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Original Article

Modulation of limb bud chondrogenesis by retinoic acid and retinoic acid receptors

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ABSTRACT An excess of retinoic acid (RA) in the mouse embryo *in utero* produces hypochondrogenesis and severe limb bone deformities. Since one of the RA receptors — RAR-ß2, is specifically induced in the limb bud cells upon treatment of embryos with teratogenic doses of RA, we investigated if this receptor played a role in teratogenesis by regulating the process of chondrogenesis. In micromass cultures of mouse limb bud mesenchymal cells, we found that a downregulation of RAR-ß2 as well as several other RAR isoforms by supplementation of the culture medium with specific antisense oligodeoxynucleotides stimulated chondrogenesis: cartilage nodule number, sulfated proteoglycans, and synthesis of collagen type IIB were all enhanced in a dose-dependent manner. However, only the antisense RAR-ß2 probe efficiently prevented the strong inhibitory effects of exogenous RA on chondrogenesis in these cells. The data suggest that the RAR-RA complexes play a role in position-dependent patterning of the limb skeleton in normal development and that, in particular, RAR-ß2 serves to prevent the mesenchymal cells from expressing their chondrogenic bias. Our results further strengthen the argument that RA-dependent elevation in RAR-ß2 levels plays a unique role in RA-induced teratogenesis.

KEY WORDS: *retinoic acid, receptors, limb, chondrogenesis, antisense*

Introduction

Retinoic acid (RA), a natural derivative of vitamin A, is present in detectable levels in embryonic tissues during critical developmental stages, but its endogenous role in embryonic development is still not clear (Durston *et al.*, 1989; Satre and Kochhar, 1989; Thaller and Eichele, 1987; Chen *et al.*, 1992, 1994). Recent studies show that it most probably is not the morphogen as was once believed (Noji *et al.*, 1991; Wanek *et al.*, 1991; Johnson *et al.*, 1994), and that its morphogenetic role may stem from its ability to regulate expression of genes necessary in diverse cell differentiation pathways (Gudas *et al.*, 1994).

The nuclear receptors that transduce the biological effects of RA were first described in 1987 by Petkovich *et al.* (1987) and Giguere *et al.* (1987). There are two classes of receptors, termed retinoic acid receptors (RARs) and retinoid X receptors (RXRs). Each class is represented by three genes termed α, β and γ. The sequences of RARs α, β and γ are more homologous to each other than to the RXR genes (Mangelsdorf *et al.*, 1992, 1994). Further, each of the receptors have several isoforms that differ at the N-terminus and which are generated through the use of multiple promoters and alternative splicing (Ruberte *et al.*, 1991b; Mangelsdorf *et al.*, 1994).

In situ hybridization studies utilizing mouse embryonic tissue have revealed that RAR-α, RXR-α and RXR-β have a low level but ubiquitous pattern of expression, while others exhibit stage and tissue-specific developmental patterns of expression consistent with specific, non-overlapping functions (Dolle *et al.*, 1990, 1994; Ruberte *et al.*, 1991a). In certain embryonic organs, e.g. the neural tube and the limb bud, both RAR-β and RAR-γ are expressed in a manner which implies that they individually modulate the differentiation pathway of the same cell type but at different stages of development. While RAR-β is expressed in mesenchymal precursor cells in the limb bud, RAR-γ expression is observed subsequently in the differentiated chondrocytes from which RAR-β is excluded (Ruberte *et al.*, 1991b). Mendelsohn *et al.* (1991) reported in transgenic animals that the RAR-β2 promoter was active in the apical ectodermal ridge (AER) of day 12.5 mouse limb bud and that this activity was enhanced several fold upon treatment of the mother with RA, a treatment which

Abbreviations used in this paper: AER, apical ectodermal ridge; As-ß2, antisense RARß2 oligodeoxynucleotide; AS-RXRß, antisense RXRß oligodeoxynucleotide; oligo, oligodeoxynucleotide; RA, retinoic acid; RAR, retinoic acid receptor; RXR, retinoic X receptor.

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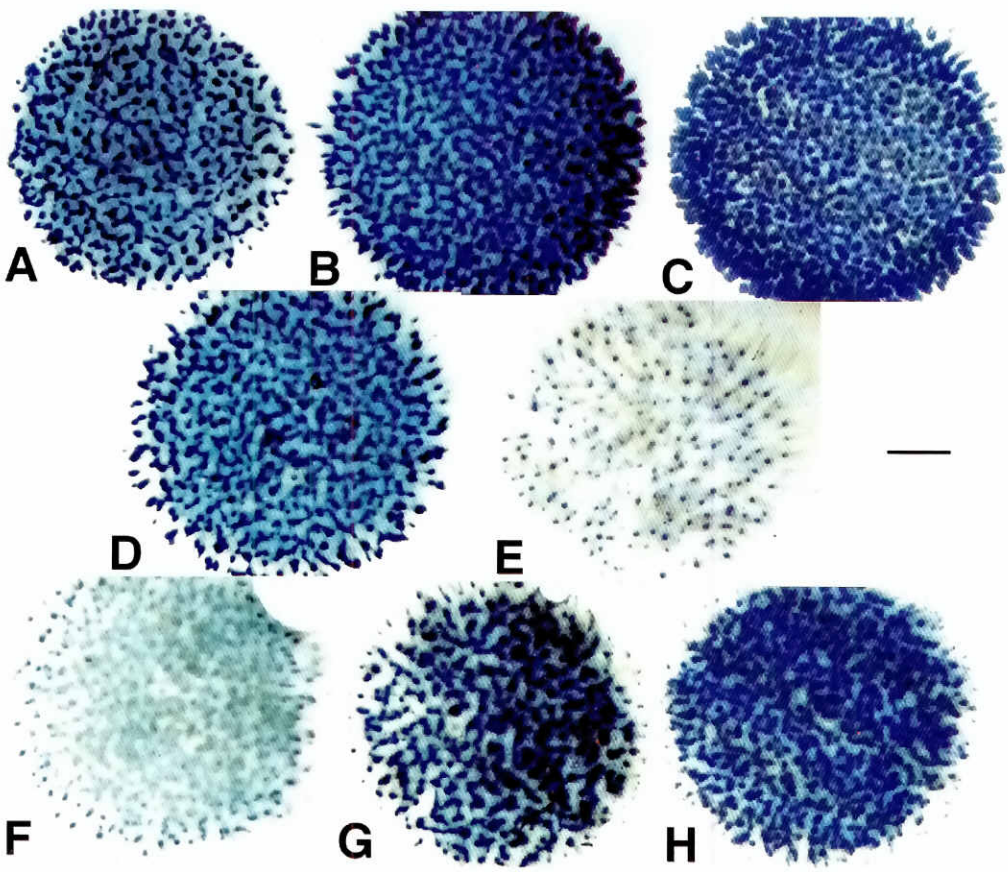


Fig. 1. Micromass cultures containing cartilage nodules at the end of 4-day culture period, stained with Alcian blue. *AS-β2 enhances chondrogenesis while RA strongly inhibits it. AS-β2 reverses the RA-induced inhibition.* **(A)** *Control, unsupplemented culture.* **(B)** *AS-β2, 6 μM.* **(C)** *AS-β2, 12 μM.* **(D)** *Sense RAR-β2, 12 μM.* **(E)** *RA, 0.3 μM.* **(F)** *RA+sense RAR-β2.* **(G)** *RA+AS-β2, 6 μM.* **(H)** *RA+AS-β2, 12 μM. Bar, 1 mm.*

results in truncation defects of the fetal limb skeleton (Kochhar, 1973). Furthermore, we have previously shown that teratogenic treatment of pregnant mice with RA specifically enhanced the synthesis of endogenous RAR-β2 mRNA by 12-fold above the level in the untreated limb bud (Harnish *et al.*, 1992). Since exogenous RA is known to inhibit chondrogenesis and at the same time enhances the expression of RAR-β2 mRNA and protein in the limb bud, we investigated in this study if the two events were causally related (Kochhar, 1973, 1977; Soprano *et al.*, 1994).

