The growth plate contains resting and proliferating chondrocytes in its upper zones (UGP) and maturing and hypertrophic chondrocytes in its lower zones (LGP), but the mechanisms by which it operates to sustain skeletal growth are not fully clear. Retinoid signaling was previously found to be nearly absent in UGP, but to be much stronger in LGP coincident with hypertrophy, extracellular matrix turnover and endochondral bone formation. To determine whether such distinct signaling levels and phenotypic events reflect different endogenous retinoid levels, the upper two-thirds and lower one-third of rabbit rib growth plates were microsurgically isolated and processed for ultrasensitive retinoid LC-tandem MS quantification. Indeed, the UGP samples contained only about a 0.6 nM concentration of all-trans-retinoic acid (atRA) that is the most active natural retinoid in tissues, whereas LGP samples contained nearly 3-fold higher atRA levels (about 1.8 nM). Perichondrium was quite rich in atRA (about 4.9 nM). Interestingly, the levels of retinol, the major but inactive atRA precursor, were similar in all tissues (1.1–1.6 μM), suggesting that the distinct atRA levels in UGP and LGP reflect different retinoid anabolic capacity. Indeed, RALDH2 and CRABP1 transcript levels were much higher in LGP than UGP samples. To determine the minimum effective atRA concentration, chondrogenic cells transfected with a retinoic acid response element (RARE)-luc reporter plasmid were treated with different concentrations of exogenous atRA, 4 retinoid signaling, and nuclear retinoic acid receptors (RARs) have essential roles in a variety of biological processes, including body patterning, cell differentiation and proliferation, and skeletal development and growth (1–3). Previous studies showed that overexpression of a constitutive active form of RARα or excess intake of atRA cause severe developmental defects, including skeletal deformities (4, 5). In addition, mouse embryos lacking RARγ or both RARα and RARγ were found to exhibit growth retardation, malformations, and homeotic transformations in their skeletons (6, 7), and transgenic mice expressing a dominant negative RAR under the control of a cartilage-specific collagen2α1 promoter/enhancer exhibited similar defects (8). These and other findings suggested that appropriate levels of atRA signaling and RAR action are required for normal skeletal development and growth, but it has remained unclear since what skeletogenic processes and steps are specifically influenced and modulated by such retinoid-dependent mechanisms.

To gain insights into these questions, other studies examined the effects of exogenous retinoids on chondrogenesis and chondrocyte function. Implantation of atRA-containing beads was found to lead to limb skeletal duplications or phocomelia depending on location and dose (9, 10), and treatment of mesenchymal chondrogenic cells with atRA caused suppression of their differentiation (11). Subsequent studies found that the ability of such mesenchymal cells to differentiate into chondrocytes does in fact require a marked decrease in retinoid signaling and action and is promoted by unliganded RARs, and RARα in particular, eliciting transcription repressor function (12, 13). In a recent report, we focused on the roles of retinoid signaling and RAR function in the mouse growth plate (14). We found that RARγ is strongly expressed in the upper resting and proliferative zones of growth plate (UGP) but is significantly down-regulated in lower maturing and hypertrophying zones (LGP). The strong RARγ expression in UGP coincided with strong expression of aggregan, a major component of cartilage extracellular matrix. Indeed, we showed that compound conditional ablation of RARγ and RARα or RARβ in cartilage causes significant reduction of aggregan expression in growth plate and that unliganded RARγ stimulates aggregan synthesis in cultured chondrocytes. A previous study using a transgenic RARE reporter mouse line showed that reporter activity was virtually undetectable in UGP zones, suggesting local absence of endogenous retinoids (15). The data suggest that RARγ may act as an implantation of atRA-containing beads was found to lead to limb skeletal duplications or phocomelia depending on location and dose (9, 10), and treatment of mesenchymal chondrogenic cells with atRA caused suppression of their differentiation (11). Subsequent studies found that the ability of such mesenchymal cells to differentiate into chondrocytes does in fact require a marked decrease in retinoid signaling and action and is promoted by unliganded RARs, and RARα in particular, eliciting transcription repressor function (12, 13). In a recent report, we focused on the roles of retinoid signaling and RAR function in the mouse growth plate (14). We found that RARγ is strongly expressed in the upper resting and proliferative zones of growth plate (UGP) but is significantly down-regulated in lower maturing and hypertrophying zones (LGP). The strong RARγ expression in UGP coincided with strong expression of aggregan, a major component of cartilage extracellular matrix. Indeed, we showed that compound conditional ablation of RARγ and RARα or RARβ in cartilage causes significant reduction of aggregan expression in growth plate and that unliganded RARγ stimulates aggregan synthesis in cultured chondrocytes. A previous study using a transgenic RARE reporter mouse line showed that reporter activity was virtually undetectable in UGP zones, suggesting local absence of endogenous retinoids (15). The data suggest that RARγ may act as an...
unliganded receptor in UGP where it would favor proteoglycan expression and matrix accumulation. Interestingly, our previous studies in chick limb growth plates indicated that the hypertrophic zone may actually be rich in retinoid signaling; when we implanted beads containing a synthetic retinoid pan-antagonist next to the growth plate, chondrocyte hypertrophy and endochondral ossification were in fact severely delayed (16). Taken together, the above studies strongly indicate that retinoid signaling and action and RAR function may significantly change in upper versus LGP zones. An obvious way in which retinoid signaling and RAR function could change is if the availability and levels of active endogenous retinoids were to be different in UGP versus LGP zones. This study was conducted precisely to tackle this and related key questions.

