25+9-cis	35,926	9,424+	
no	41,457	12,600+	18,240
no transfection	1,209		

These are the results of one experiment in duplicates (average). The same experiment was repeated four times with same conclusions. The differences are statistically significant (*$p<0.01$ for the two groups and +$p<0.05$ for the treatments).

Materials and Methods

Cells and cultures

Mesenchymal cells were dissociated from 4 day (stage 24) embryos and plated in micromass cultures at a density of $2x10^5$ cells/20 μl. The medium used was F-12 containing 10% fetal calf serum, ascorbic acid (50 μg/ml), penicillin/streptomycin, and fungizone. The vitamin D metabolites, 1,25 dihydroxyvitamin D_3 (gift from Dr. M. Uskokovic, Hoffmann-LaRoche) or KH 1060 a vitamin D analog (gift from Dr. L. Binderup, Leo Pharmaceutical Products Ltd.) and vitamin A metabolites, all trans retinoic acid (Sigma) or 9-cis retinoic acid (gift from Dr. A.A. Levin, Hoffmann-LaRoche) were added at concentrations ranging from 10^{-8} M-10^{-13} M individually or combinations of $8x10^{-10}$ M 9-cis RA with either 1,25 dihydroxyvitamin D_3 (10^{-8} M and 10^{-10} M) or the vitamin D analog (10^{-8} M- 10^{-12} M). The media was changed daily. The cultures were maintained for 2.5 days.

Cellular proliferation

Control and treated mesenchymal cells were incubated for 24 or 48 h. 1.0 μCi [^{3}H]thymidine was added to the media. After incubating for 6 h, the cells were harvested and the radioactivity was measured. A one-tailed Mann-Whitney statistical test was performed to assess whether or not the results were statistically significant.

RNA isolation

Total RNA was isolated from treated and untreated cells at the end of the experiment. The cells were dissolved with 2 ml of 4 M guanidine thiocyanate, 25 mM sodium citrate, pH 7.0 and 0.5% laurylsarcosine. The samples were layered on 2 ml CsCl (1 g/ml) and centrifuged at 36,000 rpm for 18 h at 14°C. The pellets were resuspended in 10 mM Tris, pH 7.5, 5 mM EDTA, 1% SDS, and extracted with equal volumes of 4:1 butanol:chloroform.

Dot blot-quantification of chondrogenesis

5 μg of RNA were spotted onto a nitrocellulose filter. The filter was hybridized with the cartilage specific core proteoglycan cDNA probe in order to quantitate the degree of chondrogenesis (Stirpe *et al.*, 1987; Kiss *et al.*, 1989). In the past, this message has been used as a reliable indication of chondrogenesis (Kosher *et al.*, 1986). Under the hybridization conditions used in this study, this probe interacts only with the messages for core protein (Tsonis and Goetinck, 1988). The same blot was hybridized with chicken ß-actin probe generated by PCR. The hybridization solution contained 50% formamide. Hybridizations were performed at 42°C overnight. The filters were washed 30 min at room temperature with 2xSSC, 0.1% SDS, 20 min at room temperature with 1xSSC, 0.1% SDS, and 30 min at room temperature with 0.1xSSC, 0.1% SDS. Autoradiograms showing the expression of core and ß-actin were scanned by a densitometer (LKB) and the values (absorbance units) of core protein expression were normalized. The degree of chondrogenesis in all treated cultures was then expressed as percent increase over the control. One-tailed Student's *t* test on the degree of chondrogenesis was performed.

CAT assays

Mesenchymal cells isolated from 4-day limb buds were plated and next day were transfected, using lipofectamin, with CAT plasmids containing responsive elements to either RXR-RXR homodimers of RXR-VDR heterodimers. The plasmids δSV-ost-VDRE-cat and δSV-CRBPII-cat (gift from Dr. R.M. Evans) contain the SphI-HindIII fragment of the early SV40 promoter and responsive elements for RXR-VDR and RXR-RXR homodimer formation, respectively. The plasmids were made from a basal reporter plasmid δSVCAT (Mangelsdorf *et al.*, 1991) which was used as positive control. Co-transfection with the pCH100 plasmid containing the lacZ gene was performed in order to normalize for efficiency of transfection. The cultures were treated with vitamin D (10^{-8}M), 9-cis retinoic acid (10^{-10}M), or a combined dosage of both. Two days later CAT assays were performed using the Quan-T-CAT assay system (Amersham Life Science). Biotinylated chloramphenicol and tritiated acetyl groups of coenzyme A were added to cell extracts. Subsequently streptavidin coated polystyrene beads were added, the samples were spun and the pellets containing the tritiated acetylated biotinylated chloramphenicol were collected and the radioactivity indicating signal proportional to CAT activity was counted on a scintillation counter. In each experiment we used duplicates or triplicates and each experiment was repeated four times. One tailed Student's *t* test was performed.

Acknowledgments

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