Limb bud mesenchymal cells spontaneously differentiate into chondrocytes when explanted at high cell density as micromass cultures in droplets of enriched medium (Ahrens *et al.*, 1977; Kochhar and Penner, 1992). We employed antisense technology to suppress RAR and RXR protein levels in this culture system and examined the role of these proteins in both normal differentiation of these cells into chondrocytes and RA-induced inhibition of chondrogenesis. By using morphological and specific biochemical criteria such as synthesis of sulfated proteoglycans and collagen type IIB for monitoring chondrogenesis, we found that limb bud cells in micromass cultures expressed enhanced chondrogenic potential when the levels of endogenous RARs were reduced. However, only RAR-β2 was found to be involved in mediating the inhibitory effects of RA on chondrogenesis in these cells. Our results suggest that RA-induced elevation of RAR-β2 mRNA has a specific role in mediating the teratogenic effects of RA on skeletal development.

Results

Effects of antisense RAR-β2 (AS-β2) oligodeoxynucleotide

Discrete Alcian blue-positive nodules of cartilage, uniformly distributed in the circular area of the micromass culture, were observed at day 4 of culture in the control, unsupplemented medium (Fig. 1A). The addition of AS-β2 to the medium at day 1 resulted in more densely stained and larger nodules which were more densely packed at the periphery of the treated cultures than in the control cultures (Fig. 1B,C). These changes in the appearance of the culture were not produced by the addition of sense RAR-β2 oligodeoxynucleotide (Fig. 1D), indicating that they were specific to AS-β2. Nodule count by automatic image analysis showed that AS-β2 increased the number in a dose-dependent manner; the number of nodules was increased by about 1.7-fold above control at 6 μM AS-β2 and by more than 2-fold above control at 12 μM AS-β2 (Fig. 2A).

Further evidence that treatment of the micromass cultures with AS-β2 stimulated chondrogenesis was provided by analyses of the synthesis of sulfated proteoglycans and cartilage specific collagen. Incorporation of ^{35}S-sulfate into macromolecules was enhanced by AS-β2 by 3 to 4-fold over the control level on each of the 3rd and 4th day of culture (Fig. 2B). AS-β2 also enhanced the relative expression of procollagen IIB mRNA compared to that of procollagen IIA mRNA as detected by RT-PCR analysis. The ratios of IIB/IIA mRNA were 1.51 and 11.82 in control and AS-β2 treated cultures, respectively (Fig. 3).

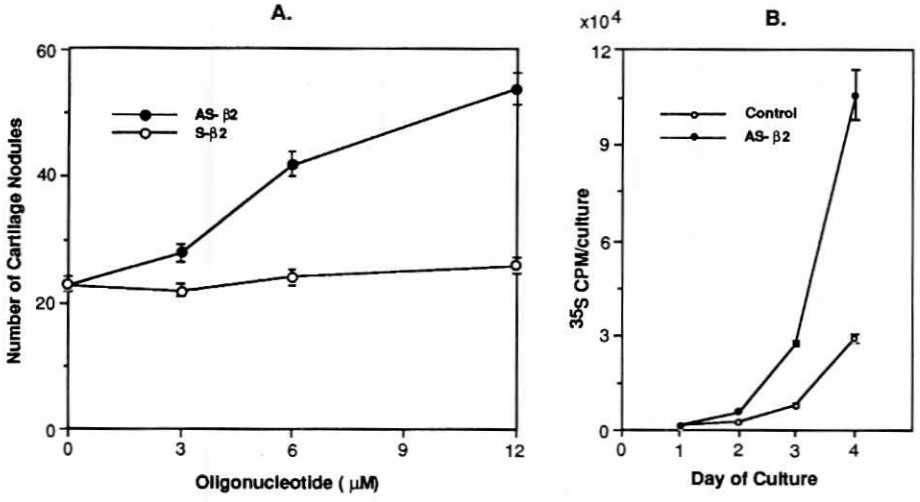


Fig. 2. AS-β2 supplied in the medium enhances chondrogenesis. (A) *Increase in number of cartilage nodules in limb bud cell micromass cultures is AS-β2 dose-dependent. Sense oligo (S-β2) had minimal effect. AS-β2 was added at 1 day after explantation and the number of cartilage nodules counted in alcian blue-stained cultures on the 4th day of culture. The data shown represents mean number of nodules in each of 5 randomly selected fields of a micromass culture. Each point represents mean±SD of 4 cultures.* **(B)** *Rate of synthesis of ^{35}S-labeled proteoglycans. ^{35}S-sulfate was supplied in fresh medium on each of the 4 days of culture and cell-associated, labeled proteoglycans monitored 18 h later. The rate is highest on day 4 at which time the synthesis in the cultures supplemented with AS-β2 is 3 to 4-fold greater than the control, unsupplemented cultures.*

Effects of other retinoid receptors

To determine if the stimulation of chondrogenesis was unique to AS-β2, we prepared antisense oligodeoxynucleotides for representative isoforms of other RARs and RXRs and monitored their effects on chondrogenesis. With the exception of antisense RXR-β, all the other antisense oligos (RAR-β1/β3, RAR-α2, RAR-γ1) stimulated chondrogenesis; the increases in cartilage nodule number and staining intensity were similar in effect to that of the AS-β2 (data not shown). ^{35}S-sulfate incorporation into proteoglycans was also enhanced by each of the antisense RAR isoform to levels similar to that of AS-β2 (Fig. 4). Again, antisense RXR-β did not stimulate proteoglycan synthesis; instead, it produced a slight inhibition in ^{35}S-sulfate incorporation.

Effect of retinoic acid

The addition of RA to cultures on day 1 inhibited the development of cartilage nodules and reduced the intensity of staining with Alcian blue, and these effects were dose-dependent (Figs. 1E and 5A). As observed previously, RA at 0.3 μM reduced the number to 38% of the control level while 0.6 μM reduced it to about 5% (Fig. 5A) (Kochhar *et al.*, 1984). Supplementation of RA-treated cultures with AS-β2 restored to normal levels the development of cartilage nodules so that the number and the staining intensity of the nodules approximated the control cultures (Figs. 1G,H and 5A). Again, sense RAR-β2 produced no such reversal (Figs. 1F, 5A). Also, no reversal of the inhibitory effects of RA was produced by RAR-β1/β3, RAR-α2, RAR-γ1, or RXR-β antisense oligos (data not shown). Thus, reversal of the RA effect on chondrogenesis was specific to only AS-β2.

AS-β2 also restored the synthesis of cartilage matrix molecules which was inhibited by RA. ^{35}S-sulfate incorporation into proteoglycans, which was reduced to 50% of the control value by RA, increased to normal levels (Fig. 5B). This effect was specific to AS-β2 since sense RAR-β2 or antisense RAR-α2 did not restore normal chondrogenesis. The ratio of procollagen IIB/IIA mRNA which was reduced to less than 1 by RA, was restored to normal levels which ranged between 6 to 8 (Fig. 3).