**EXPERIMENTAL PROCEDURES**

**Tissue Sample Preparation and Retinoid Analyses**—Tissue samples were isolated by microsurgical procedures from 3-week-old New Zealand White rabbits. All procedures except euthanasia were carried out in a dark room illuminated by yellow light to protect endogenous retinoids from isomerization and/or oxidation during tissue processing. A yellow filter was also installed in the light source of the stereomicroscope used for microsurgical dissection. The upper two-thirds and lower one-third portions were dissected from growth plates present at the costochondral junction of the second to the eighth rib. For comparison, we also isolated perichondrium adjacent to the rib growth plates, articular cartilage from the knee joints and liver, and cartilage from the ribs and femoral knee joint cartilages of 4-week-old New Zealand White rabbits as described previously (20). Briefly, cartilage fragments were collected on a wood plate, minced with a scalpel, washed twice with Hanks’ balanced salt solution, and incubated with 170 units/ml collagenase type I (Sigma) in DMEM containing 50 units of penicillin, 50 μg/ml streptomycin, and 2.5 μg/ml amphotericin B (Invitrogen) for 12 h. Tissue fragments were washed twice in Ca/Mg-free Hanks’ balanced salt solution with 0.05% trypsin, 0.01% EDTA in Hanks’ balanced salt solution for 1 h at 37 °C. During trypsin digestion, tissue fragments were vortexed for 10 s every 15 min to thoroughly remove perichondrium. Tissue fragments were collected on a wood plate, minced with a scalp, washed twice with Hanks’ balanced salt solution, and incubated with 170 units/ml collagenase type I (Sigma) in DMEM containing 50 units of penicillin, 50 μg/ml streptomycin, and 2.5 μg/ml amphotericin B (Invitrogen) for 12 h. Tissue was dispersed by pipetting, and single cells were collected by centrifugation, inoculated onto type I collagen-coated multiwell dishes, and maintained in 10% FBS, high glucose DMEM (growth medium) or 0.5% BSA (fraction V; Calbiochem), 1× ITS (insulin-transferrin-selenium; Invitrogen), 1× B27 (Gibco) and 100 μg/ml gentamicin, in a humidified 5% CO2 incubator.

**Histological Analysis**—Fragments of dissected ribs, tibia, and femur were fixed in 4% paraformaldehyde, 0.1 M NaPB (pH 7.4) overnight and decalcified in 10% EDTA (pH 7.4) for 7 days at 4 °C followed by dehydration with increasing amounts of ethanol and embedded in paraffin. Samples were sectioned at 6-μm thickness, mounted onto slides, and stained with 0.1% safranin O/0.1%fast green.

