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STABLE, POSITION-RELATED RESPONSES TO RETINOIC ACID BY CHICK LIMB-BUD MESENCHYMAL CELLS IN SERUM-FREE CULTURES

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SUMMARY

Retinoic acid (RA) has dramatic effects on limb-skeletal patterning in vivo and may well play a pivotal role in normal limb morphogenesis. RA's effects on the expression of pattern-related genes in the developing limb are probably mediated by cytoplasmic RA-binding proteins and nuclear RA-receptors. Little is known, however, about how RA modifies specific cellular behaviors required for skeletal morphogenesis. Earlier studies supported a role for regional differences in RA concentration in generating the region-specific cell behaviors that lead to pattern formation. The present study explores the possibility that position-related, cell-autonomous differences in the way limb mesenchymal cells respond to RA might have a role in generating pattern-related cell behavior. Mesenchymal cells from different proximodistal regions of stage 21–22 and 23–24 chick wing-buds were grown in chemically defined medium and exposed to 5 or 50 ng/ml of RA for 4 days in high-density microtiter cultures. The effects of RA on chondrogenesis in these cultures clearly differed depending on the limb region from which the cells were isolated. Regional differences in RA's effects on growth over 4 days in these cultures were less striking. The region-dependent responses of these cells to RA proved relatively stable in culture despite ongoing cytodifferentiation. This serum-free culture model will be useful in exploring the mechanisms underlying the region-dependent responsiveness of these cells to RA.

Key words: limb bud; retinoic acid; pattern formation; chondrogenesis; cell culture; serum-free medium.

INTRODUCTION

The vitamin A agonist, retinoic acid (RA), is a well-known skeletal teratogen (16,17). Depending on the dose and mode of delivery, exogenous RA has profound effects on limb skeletal pattern ranging from distal deletions to skeletal pattern duplications (16,17,39,43, 44). Together with evidence that developing limbs contain endogenous RA (6,42), these effects support a role for RA as a morphogen during normal limb development (9,36). On the other hand, evidence is mounting that RA is only one component of a complex cascade of morphogenic effectors in the developing limb (27,47). Nevertheless, the patterns of expression of nuclear RA-receptors and cytoplasmic RA-binding proteins in developing limbs suggest that, whatever its position in the cascade, RA's role in normal skeletal patterning is pivotal and involves regional differences in ligand-receptor interactions (8 and references therein; 37).

Inasmuch as limb morphogenesis involves the spatiotemporal coordination of growth and differentiation, regional differences in the effects of RA on these behaviors in limb cells deserve further study. Of related interest is the question of whether RA's morphogenic and teratogenic effects are due to direct effects on the cells of the limb mesoblast or whether RA acts indirectly by altering the function of the overlying ectoderm, specifically, the apical ectodermal ridge (19,38,45). It is thus of particular interest to test the responses of the limb mesenchymal cells to RA in isolation from the ectoderm, and away from the complex and rapidly changing environment of the developing limb.

Our previous studies have shown that limb mesenchyme cells respond directly to RA (29). In serum-containing medium, RA causes a dose-dependent inhibition of chondrogenesis. In serum-free medium, however, RA at physiologic doses promotes, and at higher (pharmacologic) doses inhibits chondrogenesis. Similar responses to RA have been noted in cultures of chick mandibular mesenchyme (18) where RA's effects are also stage-dependent (33). Thus, the availability of a defined medium that allows isolated chick limb mesenchymal cells to proliferate, differentiate, and respond to RA in vitro more as they do in vivo than when grown in standard serum-containing medium (29,30) provides a useful model for continuing such studies.

One view of RA's role in limb development suggests that the mesenchymal cells derive positional information from regional differences in the RA concentration to which they are exposed (9,36). From this perspective, cells from different limb regions should respond similarly to identical RA concentrations. The present results indicate that at RA concentrations in the physiologic range, the region of origin significantly influences the way limb mesenchymal cells respond to a particular concentration of RA in vitro. Furthermore, these cell-autonomous, stage- and region-related differences in response are relatively stable in culture, indicating that further