Microscopy of sectioned micromass cultures showed that the cartilage nodules in AS-β2 treated cultures, in the absence of exogenous RA, expanded in size entirely due to accumulation of large amounts of extracellular matrix materials (Fig. 6A,C). The neighboring nodules in these cultures coalesced but still retained the cartilage phenotype. RA treatment, in the absence of AS-β2, not only prevented the formation of nodules but the few nodules that did form contained closely packed cells without much matrix around them (Fig. 6B). The microscopic appearance of cartilage nodules in cultures treated with both RA and AS-β2 was essentially similar to control, untreated cultures. (Fig. 6D).

Transmission electron microscopy of control and AS-β2 treated cultures confirmed the stimulatory effect of AS-β2 on extracellular matrix synthesis. Cytological features of AS-β2 treated chondrocytes were typical of normal control chondrocytes except for the presence of an increased number of perinuclear vacuoles indicative of enhanced synthetic activity. The volume of extracellular matrix around the AS-β2 treated chondrocytes was also much larger than the control chondrocytes (Fig. 7A,B).

Modulation of RAR-β levels

In Western blot analysis, the monoclonal antibody to mouse RAR-β detected a band with relative molecular weight of 50 kDa in limb bud mesenchymal cells (Fig. 8A, lane 1). Exposure of micromass cultures to 0.3 μM RA greatly enhanced the levels of RAR-β within 3 h of treatment (Fig. 8A, lane 2). Simultaneous exposure of cells to RA and AS-β2 decreased RAR-β levels to 40% of the level in cells treated with RA alone (Fig. 8A, lane 3; Fig. 8B).

Cell proliferation

To investigate if RA was modulating chondrogenesis by controlling cell proliferation, control and RA cultures were pulse-labeled with ^{3}H-thymidine. The rate of thymidine incorporation into DNA in control cultures on each of the first 3 days was much greater than in the RA cultures (Fig. 9A). The control rate suddenly declined on day 4, indicating a lower level of DNA synthesis and presumably of cell proliferation in differentiated cultures. Beginning on day 1 when RA was first added, RA cultures showed a steady decrease in the rate of thymidine incorporation into DNA (Fig. 9A). RA did not affect cell viability which remained at 91±5% in both control and RA cultures.

We also wished to determine if AS-β2 reversed the RA-

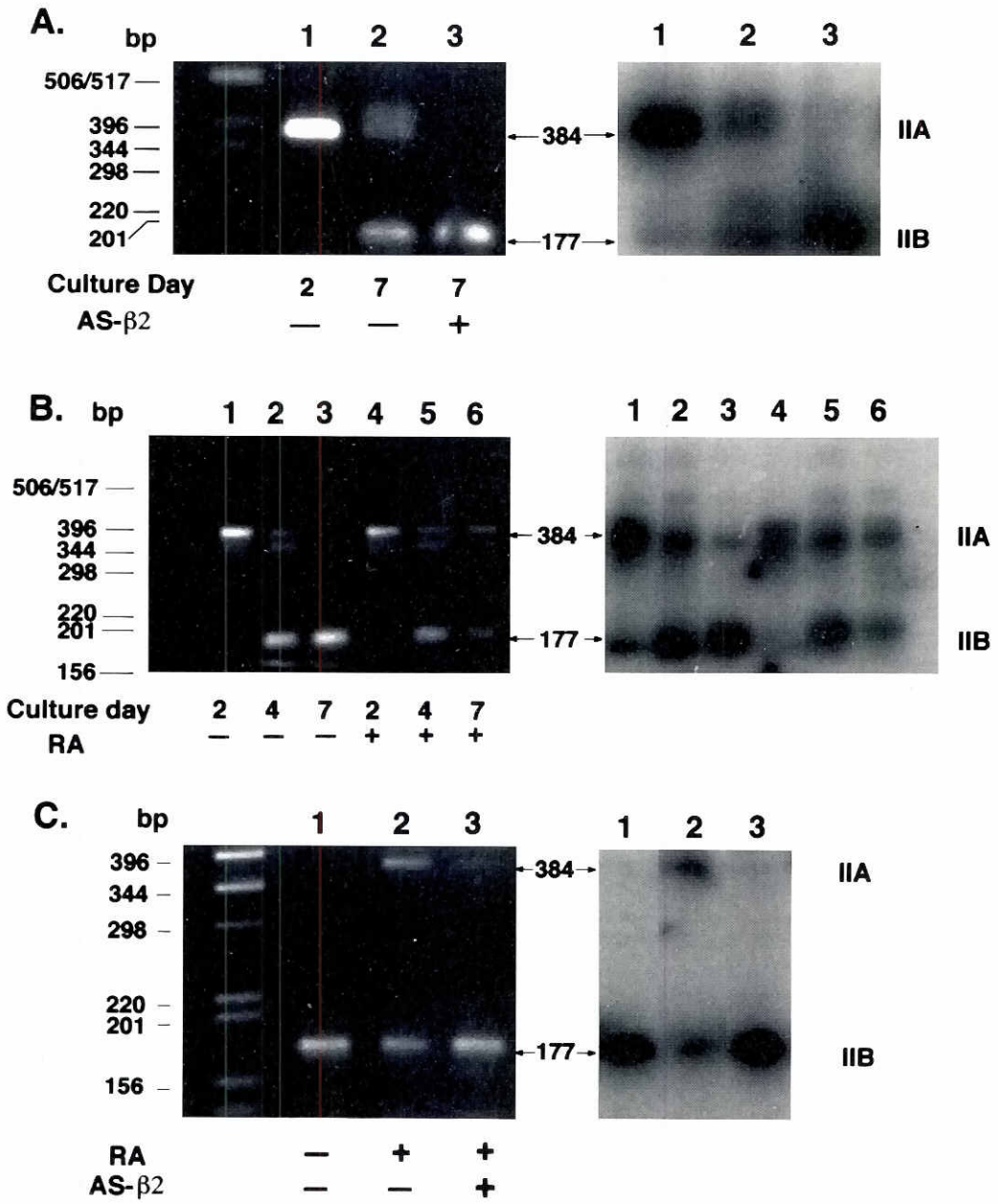


Fig. 3. Relative levels of synthesis of procollagen type IIA and cartilage-specific procollagen type IIB mRNAs in limb bud cells is influenced by AS-β2 and RA. *Cells were treated on day 1 and harvested on day 2, 4, or 7 of culture. Total extracted RNAs were submitted to RT-PCR analysis (left panels) and Southern blot hybridizations of the RT-PCR products (right panels). The bands at 384 bp (type IIA) and 177 bp (type IIB) were quantified and IIB/IIA ratios determined.* **(A)** *AS-β2 treated cultures. IIA mRNA synthesis was dominant on day 2. Both IIA and IIB mRNAs were detected on day 7. In untreated cultures the ratio IIB/ IIA was 1.51 while the ratio was greatly enhanced to 11.82 in AS-β2 treated cultures.* **(B)** *RA-treated cultures. In the absence of RA, mRNA expression shifted from IIA to IIB as chondrogenesis progressed from day 2 to day 7 of culture; IIB/IIA ratios were 0.35 (day 2, lane 1), 1.33 (day 4, lane 2), and 6.07 (day 7, lane 3). In RA-treated cultures, the IIB/IIA ratios remained about 1 throughout the culture period (lanes 4, 5, and 6 ratios were 0.4, 1.37 and 0.92, respectively).* **(C)** *Day 7 cultures treated with both RA and AS-β2. AS-β2 restored the synthesis of IIB mRNA to control levels (IIB/IIA ratio was 7.98 in lane 3).*

induced chondrogenic inhibition by restoring the normal rate of cell proliferation. Due to the altered nucleotide pools in the antisense treated cultures, we chose to assess cell proliferation by direct cell count rather than thymidine incorporation. Day 4 cultures were trypsinized and total number of cells counted in a hemacytometer (Fig. 9B). Control cultures contained an average of $5x10^6$ cells, and this number was reduced by RA to 50%. The supplementation with AS-β2 produced no significant change in cell number in control or in RA cultures (Fig. 9B).