**Measurement of Alkaline Phosphatase Activity**—Alkaline phosphatase (APase) activity was measured as described previously (19). Briefly, cartilage fragments were rinsed in ice-cold TBS (0.9% NaCl in 3 mM Tris–HCl (pH 7.4)) and homogenized in 0.9% NaCl and 0.2% Triton X-100. Samples were clarified by centrifugation, and the supernatants were mixed with 1 volume of 1 M Tris–HCl (pH 9.0) containing 1 mM p-nitrophenolphosphate and 1 mM MgCl2. The reaction was stopped by the addition of 0.25 volume of 1 N NaOH, and hydrolysis of p-nitrophenolphosphate was monitored as change in absorbance at 410 nm. Aliquots of cell lysates were diluted 10-fold with saline and used for measurement of protein content using a Modified Lowry protein assay kit (Theremo Scientific). One unit of APase activity corresponds to the hydrolysis of 1 μmol of p-nitrophenolphosphate/30 min per mg of protein at pH 9.0.

**Reverse Transcription-PCR (RT-PCR) and Quantitative Real Time PCR**—Total RNA was isolated by the guanidine isothiocyanate method and then treated with Turbo DNase I (Ambion). First-strand cDNA was synthesized from 1 μg of total RNA with 1 μM random 9-mer primer (PerkinElmer Life Sciences) using AffinityScript reverse transcriptase (Stratagene) at 42 °C for 1 h. Subsequent amplification was performed with Takara PrimeStar HS premix (Takara Mirus Bio Inc., Madison, WI) for 20–30 cycles under the following conditions: 95 °C for 10 s for denaturation and 60 °C for 1 min for annealing and extension. Quantitative real time PCR was performed on Applied Biosystems 7900HT Sequence Detection Systems running SDS 2.1 software using PowerSYBR Green PCR Master Mix (Applied Biosystems) following the manufacturer’s instructions. Primer sequences used in this study are shown in Table I.

**Isolation and Culture of Primary Rabbit Chondrocytes**—Growth plate and articular cartilages were isolated from the ribs and femoral knee joint cartilages of 4-week-old New Zealand White rabbits as described previously (20). Briefly, cartilage fragments were washed twice in Ca/Mg-free Hanks’ balanced salt solution containing penicillin/streptomycin and treated with 0.05% trypsin, 0.01% EDTA in Hanks’ balanced salt solution for 1 h at 37 °C. During trypsin digestion, tissue fragments were vortexed for 10 s every 15 min to thoroughly remove perichondrium. Tissue fragments were collected on a wood plate, minced with a scalp, washed twice with Hanks’ balanced salt solution, and incubated with 170 units/ml collagenase type I (Sigma) in DMEM containing 50 units of penicillin, 50 μg/ml streptomycin, and 2.5 μg/ml amphotericin B (Invitrogen) for 12 h. Tissue was dispersed by pipetting, and single cells were collected by centrifugation, inoculated onto type I collagen-coated multiwell dishes, and maintained in 10% FBS, high glucose DMEM (growth medium) or 0.5% BSA (fraction V; Calbiochem), 1× ITS (insulin-transferrin-selenium; Invitrogen), 1× B27 (Gibco) and 100 μg/ml gentamicin, in a humidified 5% CO2 incubator.
DMEM/F12 1:1 (retinoid-free medium). Medium was changed every other day.

**Transient Transfection Assay**—For transient transfection assay, freshly isolated chondrocytes were seeded at a density of \(2 \times 10^4\) cells/6-mm well and maintained in growth medium for 24 h. Cells were then switched and maintained for 24 h in retinoid-free medium. Retinoid-free medium was used until the end of the transfection experiment. One day later, cells were transfected with 0.1 \(\mu g/\)well retinoic acid response element luciferase reporter vector (RARE-luc) (Panomics) and the collagen2\(\alpha1\) enhancer-derived Sox activity monitoring \(4 \times 48\)p89-luc reporter (21) or the aggrecan enhancer-derived p89luc reporter (22) by using 0.1 \(\mu g/\)well reagent and 0.2 \(\mu l\) of Lipofectamine LTX (Invitrogen). Transfection efficiency averaged 5–10% as measured by a general lacZ reporter plasmid. Six hours later, medium was replaced, and then the indicated amounts of RA were added to cultures. Twenty-four hours later, cells were subjected to luciferase assay (Promega Luciferase Assay kit).

