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Retinoic Acid Treatment Elevates Matrix Metalloproteinase-2 Protein and mRNA Levels in Avian Growth Plate Chondrocyte Cultures

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Abstract Matrix metalloproteinases (MMPs) play a crucial role in tissue remodeling. In growth plate (GP) cartilage, extensive remodeling occurs at the calcification front. To study the potential involvement of MMPs in retinoic acid (RA) regulation of skeletal development, we studied the effect of *all-trans*-RA on MMPs levels in mineralizing chicken epiphyseal chondrocyte primary cultures. When treated for 4 day periods on days 10 and 17, RA increased levels of an ~70 kDa gelatinase activity. The N-terminal sequence of the first 20 amino acid residues of the purified enzyme was identical to that deduced from chicken MMP-2 cDNA. Time-course studies indicated that RA elevated MMP-2 activity levels in the cultures within 16 h. This increase was inhibited by cycloheximide and was enhanced by forskolin. The increase in MMP-2 activity induced by RA was accompanied by an increase in MMP-2 mRNA levels and was abolished by treatment with cycloheximide. This upregulation of MMP levels by RA in GP chondrocytes is consistent with its effects on osteoblasts and osteosarcoma cells and opposite its inhibitory effects on fibroblasts and endothelial cells. It may well be related to the breakdown of the extracellular matrix in the GP and would be governed by the availability of RA at the calcification front where extensive vascularization also occurs. J. Cell. Biochem. 68:90–99, 1998.

Key words: retinoic acid; matrix metalloproteinases; chondrocytes; mRNA levels; growth plate

Matrix metalloproteinases (MMPs) are a family of enzymes of great importance in tissue remodeling during development, wound healing, involution of organs [Woessner, 1991], and other pathological conditions such as tumor invasion and metastasis [Liotta et al., 1991] and arthritis. The MMP family includes but is not limited to interstitial collagenase (MMP-1), neutrophil collagenase (MMP-8), gelatinase A (72 kDa type IV collagenase) (MMP-2), gelatinase B (92 kDa type IV collagenase) (MMP-9), stromelysin-1 (MMP-3), stromelysin-2 (MMP-10), and matrilysin (PUMP) (MMP-7) [Matrisian, 1992]. Each is synthesized and secreted as a latent proenzyme requiring the removal of an 80 amino acid N-terminal domain for activation. Regulation of the expression and activation of these zymogens, together with the expression of tissue inhibitors of MMPs (TIMP-1 and TIMP-2), are key steps in the remodeling of the extracellular matrix that occurs in both normal and pathological processes. Combinations of the MMPs can degrade almost all matrix proteins. Thus, their participation in and contribution to joint destruction in rheumatoid and osteoarthritis are thought to be linked [Woolley et al., 1977; Okada et al., 1986; McCachren, 1991; Wilhelm et al., 1993].

MMPs also contribute to cartilage matrix breakdown in preparation for calcification of the epiphyseal growth plate [Howell and Dean,

Abbreviations: GP, growth plate; GuSCN, guanidinium thiocyanate; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; MMP, matrix metalloproteinase; PMA, phorbol myristate acetate; RA, retionic acid; RT-PCR, reverse transcriptase–polymerase chain reaction; TIMP, tissue inhibitor of metalloproteinase.

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1992]. During endochondral bone formation, chondrocytes undergo a progression of maturational changes in both the cells and the surrounding matrix [Hunziker, 1994; Brighton, 1978; Poole, 1991; Buckwalter et al., 1986]. Resting chondrocytes begin to proliferate, mature in their expression of cartilage-specific proteins, enlarge in size and become hypertrophic, initiate mineral deposition in the extracellular matrix, and finally are replaced by bone cells at the chondroosseous junction. Alterations in extracellular matrix synthesis include 1) production of type X collagen exclusively in the hypertrophic zone [Schmid and Linsenmayer, 1985], 2) the loss of synthetic capacity for type II and IX collagens [Oshima et al., 1989; Linsenmayer et al., 1991], and 3) a decrease in the amount of proteoglycan/glycosaminoglycans [Reddi et al., 1978]. Additionally, the presence of the pericellular cleavage product of type II procollagen, chondrocalcin, within the hypertrophic zone can be detected [Poole, 1991; Alini et al., 1992]. The degradation of type II collagen is associated with a net increase in the synthesis of collagenase MMP-1, 72 kDa gelatinase, and stromelysin-1, above that of tissue inhibitor of metalloproteinase-1 [Brown et al., 1989; Dean et al., 1985]. Abrupt reduction in type X collagen levels, once mineral formation has commenced in the lower hypertrophic zone [Alini et al., 1994], can also be explained by the presence of interstitial collagenases such as 72 kDa gelatinase activity [Sires et al., 1995]. Still unresolved are whether proteoglycans are lost from the matrix before or after the onset of calcification and whether the degree of sulfation of proteoglycan changes within the area of cartilage calcification [Reddi et al., 1978; Poole, 1991; Alini et al., 1992; Farquharson et al., 1994; Shinomura and Kimata, 1992]. It is generally agreed that a net reduction in proteoglycan content per unit wet weight of tissue takes place as cells enlarge in size and matrix volume becomes considerably reduced. The major proteoglycan in cartilage, aggrecan, contains a cleavage site for MMPs-1, -2, -3, -7, -8, and -9 as well as an uncharacterized proteolytic activity termed "aggrecanase" [Flannery et al., 1992; Fosang et al., 1992; Hughes et al., 1995]. Actions of these proteinases could cause increased degradation of proteoglycans during cartilage resorption.