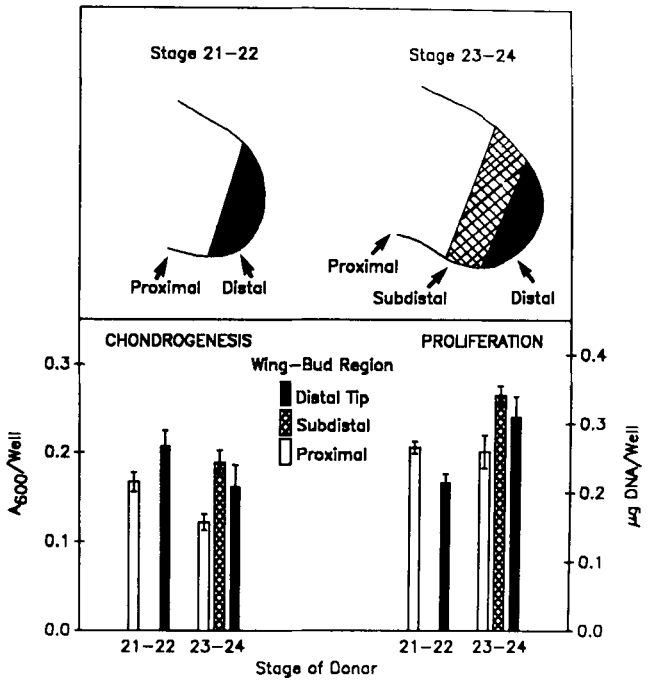

FIG. 1. *Above*, schematic representation of the wing-bud regions from which the mesenchyme was isolated in these studies. *Below*, defined medium (DM) control cultures. Quantitative estimates of chondrogenesis (A_{600}/well) and proliferation (μg DNA/well) in 4-day, serum-free, microcultures of mesenchyme from different regions of stage 21–22 and stage 23–24 chick wing buds. Values are mean ± standard error.

studies of factors underlying regional differences in the way these cells respond to RA will be possible using this experimental model.

MATERIALS AND METHODS

Tissue isolation. White Leghorn chick embryos were collected in cold Tyrode's solution (Sigma, St. Louis, MO) and staged according to Hamburger and Hamilton (13). Stage 23–24 wing-buds were divided into three regions (Fig. 1): the proximal region, containing a visible precartilage condensation; the distal 250-μm tip, containing rapidly dividing (40), undifferentiated mesenchyme known to be mainly chondrogenic and free of myogenic cells (1,25,26,28); and the intervening subdistal region containing a mixture of rapidly dividing, undifferentiated mesenchymal precursors of both chondrogenic and myogenic cells. In stage 21–22 wing buds, proximal precartilage condensations are not yet visible and thus cannot serve as a landmark to separate proximal and subdistal regions. These buds were divided into only the distal 250-μm tips and the remaining proximal portions (Fig. 1).

Microtiter cultures. High density microcultures of mesenchyme from each region were established as described previously (30) with some modifications. The defined medium (DM) consisted of Dulbecco's modified Eagle's medium (DMEM)/F12 (1:1, MediaTech, Herndon, VA) supplemented with 5 μg/ml bovine insulin (Collaborative Research, Waltham, MA), 5 μg/ml chicken transferrin (conalbumin), 100 n*M* hydrocortisone, 50 μg/ml L-ascorbic acid (all Sigma), and antibiotics (GIBCO, Grand Island, NY). The cultures were grown in half-well microtiter culture plates (Corning, Corning, NY), which were inoculated with 2.5×10^5 cells in 10 μl of DM. After a 1-h attachment period, the cultures were each carefully flooded with an additional 290 μl of the appropriate medium, followed by daily medium changes. Crystalline all-*trans* retinoic acid (Sigma) was prepared as a 0.5-mg/ml freezer stock in absolute ethanol and stored as aliquots under nitrogen gas at −80° C. On the day the cultures were seeded, an aliquot was diluted with DM to 50 and 500 ng RA/ml (10× stocks), and added at appropriate times and at each subsequent medium change to final concentrations of 5 or 50 ng/ml. These concentrations were selected based on previous dose-response studies using mesenchyme from whole limb buds (29,30). Under conditions like those in the present study, RA at 5 ng/ml maximally stimulates, and at 50 ng/ml inhibits, in vitro chondrogenesis. The cultures were grown for 0, 12, 24, or 48 h before adding the RA, then harvested after 4 days. Control cultures were grown in DM alone.