**Proteoglycan Synthesis**—Freshly isolated chondrocytes were seeded at a density of \(3 \times 10^4\) cells/6-mm well and maintained in growth medium for 2 days. Cells were then maintained in retinoid-free medium for an additional 24 h and treated with indicated concentrations of atRA for 24 h. Cells were labeled with 10 \(\mu Ci/ml\) of sulfate during the last 20 h of culture. Proteoglycan synthesis was determined by measuring incorporation into cetylpyridinium chloride-precipitated macromolecules after protease digestion.

### RESULTS

**Quantification of Endogenous Retinoids**—To determine whether the upper immature and lower hypertrophic portions of growth plate contain different endogenous retinoid levels and isomers, we isolated such portions from the costochondral growth plates of juvenile rabbit ribs by microsurgical procedures, using a stereomicroscope equipped with a yellow filter light source. Those growth plates were chosen as an experimental system because they are translucent and readily distinguishable from adjacent opaque bone (Fig. 1A). The growth plates were surrounded by a tightly adhering perichondrium (Fig. 1A, black arrowheads) that was also removed microsurgically and harvested for comparative analysis of retinoid content (see below). Interestingly, after removing the perichondrium, we noticed a clear tissue indentation along the entire perimeter (Fig. 1A, white arrowheads) that turned out to represent the location of the anatomical separation of growth plate from the ventral portion of the rib composed of resting permanent cartilage (Fig. 1B, white arrowhead). This anatomical arrangement was consistent from the second to the eighth rib and was used as a guide during dissection. Thus, we then proceeded to isolate the upper two-thirds portion of growth plate containing resting and proliferating chondrocytes (Fig. 1B, LGP) and the lower one-third portion containing maturing and hypertrophic chondrocytes (Fig. 1B, LGP) from those ribs and multiple animals. In addition to perichondrium, we also isolated articular cartilage and growth plate from the tibias of the same animals for further comparative analysis of retinoid content (see below). Histochemical staining with safranin O/fast green (Fig. 1, A–D) confirmed that all of the various tissues had the expected organization and staining patterns, including the lower safranin O staining of the superficial zone of articular cartilage (Fig. 1C) compared with the higher staining levels seen in the growth plates (Fig. 1, B and D). To verify further the identity and purity of the microsurgically isolated tissues, we processed small aliquots of UGP, LGP, and articular cartilage for expression of representative genes by RT-PCR and for APase activity. Expression of lubricin (PRG4), which is a marker of articular cartilage, was in fact detected in articular cartilage only (Fig. 1E). Matrix metalloprotease 13 (MMP13) expression and APase activity, which are both markers of chondrocyte hypertrophy, were most prominent in LGP (Fig. 1, E and F). Expression of aggrecan was seen in