Discussion

The process of chondrogenesis in micromass cultures parallels closely that observed in limbs and other sites in the developing embryo (Kosher *et al.*, 1986; Caplan and Pechak, 1987; Solursh, 1990; Sasano *et al.*, 1992). We show here that the process of chondrogenesis in limb bud mesenchymal cells is strongly modulated by the action of RARs. Antisense RAR supplementation of the culture medium stimulated the formation of cell aggregates resulting in an increase in cartilage nodule number. These nodules stained more intensely with Alcian blue than those in the unsupplemented cultures. Alcian blue, a cationic dye, binds quantitatively to the negative charges on the glycosaminoglycan chains of proteoglycans (Lev and Spicer, 1964; Bjornsson, 1993). Furthermore, the enhancement of proteoglycan synthesis by antisense RAR was confirmed quantitatively by the increase in $[^{35}S]$-sulfate incorporation into proteoglycans.

More specific evidence that AS-β2 did indeed enhance chon-

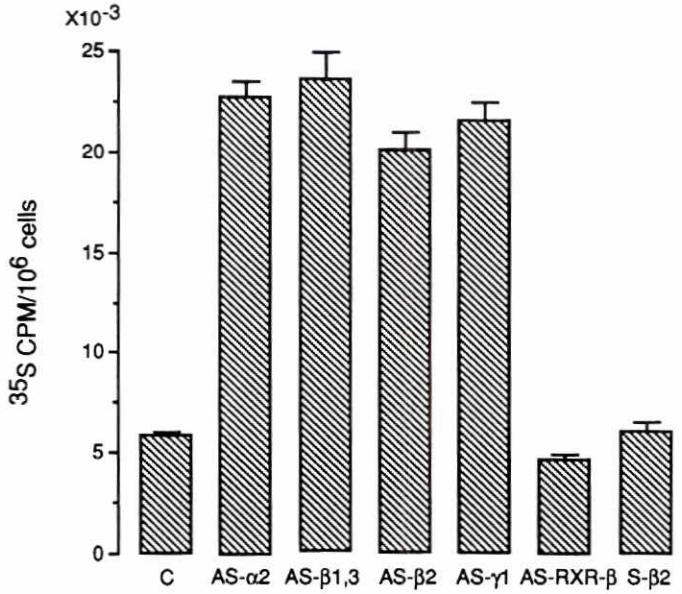


Fig. 4. Effect of antisense oligonucleotides (AS) for individual isoforms of RARs (α, β, γ) and RXR-β on synthesis of sulfated proteoglycans in limb bud cell micromass cultures. *The oligonucleotides (6 μM) were added to the medium at 1 day after the day of explantation. ^{35}S-sulfate was supplied in fresh medium for the last 18 h of the 4 day culture period. All antisense RARs (AS-α2, AS-β2, AS-γ1) produced 3 to 4-fold stimulation of the proteoglycan synthesis. The synthesis level in control* **(C)** *cultures was not changed by sense RAR-β2 oligonucleotide (S-β2). In contrast, synthesis was slightly inhibited by antisense RXR-β oligonucleotide (AS-RXR-β).*

drocyte differentiation was obtained by comparing the relative expression of the two alternatively spliced transcripts of COL2A1 gene in control and AS-β2 supplemented cultures. These two forms are differentially expressed during development and chondrogenesis with IIA being expressed by prechondrocytes (or chondroprogenitor cells) while the IIB is almost strictly associated with advanced stages of chondrocyte differentiation (Sandell *et al.*, 1994). Our results show for the first time that in micromass culture the conversion of mesenchymal cells into chondrocytes was accompanied by a major switch in the expression of the two transcripts from IIA in day 2 cultures in which the majority of the cells are still mesenchymal cells to IIB in day 7 cultures in which chondrocytes become the dominant cell population. AS-β2 supplementation, in keeping with its stimulatory effects on other parameters, enhanced the proportion of IIB over IIA transcripts by about 3-fold.

Inhibition of chondrogenesis by RA such as shown here is well known but the mechanism remains unresolved (Kochhar, 1977; Lewis *et al.*, 1978; Kochhar *et al.*, 1984; Zimmermann and Tsambaos, 1985; Kistler, 1987; Von Schroeder *et al.*, 1994). RA treatment did not produce cytotoxicity or lower cell viability; in fact, at lower concentrations and under serum-free conditions, RA has been shown to promote growth and stimulate chondrogenesis (Ide and Aono, 1988; Paulsen *et al.*, 1994). Cell proliferation was reduced by RA but this may be unrelated to chondrogenesis since the chondrogenic inhibition was ameliorated by AS-β2 without any significant restoration of the normal level of cell proliferation. Our findings that a downregulation in the synthesis of two independent markers of chondrocyte differentiation, i.e., sulfated proteoglycans and collagen IIB, by RA and the simultaneous restoration of their synthesis by AS-β2 indicated