Chondrogenesis. Cartilage differentiation in RA-treated and control cultures was assessed colorimetrically by a modification of the method of Hassell and Horigan (14) as described previously (29,30). Briefly, the net absorbance at 600 nm (A_{600}) per well was measured in 4 *M* guanidinium HCl (Sigma) extracts of alcian blue stained cartilage matrix from fixed 4-day cultures. Alcian blue (ICN, Cleveland, OH) binds quantitatively to sulfate groups in the cartilage matrix (21) and the bound and extracted stain in micromass cultures of limb-bud mesenchyme is proportionate to accumulated sulfated proteoglycan (2,20).

Growth. DNA accumulation was measured in companion 4-day cultures seeded from the same cell suspensions used for the chondrogenesis studies. DNA content in a sonicate of the cell layer from each culture well was quantitated using the Hoechst 33258 (Polysciences, Warrington, PA) fluorescence enhancement method of Brunk et al. (4), as in previous studies (29,30). DNA accumulation in defined medium is known to be similar to, but slightly less than, that in standard serum-containing medium (30). The moderate baseline growth rate is expected because the cultures were essentially confluent at seeding.

Data analysis. Each data point (A600/well and μg DNA/well) in Figs. 1, 2 and 4 represents the mean and standard error for four to six separate experiments, including a minimum of two and typically three separate cultures in each experiment, for each condition. Each data point thus represents a minimum of 10, and more typically 12 to 16 cultures. To collect sufficient tissue from each subregion (the distal tip being the smallest) a minimum of 15 dozen embryos were routinely used for each experiment. Inasmuch as two stages were included in each age group, there was some

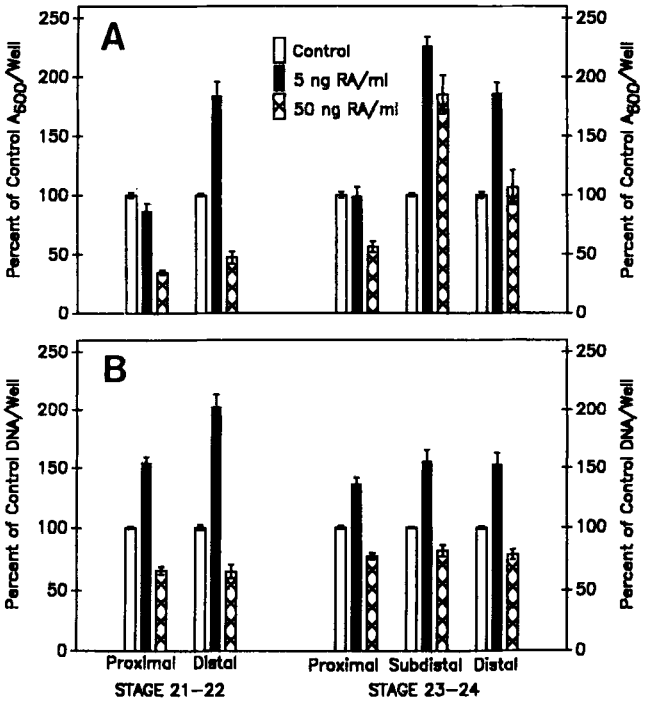

FIG. 2. Effects of retinoic acid (RA) treatment, beginning at seeding, on stage 21–22 and stage 23–24 wing-bud mesenchyme in serum-free microculture. Quantitative estimates of (*A*) chondrogenesis (A_{600}/well) and (*B*) proliferation (μg DNA/well) in 4-day, serum-free, microcultures of mesenchyme from different regions of chick wing buds. Values are mean percent of corresponding DM controls ± standard error.