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**Table 1**

| Gene name | NCBI or Ensemble accession no. | Sequence |
|-----------|--------------------------------|----------|
| Hpert     | AF020294                       | 5'-ATGAGACAGACGATGCACACGGTCTCCGT-3' |
| PRG4      | ENSCUG00000027187              | 5'-GTGGTCGATTGCTGTTGGGACATCATCC-3' |
| MMP13     | AF059201                       | 5'-TGGTGCTCTGCCACGAAATGGAG-3' |
| Agc       | L38484                         | 5'-CCCTACCCCGAGAACTCAAGGAG-3' |
| RALDH2    | ENSCUTC00000016946             | 5'-GGCAAGGCTCCTCTAATGAACCA-3' |
| Cyp26a1   | ENSCUTC0000000178              | 5'-GAGAAATTTCCACGACGATCTCAG-3' |
| CRABP1    | ENSCUTC0000000431              | 5'-TCTACTCAAAACCTCCACACAGCT-3' |
| CRABP2    | ENSCUTC0000002724              | 5'-GGACGCTCTGCTGCAACACCATT-3' |
| RBP       | ENSCUTC00000010524             | 5'-CCTGCCTGGGGTCTGTAATCTGACT-3' |
| ADAMTS-4  | AF247707                       | 5'-CTCCACGATGATTCTGCTTGGAG-3' |
| Col2A1    | D83228                         | 5'-CTCCACGATGATTCTGCTTGGAG-3' |
| Runx2     | ENSCUTC0000001739             | 5'-CCCTACCCCGAGAACTCAAGGAG-3' |
every tissue (Fig. 1F), signifying that differences in other genes were genuine.

To analyze endogenous retinoids quantitatively and qualitatively, we used an ultrasensitive procedure that relies on HPLC and MS/MS (18). For identification of different retinoids, commercially available retinoid standards including 13-cis-RA (13cRA), 9-cis-RA (9cRA), and atRA were analyzed in parallel; a representative chromatogram of their separation is shown in Fig. 2A. As an additional reference, we isolated and processed endogenous retinoids from liver (isolated from the rabbits above), a tissue rich in retinoids. Chromatograms of liver samples invariably displayed two major peaks corresponding to 9,13-dicRA and atRA (Fig. 2B) (17, 18). Artificial peaks of degradation or isomerization products were not detected in any sample, confirming that tissue isolation and retinoid extraction and handling were done under appropriate analytical conditions.

Aliquots of UGP, LGP, perichondrium, and AC were then analyzed in a similar manner. UGP samples displayed fairly low overall levels of retinoids (Fig. 2E) that were at the lower limit of resolution of our analytical methods and equipment, thus accounting for the background noise that was absent in chromatograms of retinoid-rich tissues such as liver (Fig. 2B). The most prominent peak was atRA that was present at approximately 0.6 pmol/g of wet tissue (Fig. 2E); assuming that the specific gravity of water-rich cartilage is equivalent to that of water, the atRA concentration was approximately 0.6 nM. In LGP samples, however, retinoid content was higher, and the chromatograms displayed two prominent peaks of 9,13-dicRA and atRA (Fig. 2F) that, once normalized to wet weight, amounted to ~2.0 pmol/g and 1.8 pmol/g (~2.0 and 1.8 nM, respectively) (Fig. 2F). atRA was conspicuous in AC also (~2.2 pmol/g) (Fig. 2D) but was most evident in perichondrium (~4.9 pmol/g) (Fig. 2C) as expected from previous studies (16). We

FIGURE 1. Isolation and characterization of growth plate and AC. A and B, anatomical appearance (A) and safranin O-stained histological section (B) of the chondro-osseous junction in 3-week-old rabbit rib. Following removal of perichondrium (A, black arrowheads; B, asterisk), a groove becomes evident (A, white arrowheads; B, white arrowhead) that corresponds to the boundary between resting cartilage (on the left) and growth plate cartilage (on the right). Upper two-thirds portion of growth plate (UGP) and lower one-third portion (LGP) are indicated. C and D, safranin O-stained histological sections of AC (C) and growth plate (D) in tibias from the same rabbits. Note that the upper half of AC stains less than the bottom portion. E, RT-PCR analysis of gene expression in AC, UGP, and LGP samples: PRG4, proteoglycan 4 (also called lubricin); MMP13 (also called collagenase 3); Agc, aggrecan; and Hprt, hypoxanthine guanine phosphoribosyltransferase (used for normalization). F, APase activity in AC, UGP, and LGP samples. *1 unit = 1 μM p-nitrophe-nylphosphate hydrolysis/30 min per mg of protein.