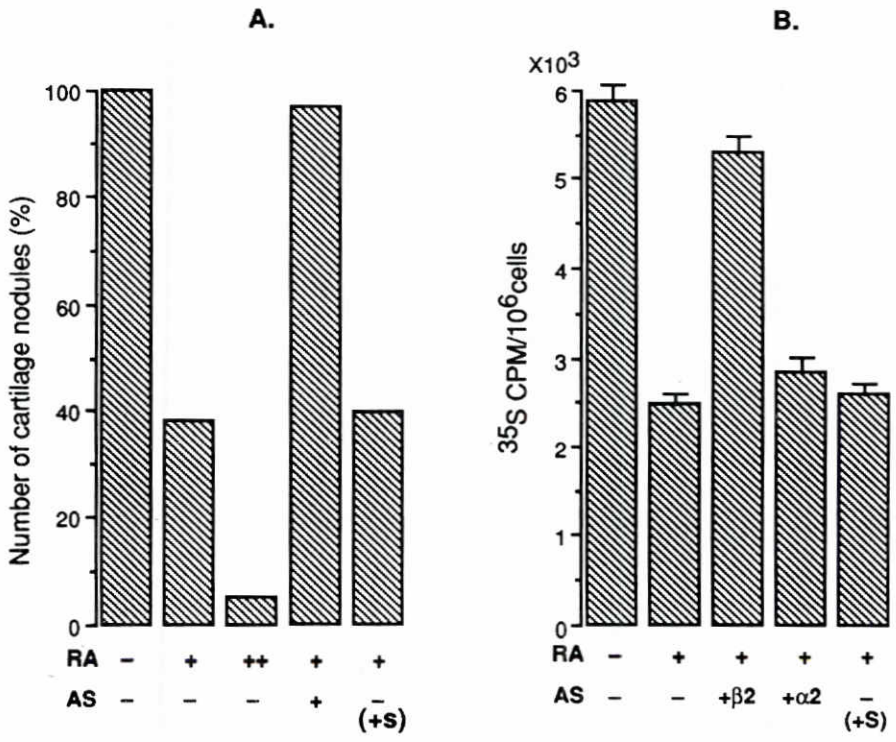


Fig. 5. Reversal of RA-induced inhibition of chondrogenesis by antisense AS-β2 (AS). (A) *Effect on number of nodules. RA at 0.3 μM (+) or 0.6 μM (++) was added on day 1 to the cultures alone or simultaneously with 6 μM of either AS or sense (S) RAR-β2 oligonucleotide. Cartilage nodule number in control, unsupplemented cultures on day 4 is taken as 100%.* **(B)** *Effect on ^{35}S-sulfate incorporation. RA (0.3 μM) was added without or with AS-β2 (6 μM) on day 1 of culture. ^{35}S-sulfate incorporation into proteoglycans was monitored on day 4. AS-β2 restored the synthesis to control level, while AS-α2 or sense (S)-β2 did not.*

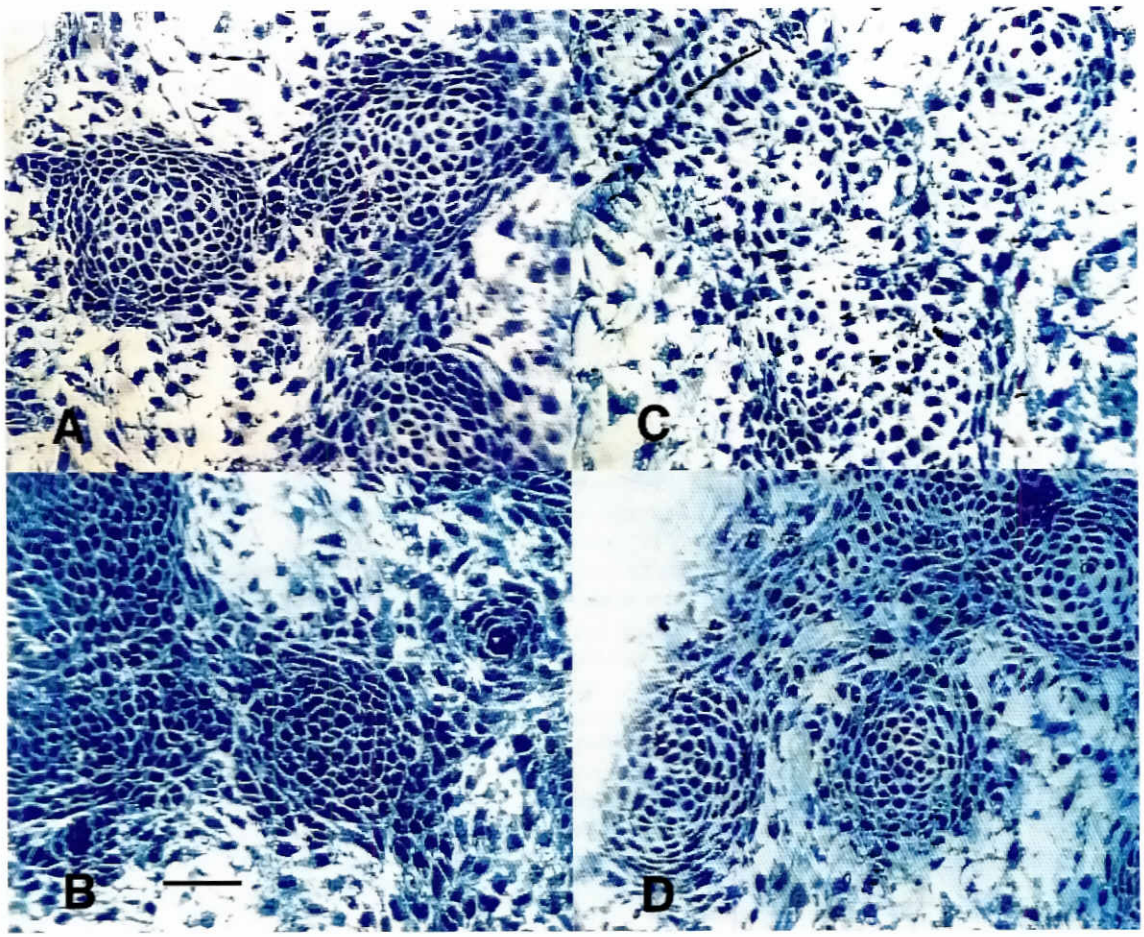


Fig. 6. Microscopy of sectioned micromass cultures, stained with Azure II. (A) *Control, unsupplemented culture. A field with three cartilage nodules and internodular mesenchymal cells.* **(B)** *RA-treated culture. The field shown had three nodules which are otherwise sparsely distributed. The extracellular matrix is much reduced in amount.* **(C)** *AS-β2 treated culture. The three coalesced nodules show cartilage phenotype, but contain dramatically increased amount of extracellular matrix.* **(D)** *Culture treated with both RA and AS-β2. Normal nodular phenotype is restored. Bar, 50 μm.*

that a preceding step in this differentiation pathway was involved.

The negative control of chondrogenesis by RA and RAR-β2 in micromass cultures may operate similarly in skeletal morphogenesis in the developing limb. RA administration to mice at midgestation produces skeletal dysplasia in fetuses which show long bone reduction and digital deformities (Kochhar, 1973). Within 3-4 h of RA administration, the levels of RAR-β2 transcripts in the AER and the central core mesenchyme are increased which are 10-20 fold higher than the pretreatment levels (Mendelsohn *et al.*, 1991; Harnish *et al.*, 1992). The AER is an inducer of distal limb outgrowth of mesenchyme underlying the AER (Saunders and Gasseling, 1968). Among other functions, AER serves to suppress chondrogenesis in the subjacent mesenchyme in the so-called "progress zone" which is an important event in pattern formation (Solursh *et al.*, 1981; Summerbell, 1976). RA-induced overexpression of RAR-β2 in the AER may constitute one mechanism by which RA produces abnormalities in the skeletal patterning.

In view of our results where dramatic enhancement in chondrogenesis in micromass culture occurred in response to a suppression in the level of RARs, one could envision that normal function of the retinoids in limb development would be to define regionally restricted cell differentiation domains. The presence of RAR-β2 expressing cells lining the periphery of the digital cartilage segments and the absence of this receptor from cartilage anlage of long bones which eventually forms in the central core of the mouse limb supports this suggestion (Mendelsohn *et al.*, 1991). Other evidence for a similar role of RA in development comes from the hindbrain development in the chick and mouse embryos where the expression of the *Hoxb-1* gene is progressively restricted to only one segment, rhombomere 4 (Murphy *et al.*, 1989; Wilkinson *et al.*, 1989; Zimmer and Zimmer, 1992). It is now known that RA is responsible for this restriction by virtue of the presence of a conserved RARE in the negative regulatory region (repressor) in the *Hoxb-1* gene resulting in a sharpened segmentally restricted expression during rhombomere boundary formation (Studer *et al.*, 1994). The skeletal deformities in RA-treated fetuses may also be due to, in part, an elimination of sharp boundaries between cartilage-forming and non-cartilage forming regions in response to an overexpression of RAR-β2. Further *in situ* hybridization and immunolocalization studies will be needed to provide more definitive evidence.