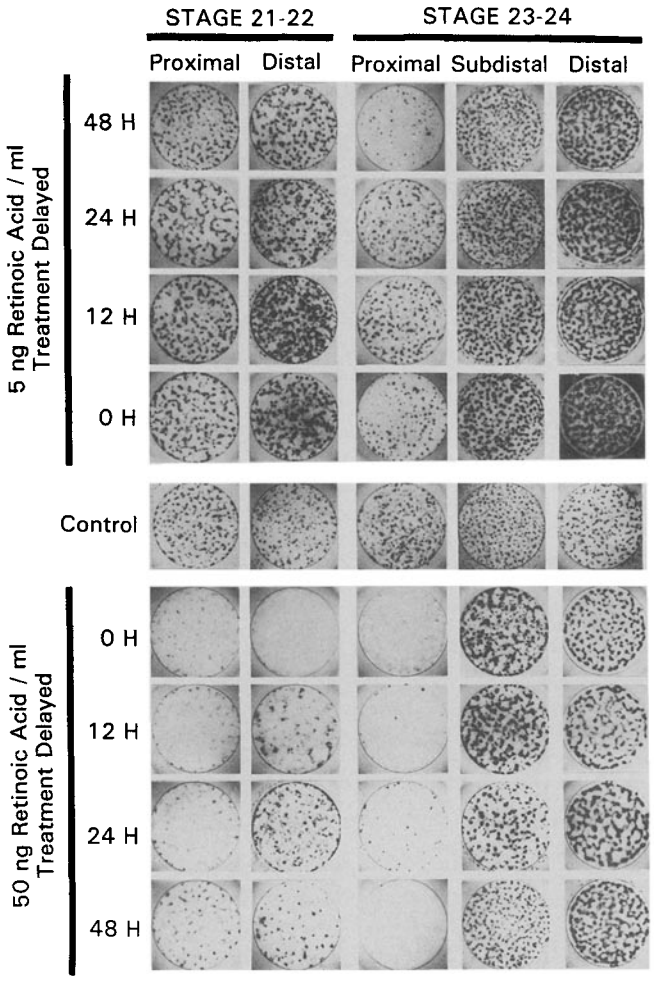

FIG. 3. Morphology of representative alcian blue-stained, 4-day, serum-free microcultures of stage 21–22 and stage 23–24 mesenchyme isolated from the chick wing-bud regions shown. Control cultures (*middle row*) from the various regions were grown in defined medium (DM). Experimental cultures were subjected to the treatment with retinoic acid (RA) at 5 ng/ml (*above controls*) or 50 ng/ml (*below controls*) beginning at the times shown after seeding. *See* Figures 1, 2, and 4 for quantitative estimates of chondrogenesis and DNA accumulation that accompany the morphology shown. Actual well diameter is 4.5 mm.

unavoidable age variation in the chicks donating tissue from experiment to experiment, and thus some variation in the control values. The percent-of-control values used in Figs. 2 and 4 thus provide an internal control for experiment-to-experiment variation. Computerized *t* tests were performed on the data to compare values obtained for cells from each region and in different media. Probability (*P*) values thus obtained which were less than 0.05 were taken as indicative of significant differences.

RESULTS

Stage- and Region-Dependent Differences in the Defined Medium Controls

Chondrogenesis. As measured by alcian blue staining, the level of cartilage differentiation (chondrogenesis) in 4-day, serum-free cultures of mesenchyme from different regions of stage 21–22 wing buds was only slightly greater ($P < 0.03$) in distal tip than in proximal cultures (Fig. 1). At stage 23–24, chondrogenesis from proximal mesenchyme was still less than that from distal or subdistal mesenchyme, which exhibited similar levels of chondrogenesis. Chondrogenesis was greater in proximal stage 21–22 cultures than in proximal stage 23–24 cultures ($P < 0.002$), which exhibited the least chondrogenesis of the regions tested. The levels of chondrogenesis in control cultures of mesenchyme from distal and subdistal stage 23–24 and distal stage 21–22 wing-bud regions were similar.

Growth. In 4-day control cultures of stage 21–22 wing mesenchyme, DNA accumulation was slightly less ($P < 0.01$) in the distal tip cells than in the proximal cultures (Fig. 1). For stage 23–24 limb mesenchyme, distal and subdistal cultures exhibited similar levels of growth accumulation. At this later stage, growth was slightly greater in subdistal than in proximal cultures ($P < 0.05$), whereas that in the distal cultures did not differ significantly from the other regions (Fig. 1). In comparing the stages, it was evident that growth was greater in distal and subdistal stage 23–24 cultures than in distal stage 21–22 cultures ($P < 0.05$), whereas that in the proximal cultures from both stages was similar.