FIGURE 2. Separation and quantification of endogenous retinoids. A–F, representative selected reaction monitoring chromatograms of retinoid standards (A) and endogenous retinoids extracted from samples of 3-week-old rabbit liver (B), perichondrium (C), articular cartilage (D), UGP (E), and LGP (F). Retention times of atRA, 9cRA, 9,13-dicRA, and 13cRA are indicated.
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### TABLE 2
Quantification of retinoic acid (RA), retinol (ROL), and retinyl esters (RE) in 4-week-old rabbit liver, rib cartilage perichondrium, AC, UGP, and LGP

| Tissue          | RA (pmol/g tissue) | ROL (nmol/g tissue) | RE (nmol/g tissue) | No. of samples |
|-----------------|-------------------|---------------------|-------------------|---------------|
| Liver           | 101.5 ± 5.0       | 5.5 ± 0.6           | 97.7 ± 5.7        | 10            |
| Perichondrium   | 2.3 ± 0.3         | 1.6 ± 0.4           | 0.61 ± 0.13       | 7             |
| AC              | 0.6 ± 0.1         | 1.1 ± 0.2           | 0.03 ± 0.01       | 4             |
| UGP             | 1.8 ± 0.2         | 1.7 ± 0.2           | 0.20 ± 0.11       | 4             |
| LGP             | 5.0 5.5           | 0.3 1.6             | 0.2 0.08          | 4             |

* p < 0.003 perichondrium vs. LGP, AC, UGP, or UGP vs. LGP.

should note that although the biological relevance of atRA in skeletal and nonskeletal tissues is widely known and clear, the biological activity and significance of 9,13dcRA are not (23).

We also measured retinol and retinyl esters in all the samples (Table 2). The levels of retinyl esters, a storage form of retinoids, did not vary significantly from tissue to tissue (Table 2). Interestingly, the levels of retinol, a major but inactive atRA precursor, were also similar in each tissue and averaged about 1.1–1.7 nmol/g (Table 2), indicating that the differential levels of atRA in UGP, LGP, and other tissues may reflect differences in retinoid metabolism. To test this possibility, we compared the expression of gene products involved in retinoid synthesis, transport and degradation by real time PCR (Fig. 3). Expression of RALDH2, a key enzyme for atRA synthesis (24), was highest in perichondrium, was appreciable in LGP, and was barely detectable in UGP, resting rib cartilage, and AC (Fig. 3, A–C), strongly indicating that retinoid metabolism varied from tissue to tissue and was most active in perichondrium. Indeed, expression levels of Cyp26a1 (one of the enzymes regulating atRA degradation) and CRABP-I and CRABP-II (the main cellular atRA-binding proteins) were most prominent in perichondrium, LGP, and AC but were low in UGP and resting cartilage (Fig. 3, D–F). CRABP-II expression was appreciable in UGP and resting cartilage as well, but CRABP-I was not (Fig. 3, E and F). The latter two tissues exhibited the highest levels of expression of retinol-binding protein, possibly reflecting (and accounting for) their fairly low retinoid signaling activity and metabolism (Fig. 3G).

**Minimum Effective atRA Dose in Chondrocytes**—A wealth of studies have documented the ability of exogenous retinoids to modulate cartilage development and chondrocyte phenotype, but what has remained unclear is whether endogenous retinoid levels are within ranges to exert physiologically relevant responses and effects. Thus, based on our results on endogenous retinoids above, we set out to address this question. Rib growth plates were dissected from 4-week-old rabbits and dissociated into cell suspensions by enzymatic treatment, and the resulting cells were seeded in monolayer culture in standard serum-containing growth medium. Because hypertrophic chondrocytes are very fragile and are irreversibly damaged by the dissociation procedure, the cultures were largely composed of proliferating and prehypertrophic chondrocytes. Cultures were then switched to retinoid-free medium for 24 h and tested for responsiveness to different concentrations of exogenous atRA ranging from 0.03 nM to 1 μM. To monitor responsiveness, the cells were transfected with a sensitive RARE-luc reporter plasmid. Companion cultures were transfected with a collagen2α1 enhancer-derived reporter plasmid (termed 4×48p89-luc) or an aggrecan enhancer-derived reporter plasmid (4×A1-p89luc) to monitor and assess effects on chondrocyte phenotype. Average transfection efficiencies were 9–22%. We observed no changes in the activities of any of the three reporter plasmids when the cells were treated with doses up to 1 nM atRA (Fig. 4). However, treatment with 3 nM atRA or higher concentrations significantly increased RARE-luc activity and decreased the activities of both collagen2α1 4×48p89-luc and aggrecan 4×A1-p89luc reporters (Fig. 4, A–C).
To link changes in reporter activity to actual chondrocyte function, parallel cultures were labeled with $^{35}$SO$_4$, and the amount of incorporated $^{35}$SO$_4$ in cetylpyridinium chloride-precipitated macromolecules was measured as an indicator of proteoglycan synthesis. We found that treatment with atRA decreased proteoglycan synthesis in a dose-dependent manner (Fig. 4D). The decrease was appreciable and reproducible at 3 nM atRA and was statistically significant at 10 nM atRA.