It is interesting that the antisense oligodeoxynucleotides for the other major RAR-β isoform, RAR-β1/β3, as well as certain isoforms of RAR-α and RAR-γ which we tested were also able to stimulate chondrogenesis. These results would indicate that all RARs are equally involved in the negative regulation of chondrogenesis, at least in micromass cultures (Motoyama and Eto, 1994). Such functional redundancy among RAR subtypes may

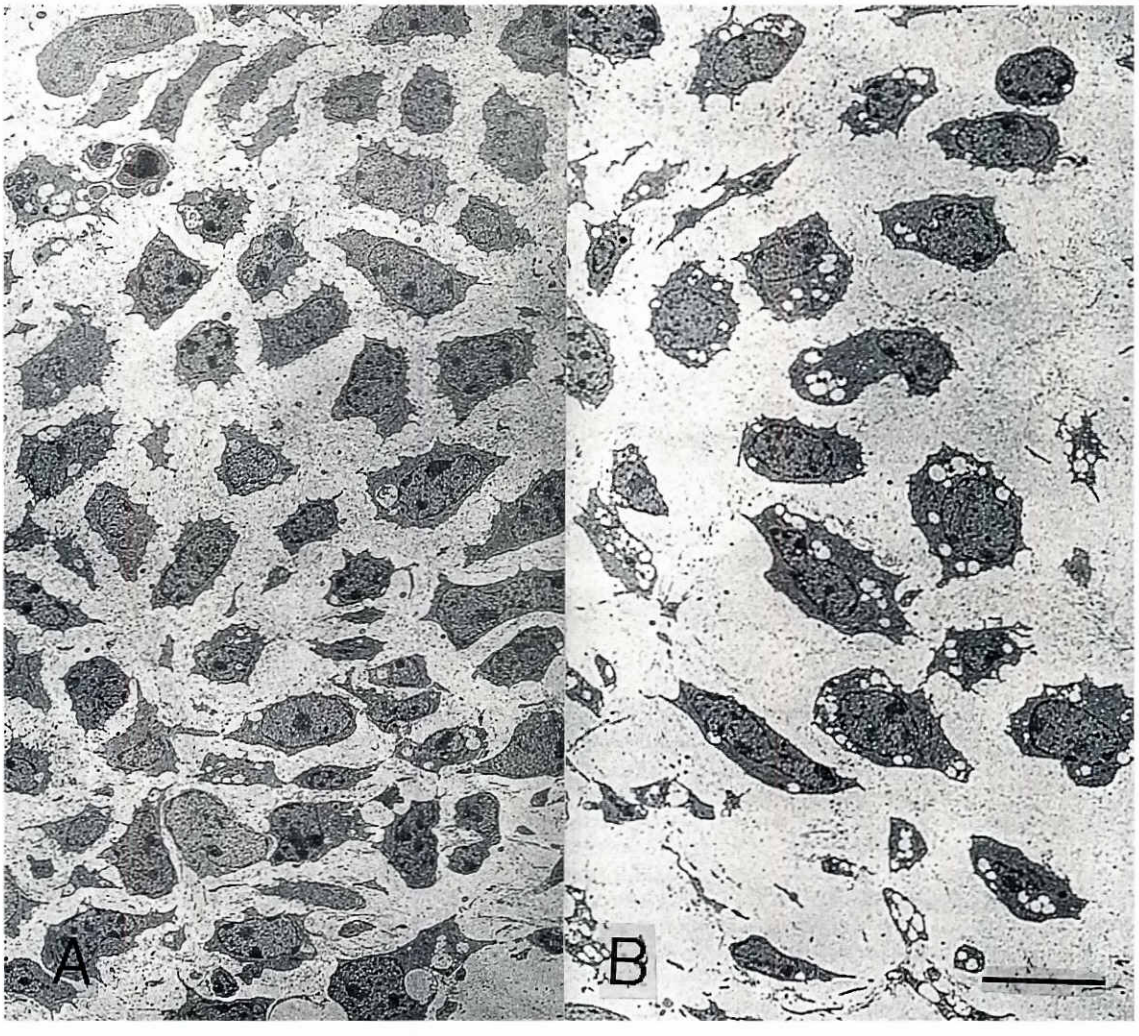
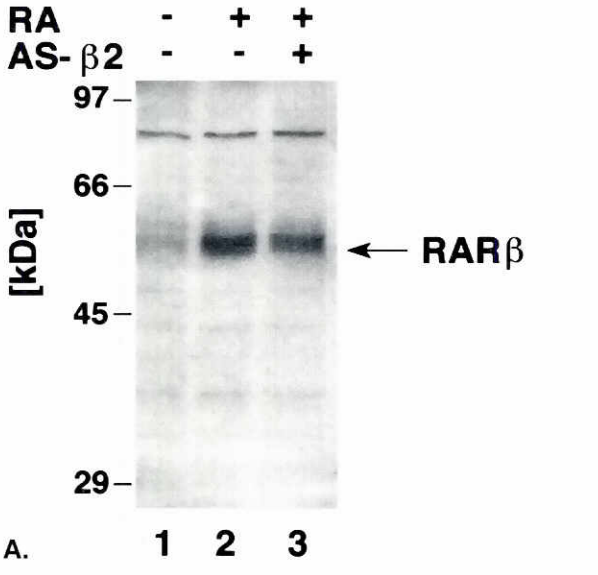


Fig. 7. Transmission electron microscopy of micromass cultures. **(A)** *Control, unsupplemented culture, same as shown in Fig. 6A.* **(B)** *AS-β2 treated culture, same as shown in Fig. 6C. Chondrocytes with an increased number of cytoplasmic vacuoles are separated by a greater volume of extracellular matrix than the chondrocyte in control cultures. Bar, 10 μm.*

explain why no skeletal defects were observed in transgenic mice lacking one or even two RARs and RXRs (Chambon, 1994). It is significant, however, that none of the other antisense oligos except AS-β2 were able to reverse the RA-induced chondrogenic inhibition, a fact which in view of its individual upregulation by RA treatment attests to a specific role for this receptor in teratogenesis. It is to be noted that antisense RXR-β oligo did not stimulate chondrogenesis. Although we have yet to examine the role of RXR-α and RXR-γ, we have observed that certain synthetic retinoids which are selective ligands for RXRs fail to inhibit chondrogenesis in the micromass cultures (Kochhar *et al.*, 1994). Indeed, recent genetic evidence from mutant mice lacking RXR-α gene suggests that eye development and cardiac morphogenesis may be the targets for response pathways controlled by RXRs (Kastner *et al.*, 1994; Sucov *et al.*, 1994).