Region-Dependent Differences in the Effects of Retinoic Acid

Chondrogenesis. Treatment with 5 ng/ml RA from the time of seeding and at each medium change enhanced chondrogenesis over control values ($P < 10^{-8}$) in 4-day cultures of distal stage 21–22 and 23–24, and subdistal stage 23–24 wing-bud mesenchyme (Figs. 2 *A* and 3). This same treatment inhibited chondrogenesis in cultures of proximal wing-bud mesenchyme from stage 21–22 ($P < 0.025$) but not stage 23–24 embryos (Fig. 2 *A*). At 50 ng/ml, RA inhibited chondrogenesis in cultures of both proximal and distal stage 21–22, and proximal stage 23–24 wing-bud mesenchyme ($P < 10^{-11}$). Overall, this concentration had no significant effect on chondrogenesis in distal stage 23–24 cultures, although there were occasional experiments in which a marked enhancement or inhibition in the level of chondrogenesis was noted. This suggests that for these cells the concentration-dependent shift from a stimulatory to inhibitory effect on chondrogenesis occurs at or just above 50 ng/ml, a result consistent with previous findings using whole-limb mesenchyme (29). On the other hand, RA treatment at 50 ng/ml consistently enhanced chondrogenesis in subdistal stage 23–24 cultures ($P < 10^{-5}$), making this region unique among those examined.

Growth. When RA was added at seeding and replaced at each medium change, 5 ng/ml RA enhanced DNA accumulation in cultures of mesenchyme from all stages and regions tested ($P < 10^{-4}$; Fig. 2 *B*). In general, distal and subdistal cultures showed greater growth enhancement by 5 ng RA/ml than did the proximal cultures ($P < 0.05$). Growth in the distal and subdistal stage 23–24 cultures treated from seeding with 5 ng RA/ml was not significantly different. The greatest enhancement of growth was seen in the cultures of distal tip mesenchyme from stage 21–22 wing buds treated from seeding with 5 ng RA/ml (Fig. 2 *B* and Fig. 4 *B,D*). At 50 ng/ml, RA inhibited growth in cultures of all stages and regions examined ($P < 3 \times 10^{-4}$). In general, younger and more proximal tissues were more sensitive to this effect, and inhibition of growth by 50 ng RA/ml was typically less, when expressed as a percent of control, than the inhibition of chondrogenesis in companion cultures ($P < 0.02$).