While many other MMPs are synthesized, mainly upon stimulation, MMP-2 is constitutively synthesized by many types of cells. MMP-2 has been shown to degrade native collagen type IV and V and also collagen type I [Aimes and Quigley, 1995]. It has been implicated in angiogenesis and in the invasion of metastatic cells across basement membranes [Stetler-Stevenson, 1990]. Indeed, in many metastatic cancer cell lines [Brown et al., 1990; Pyke et al., 1992] and Rous sarcoma virus–transformed fibrobasts [Chen et al., 1991], there are increased MMP-2 expression and activation. Transfection of nontumorigenic cells with Ha-*ras*, which induces the metastatic phenotype, also increases the level of MMP-2 [Collier et al., 1988]. However, due to the lack of a proper model system, there is little information available regarding the regulation of MMP-2 gene expression, except that it is a housekeeping gene. In this paper, we report that the level of MMP-2 expression in chicken growth plate (GP) chondrocyte primary cultures is upregulated by retinoic acid (RA) treatment. This culture system may serve as a good model to study the regulation of MMP-2 gene expression.

MATERIALS AND METHODS

Materials

All-trans-retinoic acid, phorbol-12-myristate-13-acetate, forskolin, dexamethasone, and cycloheximide were purchased from Sigma Chemicals (St. Louis, MO). Gelatin-Sepharose was from Pharmacia Biotech (Piscataway, NJ).

Cell Culture

Chondrocytes were isolated from epiphyseal growth plate cartilage of the tibiae of 6–8-week-old hybrid broiler-strain chickens and plated in 35 mm dishes as previously described [Wu et al., 1992]. On day 7 after plating, cells were switched to a medium composed of a 1:1 mixture of Dulbecco's modified Eagle's medium with 10% fetal bovine serum and serum-free HL-1 medium (Hycore Biomedical, Portland, ME), and from day 10 onward the cells were cultured only in HL-1. The culture medium was changed every 3 or 4 days. Ascorbate was provided from day 3 onward at a final concentration of 50 µg/ml. For treatment of cells, all reagents were prepared in stock solutions with ethanol (RA) or dimethylsulfoxide (phorbol myristate acetate (PMA), forskolin, and cycloheximide) and then diluted to the desired concentration. All dishes, including the controls, received the same volume of vehicle ethanol or dimethylsulfoxide (1.0–2.0 µl per milliliter of medium).

Protease Zymogram

Proteolytic activity in the culture medium was assayed by a modification of the method of Kleiner and Stetler-Stevenson [1994]. In brief, 8% acrylamide gels polymerized with 0.2% gelatin were used. After electrophoresis, gels were washed twice with 2.5% Triton X-100 for 30 min with gentle shaking and incubated in 1% Triton X-100, 50 mM Tris, 5 mM $CaCl_2$, 0.02% sodium azide, pH 8.0, at 37°C for 18 h. Gels were stained with Coomassie brilliant blue R-250. The presence of protease activity was revealed as a negative band against the blue background.

Protein Purification and Characterization

To purify the MMP present in the culture medium, chondrocytes were cultured in 75 cm^2 flasks with serum-free HL-1 medium containing 50 nM RA. Media harvested every 3 days were pooled, concentrated severalfold by Centricon 30 (Amicon, Danvers, MA) ultrafiltration, and applied onto a DEAE-Sephadex column to remove proteoglycan. The protein present in the flowthrough was then precipitated by overnight treatment with 70% saturation of ammonium sulfate. The precipitate was reconstituted in a buffer containing 50 mM Tris, 0.5 M NaCl, 5 mM $CaCl_2$, 0.02% NaN_3, and 0.05% Brij 35, pH 7.5, dialyzed against the same buffer, and applied to a gelatin-sepharose column preequilibrated with the same buffer. After being washed with 5 bed volumes of this buffer, the gelatin-bound fraction was eluted with 7.5% DMSO eluting buffer (50 mM Tris, 1.0 M NaCl, 5 mM $CaCl_2$, 0.02% NaN_3, 0.05% Brij 35, and 7.5% dimethylsulfoxide, pH 7.5). The MMP activity in each fraction was assayed using the zymogram procedure described above. For N-terminal sequencing of the protein, fractions with MMP activity were pooled and dialyzed against Tris buffer (50 mM Tris, 5 mM $CaCl_2$, 0.02% NaN_3, 0.05% Brij 35, pH 7.5); the fractions were then vacuum-dried and reconstituted in Laemmli sample buffer. After electrophoresis (SDS-PAGE), proteins were transferred to an Immobilon P membrane using an LKB (Bromma, Sweden) 2117 Multiphor II semidry transfer apparatus. The membrane was then stained with Coomassie blue, and the bands of interest were excised for N-terminal sequencing using an Applied Biosystems (Foster City, CA) 477A protein sequencer.