Our use of antisense oligodeoxynucleotides is based on several studies which have previously demonstrated that this technology can effectively reduce levels of functional gene products essential for growth or differentiation (Heikkila *et al.*, 1987; Gewirtz and Calabretta, 1988; Wickstrom *et al.*, 1988; Bacon and Wickstrom, 1991; Soprano *et al.*, 1992; Pena *et al.*, 1993; Cosenza *et al.*, 1994). The exposure of the limb bud mesenchymal cells to AS-β2 would be expected to lower the level of the receptor protein in the cells, and this was indeed observed. Normal, untreated limb bud cells have a low but detectable levels of RAR-β mRNA and protein as reported previously (Dolle *et al.*, 1990; Mendelsohn *et al.*, 1991; Harnish *et al.*, 1992). An exposure to RA upregulated the level of RAR-β protein about 5-fold above the level of the control limb bud cells as has been observed in the limb bud *in situ* (Soprano *et al.*, 1994), and this level was decreased in AS-β2 supplemented cultures to about 40% of the level of cells exposed to RA alone. The fact that modulation of chondrogenesis was observed even in the presence of residual levels of the receptor support our previous finding that the embryological events are governed by threshold levels of the retinoid receptors (Soprano *et al.*, 1994).

The suggestion that RAR-β2 mediates the teratogenic activity of RA is challenged by the results of a recent study (Mendelsohn *et al.*, 1994). When RAR-β2 null mice were challenged with a teratogenic dose of RA on 11.5 day of gestation, typical limb reduction defects resulted which were no different from those of the wild type mice (Mendelsohn *et al.*, 1994). This observation is puzzling and raise the likelihood that there are alternative factors in the embryo which permit RA to maintain its teratogenic activity.



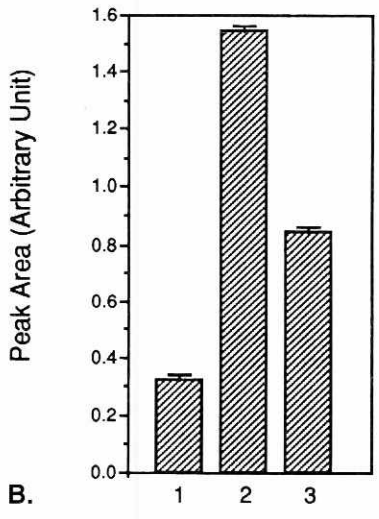


Fig. 8. Western blot analysis of RAR-β expression in limb bud cells. (A) *Cells in day 2 cultures were untreated (lane 1) or treated for 3 h with 0.3 μM RA (lane 2) or treated both with RA and AS-β2 (12 μM, lane 3). Whole cell extracts were submitted to electrophoresis and electrotransferred to nitrocellulose filters. Monoclonal RAR-β antibody detected a band of approximately 50 kDa (lane 1) which was upregulated several fold by RA treatment (lane 2). RAR-β was downregulated by AS-β2 to about 40% of the level of RA-treated cells (lane 3).* **(B)** *Band quantitation by automatic image analysis.*

Materials and Methods

Cultures

Micromass cultures were prepared from limb buds of day 11 embryos (40±2 somites) of ICR mice as previously reported (Ahrens *et al.*, 1977; Kochhar and Penner, 1992). Limb buds were incubated at 37°C for 10-20 min in calcium- and magnesium–free saline containing trypsin and EDTA (0.1% each) and dissociated by trituration. The enzyme solution was diluted with equal volume (1 mL) of the culture medium consisting of Eagle's MEM plus 10% fetal bovine serum (GIBCO). The cells were collected by centrifugation, resuspended in 1 mL culture medium and filtered through 20 μm mesh nylon macroporous filters (Spectrum, Los Angeles, CA, USA) to remove clumps and epithelial fragments. The cell number was determined by a hemacytometer and adjusted to a final concentration of $2x10^5$ cells per 20 μL. Twenty microliters of cell suspension was placed in the center of each well of a 24-well tissue culture dish and incubated at 37°C for 2 h in a humidified chamber in an atmosphere of 5% CO_2 in air to allow cell attachment. One mL of culture medium containing 150 μg/mL ascorbic acid was added to each well and incubation continued overnight without any further treatment.

On day 1 of culture (24 h after explantation), the cultures were treated in fresh medium with either antisense oligodeoxynucleotide (oligo) or sense oligo in the presence or absence of all-trans retinoic acid (RA). The oligos were dissolved in distilled water and added at various concentrations to the culture medium. RA (Sigma) was dissolved in ethanol before addition at various concentrations to the culture medium. For microscopy, the cultures were terminated on day 4. For other experiments, they were terminated on each day of the 4 day culture period as outlined below; in some cases, the cultures were maintained for 7 days with one change of fresh medium on day 4.

The use of charcoal-treated serum (to remove small molecular weight lipid soluble materials including retinoids) instead of the whole serum as

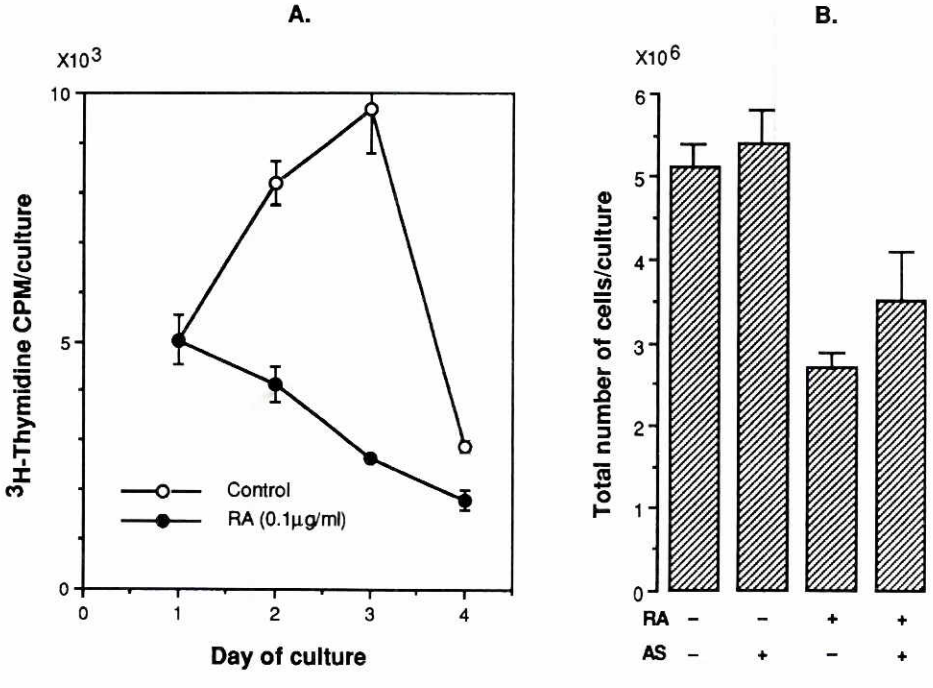


Fig. 9. AS-β2 does not change cell proliferation rate in control or RA-treated cultures. (A) *^{3}H-thymidine incorporation. The cells were exposed to ^{3}H-thymidine for 5 h before harvesting on each day of culture. Each point represents mean (±SD) of 4 micromass cultures. Proliferation rate peaks on day 3 in control cultures and then declines. RA induces a depression in proliferation rate throughout the culture period.* **(B)** *Cell number at the end of 4 day culture period. The addition of AS-β2 (6 μM) alone produced no significant change while RA (0.3 μM) alone or in the presence of AS-β2 reduced cell multiplication in micromass cultures.*

supplement in the culture medium did not alter any of the reported morphological or quantitative parameters.