Effects of delaying the start of RA exposure. Considering that

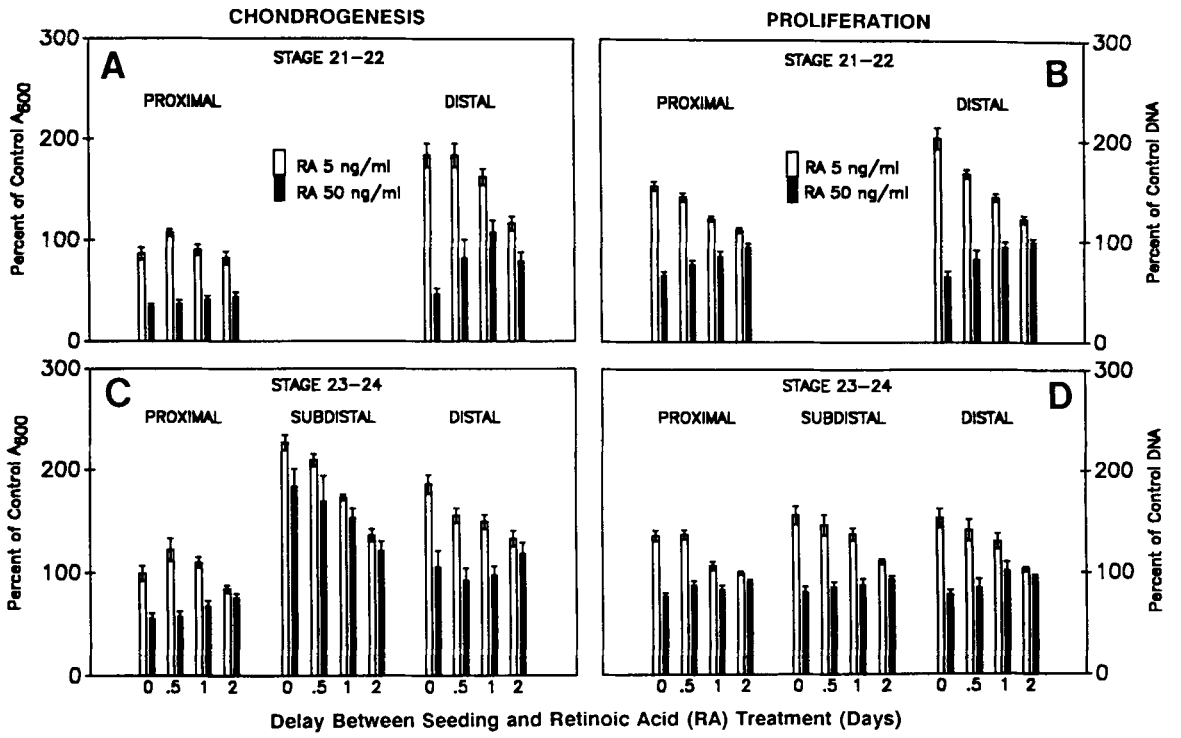

FIG. 4. Effects of delaying treatment with 5 or 50 ng RA/ml for 12, 24, and 48 hours on chondrogenesis (A_{600}/well; *A,C*) and on DNA accumulation (μg/well; *B,D*) at 4 days in serum-free microcultures of mesenchyme from different regions of stage 21–22 (*A,B*) and 23–24 (*C,D*) chick wing buds. Values are mean percent of corresponding DM controls ± standard error.

limb skeletogenesis occurs in a proximal-to-distal progression (34), the regional differences in the way these cells respond to RA in vitro might reflect their progress along the path from mesenchyme to cartilage. Inasmuch as these cells are known to progress along this path in this medium (29,30), delaying the onset of RA treatment might be expected to expose the cells to RA at a point further along the chondrogenic path and thus to elicit more "proximal-like" responses from cells from the more distal limb regions as they age in vitro. In that instance, delayed RA treatment of mesenchyme from stage 23–24 limbs would be expected to cause distal cells to respond more like subdistal cells, and subdistal cells to respond more like proximal cells. Figures 3 and 4 show that this was not the case. Although there were often reductions in RA's effects associated with delaying the onset of treatment, there were no notable qualitative switches in the responses of more distal cells to patterns resembling those of cells from more proximal regions. Thus the effects of delaying the onset of RA exposure seem more dependent on the duration of exposure than on the cells' progress along the path of differentiation at the time of exposure. This observation suggests that the region-dependent differences in the responses of these cells to RA are relatively stable in vitro despite ongoing cytodifferentiation. It also raises the possibility that certain components of positional information remain intact in the face of changes in gene expression that normally accompany differentiation.

Regional correspondence between RA effects on chondrogenesis and growth. There is a reasonable correspondence between the effects of RA on chondrogenesis and on growth for most of the limb regions and RA concentrations tested. This is not entirely unexpected because high cell densities and cell-to-cell contact are required for in vitro chondrogenesis from limb mesenchyme (5,46). However, mesenchymal cells obtained from the stage 23–24 subdistal region were a notable exception to this pattern. Figures 2 and 3 both show a consistently greater enhancement of chondrogenesis by RA at either 5 or 50 ng/ml in subdistal cultures than would be predicted from examining the RA's effects on cultures of the proximal or distal tip mesenchyme. Furthermore, 50 ng RA/ml inhibited growth in cultures from all regions and inhibited chondrogenesis from all but the stage 23–24 subdistal cultures. In these cultures, 50 ng RA/ml nearly doubled chondrogenesis while at the same time inhibiting growth. These findings suggest that some unique intrinsic property of the subdistal cells allows them to mount a more positive chondrogenic response to RA exposure. They further raise the possibility that RA has some separable effects on growth and differentiation.

DISCUSSION

At issue in the present study is the question of what factors underlie the region-specific patterns of cellular proliferation and differentiation in the developing limb that ultimately result in the development of normal limb-skeletal pattern. Numerous previous studies have indicated a role for RA in regulating normal and abnormal limb-skeletal patterning. The present study extends earlier analyses of RA's dose-dependent effects on serum-free microcultures of whole limb mesenchyme (29,30) to examine whether the position of the cells in the developing limb affects their response to RA. Chick wing-bud mesenchymal cells responded differently to RA depending as much on the age and region of the limb buds from which they

were isolated as on the RA concentration to which they were exposed. For both stages tested, distal and subdistal cells generally showed greater stimulation and less inhibition of chondrogenesis and growth by RA than did cells isolated from more proximal limb regions. Subdistal mesenchyme proved to be uniquely responsive to RA in terms of chondrogenic stimulation at both concentrations tested (Fig. 2 *A*).