**Differential Phenotypic Responses of Articular and Growth plate Chondrocytes to atRA**—Articular chondrocytes are permanent cells that exhibit a stable phenotype and last throughout life, whereas growth plate chondrocytes are highly dynamic, become hypertrophic, and are eventually replaced by bone cells. In a previous study with chick chondrocytes, we showed that the responses of cells to exogenous atRA depend on the degree of maturation of the cells (25). To extend those studies to mammalian chondrocytes and to test their responses to retinoid concentration ranges seen in vivo above, we isolated growth plate chondrocytes from rabbit ribs and articular chondrocytes from femurs of the same animals and seeded each population in monolayer culture. As to be expected, the articular chondrocytes were appreciably smaller in average size (Fig. 5A) than their growth plate counterpart (Fig. 5B). Basal gene expression levels of MMP13 and Runx2, which are both markers of hypertrophic chondrocytes, were higher in growth plate than AC (Fig. 5, C and D). These two genes were dose-dependently up-regulated in growth plate chondrocytes by as low concentration of atRA as 3 nM but remained largely unaffected in articular chondrocytes even at higher doses (Fig. 5, C and D). Expression of Cyp26a1, a known direct target of retinoic signaling, was induced in similar patterns and extents in both growth plate and AC (Fig. 5E), indicating that the differential effects on MMP13 and Runx2 were selective and maturation-dependent.
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DISCUSSION

Different concentrations of endogenous retinoids have been found in a variety of developmental systems, including limbs, lungs and skin, where they play critical roles in patterning, cell proliferation, and cell differentiation (26–28). Our study is the first to show that the concentrations of endogenous atRA change in the growth plate as well and are nearly 3-fold higher in lower maturing/hypertrophying portions of rabbit growth plate than upper resting/proliferating portions. There are several conceivable mechanisms by which this differential distribution of retinoids could be brought about in the growth plate. One obvious possibility is that the upper and lower portions may have differential retinoid anabolic capacity. This is clearly sustained by our observation that gene expression of RALDH2 is higher in the lower hypertrophying portion than upper immature portion. A second and related possibility is that the observed endogenous levels of retinoids reflect not only anabolic activities, but also diffusion of retinoids from surrounding sources. Although the growth plate is largely avascular, the hypertrophic zone abuts the endochondral bone and marrow that are rich in blood vessels. In addition, the hypertrophic zone is surrounded by periosteum that is engaged in intramembranous bone formation and is also rich in blood vessels. Thus, it is possible that higher levels of retinoids in the hypertrophic zone could at least in part be due to higher delivery of active retinoids and/or retinoid precursors from adjacent blood vessels. Our data show that the hypertrophic zone expresses higher levels of CRABPs that could also facilitate diffusion and accumulation of the hydrophobic retinoids (27).