Oligodeoxynucleotides

All antisense and sense oligodeoxynucleotide phosphorothioates were synthesized as 18 mers utilizing an ABI 380B synthesizer on a scale of 1.0 μM per synthesis by the Oligonucleotide Synthesis Laboratory at Temple University School of Medicine. Antisense oligodeoxynucleotide phosphorothioate sequences were complementary to the mRNA sequences which encode amino acids 3 to 8 of mouse RAR-β2 (AS-β2) [5'CAGAACATCCATACAGTC3'] (Zelent *et al.*, 1991), mouse RAR-β1, 3 (AS-β1, 3) [5'GCATGCGTGGCTGCTGGT3'] (Zelent *et al.*, 1991), mouse RAR-γ1 (AS-γ1) [5'GAGTCTCTCCTTATTGGT3'] (Kastner *et al.*, 1990), mouse RAR-α2 (AS-α2) [5'CCCGACTTCCACACTCTC3'] (Leroy *et al.*, 1991) and mouse RXR-β (AS-RXR-β) [5'GGAGCCCAGTGGCGGGGG3'] (Mangelsdorf *et al.*, 1992). The sense oligodeoxynucleotide phosphorothioate corresponded to the nucleotide sequence encoding amino acids 3 to 8 of mouse RAR-β2 (S-β2) [5'GACTGTATGGATGTTCTG3'].

Assessment of chondrogenesis

Cartilage nodules

The cultures were fixed for 30 min in 10% formalin containing 0.5% cetyl pyridinium chloride and stained with Alcian blue at pH 1.0 as described (Kochhar and Penner, 1992). Under an inverted microscope (Leitz Diavert), each micromass culture was frame-grabbed and stored as a digital image using a color video camera (Sony DXC 151) and a capture board (Computer Friends, Inc.) contained in a Quadra 800 computer (Apple Macintosh). Total nodule number in a culture was obtained by an automatic image analysis program (N.I.H. Image-1.55, Wayne Rasband), and mean±S.D. values were determined using 4 replicate cultures per treatment. Spot micromass images for visual presentation were photographed using a Leitz Orthomat camera atop a Leitz Diavert microscope. The photographic slides were then scanned using a digital slide scanner (Barney Scan) and the digital images combined using Adobe Photoshop and printed on a dye sublimation printer (Phaser II SDX, Tektronix, Inc.).

Microscopy

The cultures were fixed *in situ* for 2 h in 1% glutaraldehyde-paraformaldehyde dissolved in 0.1 M cacodylate buffer at pH 7.3 (Karnovsky, 1965), postfixed in 1% osmium tetroxide, and embedded in Spurr resin. The embedded cell layer was separated from the plastic substratum and sectioned using an LKB-Huxley ultramicrotome. Thick sections were mounted on glass slides, stained with Azure II, and digital images obtained using a Nikon Biophot microscope, grouped and printed as mentioned above. The thin sections were examined with a Zeiss 109 transmission electron microscope.

[^{35}S] Sulfate incorporation

The cultures were incubated for 18h in fresh medium containing 5-20 μCi/ml carrier-free $H_2{}^{35}SO_4$ (ICN Biomedicals, Irvine, CA, USA), washed three times with phosphate-buffered saline (PBS) containing 100 μg/ml sodium sulfate in an ice-bath, and air-dried. Under these conditions, >95% of the label incorporated in the cultured limb bud cells is recovered as a component of high molecular weight proteoglycans (Kochhar *et al.*, 1984). The cell layer was scraped off the dish in 1 mL of 10% TCA containing serum albumin (1 mg/mL) as carrier, sonicated for 1 min and allowed to remain in the ice-bath for 30 min. The sonicate was spotted on filter paper discs (GN6, 0.45 μm pore size, Gelman Sciences) moistened with 10% TCA on a filter manifold, and washed with an excess of 10% TCA to remove the free label. The filters were air-dried and counted in 10 mL Scintiverse (Fisher) in a liquid scintillation counter.

Collagen II synthesis

Collagen II is the product of a single copy gene, COL2A1, which produces two pro-α1(II) collagen mRNA transcripts by alternative splicing. One of these transcripts is missing exon 2 which codes for much of the NH_2-terminal propeptide of fibrillar collagens. The resultant peptide, termed collagen IIB, is a specific marker for chondrocytes in contrast to the product of the other transcript containing exon 2 (collagen IIA) which is expressed in skeletal precursors and other non-chondrogenic cell lineages (Ryan and Sandell, 1990; Sandell *et al.*, 1991, 1994; Ng *et al.*, 1993). We used reverse transcriptase-polymerase chain reaction (RT-PCR) to detect the relative expression of the two mRNAs obtained from each micromass culture.

Total cellular RNA was extracted from the cultured cells by guanidinium thiocyanate method (Chomczynski and Sacchi, 1987) and 5 μg of this RNA used to synthesize cDNA with random oligonucleotide primers and reverse transcriptase in a 15 μL reaction mixture (First-Strand cDNA synthesis kit, Pharmacia). 5 μL of the cDNA synthesis reaction mixture was then amplified (GeneAmp PCR Core Reagents kit, Perkin Elmer Cetus) using a pair of oligonucleotide primers corresponding to mouse COL2A1 exon 1 (5' GAGGGCCAGGAGGTCCTCTGG 3') and exon 4 (5' TCGCGGTGAGCCATGATCCGC 3') to amplify both IIA and IIB cDNA simultaneously. PCR was carried out using a 0.5-min denaturation (94°C), 0.5-min annealing (50°C), and 0.5-min extension (72°C) for 30 cycles. PCR products were separated by gel electrophoresis in 2% agarose gel, transferred to Hybond-N (Amersham) nylon filters, and probed with a fragment of mouse COL2A1 cDNA clone containing exon 1-4 sequence. Quantitation of the bands was carried out as described for the western blot analysis below.

Cell proliferation

[^{3}H] Thymidine incorporation

The cultures were supplied with fresh medium containing [^{3}H] thymidine (1.0 μCi/ml, specific activity 35 Ci/mmol, ICN Biomedicals, Irvine, CA, USA), and incubation continued for 5h. The radioactive medium was removed and the cells washed three times with PBS in an ice bath; the washes contained cold thymidine (100 μg/ml). The cultures were sonicated in 1 mL of 1% TCA and processed for counting by the filter method mentioned above in the [^{35}S] sulfate incorporation experiment.

Cell number

At the end of the 4 day culture period, the micromass cultures were dissociated by replacing the culture medium with 1 mL/well of calcium- and magnesium-free saline containing trypsin/EDTA (0.1% each). After 30 min at 37°C, the digest was triturated and cell count made using a hemacytometer.

Western blot analysis of RAR-β levels

The cells cultured for 2 days in control media were further incubated for 3 h in fresh medium in the presence or absence of 0.3 μM RA, or in the presence of both RA and 12 μM AS-β2. They were washed two times with ice-cold PBS containing dithiothreitol (1 mM), phenylmethylsulfonyl fluoride (1 mM), Leupeptin (0.5 μg/ml), Pepstatin A (0.5 μg/ml) and Aprotinin (0.5 μg/ml). The cells were scaped off the dish in the lysis buffer, homogenized, and 100 μg protein of the homogenate was analyzed by Western blot utilizing the monoclonal mouse RAR-β antibody (Affinity Bioreagents, Inc., Neshanic Station, NJ, USA) and the "Enhanced Chemiluminescence" (ECL) kit (Amersham) as described previously (Soprano *et al.*, 1994). The band densities were quantitated by means of a flat bed scanner (Scan Maker 600 ZS, Microtex) using Scan Analysis Biosoft-2.21 and the automatic image analysis program (N.I.H. Image – 1.55, Wayne Rasband).

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