Similar effects of RA at concentrations below 25 ng/ml on distal and proximal limb mesenchyme cultures were reported previously (15). In that study, however, the culture medium was poorly defined, containing serum and tissue extracts. These are potential sources of unknown amounts of retinoids and other factors likely to influence cell behavior. Serum, for instance, is known to affect the response of mesenchymal cells from whole chick limbs to RA in vitro (29). It was thus unclear in the earlier study whether the effects resulted from the added RA or from its interaction with one or more unknown medium components. The present study confirms, using a chemically defined medium, the stimulatory effect of low levels of RA on the chondrogenesis and growth of distal limb-bud mesenchymal cells in culture. More importantly, it is the first to report the unique RA-responsiveness of the isolated subdistal tissue, the regional differences in the effects of a higher RA concentration (50 ng/ml), and the in vitro stability of the region-dependent responses.

Many studies of the role of retinoids in skeletal pattern formation have been carried out at the organismal, organ (limb), and molecular levels (8–12,16,17,27,43–45). Studies at the cellular level have been less numerous, perhaps because appropriate in vitro models were not available. As a result, it has been difficult to forge direct mechanistic links among the different hierarchical levels. Approaches are therefore needed to begin asking whether and to what extent RA's effects on limb mesenchyme reflect position-specific, cell-autonomous differences in the way the cells respond to retinoids. The present findings indicate that, at least along the proximodistal axis, there are indeed detectable, region-specific, and cell-autonomous differences in the way these cells respond to RA; differences that are maintained even after tissue dissociation. Preliminary studies indicate that similar differences exist along the anteroposterior axis of these limbs as well (31,32). The nature of these position-related, cell-autonomous differences, and their significance in pattern formation, remains to be determined, but some possibilities deserve immediate consideration.

Limb-skeletal development occurs in a proximodistal progression (34), suggesting that the proximodistal differences in chick wing mesenchymal responses to RA in vitro are related to the relative progress of these cells along the path to mature cartilage. Allowing the cells to progress further along this path in vitro prior to RA exposure, however, does not qualitatively alter the responses to RA (Fig. 4). The region-specific, cell-autonomous components of RA responsiveness seem to be relatively stable despite ongoing cytodifferentiation in vitro. Therefore, regional differences in factors other than the stage of differentiation must be involved.

Inasmuch as RA is thought to directly affect gene expression through specific nuclear RA receptors [RARs and RXRs; (8,10,12,23)], region-dependent differences in RA responsiveness could involve regional differences in nuclear receptor expression. Higher proximal than distal expression of RAR-β, for example, has been demonstrated in chick-wing buds between stages 20 and 27 (35,37). In addition, cytoplasmic RA-binding proteins (CRABPs) are expressed at higher levels distally than proximally (8,22). It is worth noting here that the subdistal region, which exhibited unique chondrogenic responsiveness to RA in vitro, also exhibits a unique level of CRABP expression in vivo (22). CRABPs may regulate the amount of RA encountered by the nuclear RARs (22), facilitate RA metabolism (11,24), or both if the metabolism facilitated is RA degradation (3). Owing to the rapid diffusion of many retinoid metabolites across cell membranes, it is also conceivable that regional differences in retinoid metabolism yield the regional differences in responsiveness to RA.

Mechanistic interrelationships between RA concentrations, retinoid metabolism, and levels of expression of RARs, RXRs, and CRABPs remain to be determined for the developing limb. As greater detail is revealed regarding expression patterns and functions of various isoforms of these and other pattern-related genes [e.g., certain homeobox-containing genes (7,41,48)], knowledge of the way these cells respond to morphogenic signals when isolated in vitro will become increasingly useful for testing mechanistic models. The apparent stability of the region-specific differences in the responsiveness of these cells to RA in vitro will be particularly useful in this regard.

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