Compared with endogenous levels of retinoids in other tissues (17, 18), the concentrations of endogenous atRA we observe in growth plate cartilage are among the lowest reported to date. The concentration in UGP was ~0.6 nM that is far below that needed to exert an appreciable response as our data indicate. Previous work has shown that the Kd value of atRA binding affinity for RARs ranges from 0.6 nM to 18 nM depending on assay systems and species (29, 30). Thus, a fraction of RARs in UGP chondrocytes could possibly be present in a ligand-bound state if the available atRA were concentrated in the nuclei. Because our reporter assays show that the minimum dose of exogenous atRA able to elicit a response in immature growth plate chondrocytes is higher than 0.6 nM, it is more likely that the majority of RARs expressed in immature growth plate zones would be present as unliganded nuclear receptors, associate with co-repressors, and operate as transcriptional repressors (31, 32). This conclusion is in line with data in our recent study showing that RARγ is preferentially expressed in UGP zones and stimulates aggrecan gene expression and matrix accumulation in its unliganded repressor form (14).

In contrast to the upper zones, the lower maturing and hypertrophying zones of growth plate contain levels of atRA (~1.8 nM) that are certainly within the range of minimal effective dose in chondrocytes based on our in vitro data. This interpretation is sustained by previous in vivo data reported by others using RARE-lacZ reporter mice showing quite clearly that only hypertrophic chondrocytes are lacZ-positive and particularly those located near the boundary with subadjacent endochondral bone and marrow (15). Thus, endogenous retinoids in that location must be high enough to activate lacZ expression in hypertrophic chondrocytes. In this regard it should be noted that the lower one-third portion of growth plate that we can isolate by microsurgical dissection includes both hypertrophic chondrocytes and some prehypertrophic chondrocytes in the preceding zone (see Fig. 1B). It is practically impossible to isolate only hypertrophic chondrocytes because their zone is too small and narrow. Thus, the atRA concentration present in our one-third LGP samples may actually be somewhat diluted by the presence of prehypertrophic chondrocytes, and the true concentration of atRA in hypertrophic chondrocytes may actually be higher than 1.8 nM and well within effective physiologic ranges. atRA would be able to stimulate specific phenotypic functions in hypertrophic chondrocytes required for their normal replacement with bone cells and marrow, including expression of genes such as MMP13 and Runx2, matrix degradation, and blood vessel invasion (33, 34). Our data show that atRA concentrations in the range of 3 nM can in fact stimulate expression of those genes (Fig. 5). Activation of retinoid signaling and activity of liganded RARs would thus bring about the terminal phases of growth plate functioning and terminal chondrocyte maturation and hypertrophy and would allow for a seamless transition from hypertrophic cartilage to endochondral bone (16, 25).

The above conclusions and implications may appear to contrast with our observation that AC actually displays an average atRA content of 2.2 nM that is higher than that in LGP. Because this tissue is permanent and stable and does not undergo maturation and hypertrophy, what could be the significance of its atRA content and how could it relate to that in growth plate? One interesting possibility is that one role of atRA in AC would be to limit aggrecan and matrix content and maintain it at slightly lower levels than those observed in growth plate. Our histochemical data with safranin O confirm the well-established fact that AC contains lower levels of aggrecan-rich matrix than growth plate, particularly in its superficial zone facing the synovial cavity (35). In addition, we previously showed that proteoglycan synthesis is lower in articular than growth plate cartilage (20). The superficial zone contains a high collagen content and expresses unique components including lubricin that allow the tissue to have an antiadhesive function critical for smooth and unhindered joint movement. Because active retinoid signaling reduces aggrecan expression and matrix content, it could thus serve to lower these parameters in AC and superficial zone in particular and maintain them within needed physiologic levels. An alternative possibility is suggested by the findings that atRA can exert antiinflammatory roles and can promote tissue repair (36). Because AC is continuously engaged mechanically during movement and exposed to strong shear and compressive forces, the articular chondrocytes may need to be in constant need to repair microdamages of the tissue, and the retinoids could aid this function as well. Thus, it will be important to carry out long-term animal experiments to validate the effects and roles of endogenous atRA in joint function and determine whether modulations by retinoid agonists or antagonists may promote their roles. There is also some evidence that excessive retinoid signaling occurs in degenerative joint disease (37, 38). It would
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