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-β2 — 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-β2 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 IIIB were all enhanced in a dose-dependent manner. However, only the antisense RAR-β2 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-β2 serves to prevent the mesenchymal cells from expressing their chondrogenic bias. Our results further strengthen the argument that RA-dependent elevation in RAR-β2 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-terminal 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; Rutter et al., 1991a). In certain embryonic organs, e.g. the neural tube and the limb bud, both RAR-β and RXR-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, RXR-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 mice that the RAR-β 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-β2, antisense RAR-β oligodeoxynucleotide; AS-RXRβ, antisense RXR-β oligodeoxynucleotide; oligo, oligodeoxynucleotide; RA, retinoic acid; RAR, retinoic acid receptor; RXR, retinoic X receptor.

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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 increased nodules numbers 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 [35S]-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).
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). 35S-sulfate incorporation into proteoglycans was also enhanced by each of the antisense RAR isofrom 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 35S-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 only to AS-β2.

AS-β2 also restored the synthesis of cartilage matrix molecules which was inhibited by RA. 35S-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 II/B/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. 5A). 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. 5B). 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 3H-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-
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 \(5 \times 10^6\) 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, 1954; Bjornsson, 1993). Furthermore, the enhancement of proteoglycan synthesis by antisense RAR was confirmed quantitatively by the increase in [35S]-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 (8 µM) were added to the medium at 1 day after the day of explantation. 35S-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).

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 35S-sulfate incorporation. RA (0.3 µM) was added without or with AS-β2 (6 µM) on day 1 of culture. 35S-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.

Chondrocyte 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...
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, 1986). 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 isofrom, 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
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.
Materials and Methods

Cultures

Micromass cultures were prepared from limb buds of day 11 embryos (40s2 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% CO₂ 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
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 16mers 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'CAGAACATCCCTACATCTCTCC3'] (Zelent et al., 1991), mouse RAR-β1, 3 (AS-β1, 3) [5'GACATGCGTGCGCTGCTGG3'] (Zelent et al., 1991), mouse RAR-γ1 (AS-γ1) [5'GAGGCTCTCTTTATTAGCTG3'] (Kastner et al., 1990), mouse RAR-α2 (AS-α2) [5'GCCGACTTCCACACCTCTGC3'] (Leroy et al., 1991) and mouse RXR-α (AS-RXR-α) [5'GAGGCCAGTAGCAGAGGGG3'] (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'GACTGATGGAAGCAGGAT3'].

**Assessment of chondrogenesis**

**Cartilage nodules**

The cultures were fixed for 30 min in 10% formalin containing 0.5% cetylpyridinium 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 Orthomatt 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.

**[35S] Sulfate incorporation**

The cultures were incubated for 18 h in fresh medium containing 5-20 μCi/ml carrier-free H35SO4 (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 (GNS, 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 NH2-terminal portion 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 lin- eages (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 15μl 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'GAGGGCGAGAGGCGCTGCTGG3') and exon 4 (5'TCGCGGTGACCGCATGATCCGC3') 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**

**[3H] Thymidine incorporation**

The cultures were supplied with fresh medium containing [3H] thymi- dine (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 [35S] 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 scraped 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, Microtek) using Scan Analysis Biosoft-2.21 and the automatic image analysis program (N.I.H. Image – 1.55, Wayne Rasband).

**Acknowledgments**

This work was supported by N.I.H. grants HD 20925 (DMK), HD 27665 (DRS), and a March of Dimes award (DMK). DRS is a recipient of Research Career Development Award (HD 01076) from N.I.H.
References


Accepted for publication: July 1995