Total RNA Extraction

Total RNA was extracted from cultured GP chondrocytes by a modification of the acid guanidinium thiocyanate (GuSCN)–phenol-chloroform method [Chomczynski and Sacchi, 1987] with some modifications. Basically, cell monolayers were washed twice with ice-cold TMS (50 mM Tris, pH 7.5, 1.5 mM $MgCl_2$, and 10% sucrose) buffer, and then 0.4 ml of 4 M GuSCN solution was added to each 35 mm dish. After gentle agitation, the GuSCN solution was transferred to a 2 ml Eppendorf tube, and one-third volume of absolute ethanol was added to the GuSCN solution to precipitate the RNA. After 5 min on ice, the RNA was precipitated from the GuSCN-ethanol mixture by centrifugation for 5 min at 14,000 rpm (15,800*g*). The pellets were resuspended in a small volume of fresh GuSCN solution, and one-tenth volume of 2 M sodium acetate (pH 4.0), one volume of phenol, and one-fifth volume of chloroform/isoamyl alcohol (49:1, v/v) were added sequentially to this suspension. After vigorous agitation for 20 s, the tubes were centrifuged at 11,000*g* for 20 min at 4°C. Then the aqueous phase was transferred to a new tube, and 2.5 volumes of ethanol were added to precipitate RNA overnight. After centrifugation, the pellets were washed briefly twice with 70% ethanol and then resuspended in 2 M LiCl-5 mM EDTA solution. The suspension was kept on ice for 30 min and then centrifuged at 15,800*g* speed for 15 min to precipitate the RNA. The RNA precipitates were washed twice with 70% ethanol and then resuspended in 100 µl of 1 mM EDTA solution prepared in DEPC-treated water to quantitate the amount of RNA isolated. The RNA was preserved as an ethanol precipitate for later analysis.

Reverse Transcriptase and Polymerase Chain Reaction (RT-PCR)

An RT-PCR procedure to quantitate MMP-2 mRNA was developed using a Perkin Elmer (Foster City, CA) GeneAmp® EZ rTth RNA PCR kit according to the manufacturer's instructions, with some modifications. Basically, 5 µg (or less) of total RNA from each sample was reverse-transcribed using random hexamers and MMLV reverse transcriptase supplied by Epicentre Technologies (Madison, WI), according to the manufacturer's instructions. After 60 min at 37°C, one-tenth volume of 3 M sodium

acetate (pH 5.2) and 2.5 volumes of ethanol were added to the RT reaction tube. The precipitate was washed twice with 70% ethanol and suspended in deionized-distilled water. Aliquots (equivalent to 0.5 µg total RNA) of each cDNA sample were used to set the PCR reaction, according to the manufacturer's instructions. The final concentration of Mn^{2+} used was 2.5 mM. The primers used for the MMP-2 RT-PCR procedure were 5′AAA CCC CGC TGT GGT AAC CCC GAT G-OH (207–231) for the sense strand and 5′AGG GCT GTC CAT CAC CTA TGC CAC C-OH (784–760) for the antisense strand. This procedure generates a product of 578 base pairs [Aimes et al., 1994]. The primers for glyceraldehyde 3-phosphate dehydrogenase (G3PDH) were 5′GAC CAC TGT CCA TGC CAT CAC AGC C-OH (575–599) for the sense strand and 5′TCC AAA CTC ATT GTC ATA CCA GGA A-OH (1007–984) for the antisense strand. This procedure generates a product of 433 base pairs [Pnabrieres et al., 1983]. Following initial heating at 94°C for 5 min, amplification was conducted at 94°C for 30 s, 57°C for 30 s, and 72°C for 45 s for 30 cycles, followed by 10 min of extension at 72°C. A 10 µl aliquot of each sample was analyzed using 2% agarose TBE (50 mM Tris, 50 mM boric acid, 1 mM EDTA) gels. The presence of DNA in the gel was revealed by ethidium bromide staining.

RESULTS

Elevation in the Level of 70 kDa MMP Activity Upon RA Treatment

When avian GP chondrocyte cultures were treated with graded levels of RA in HL-1 medium for 4 days, beginning on day 10 or 17 of culture, an increased level of a gelatinase with a MW of ~70 kDa was observed using the gelatin-substrate gel assay (Fig. 1A). As seen in

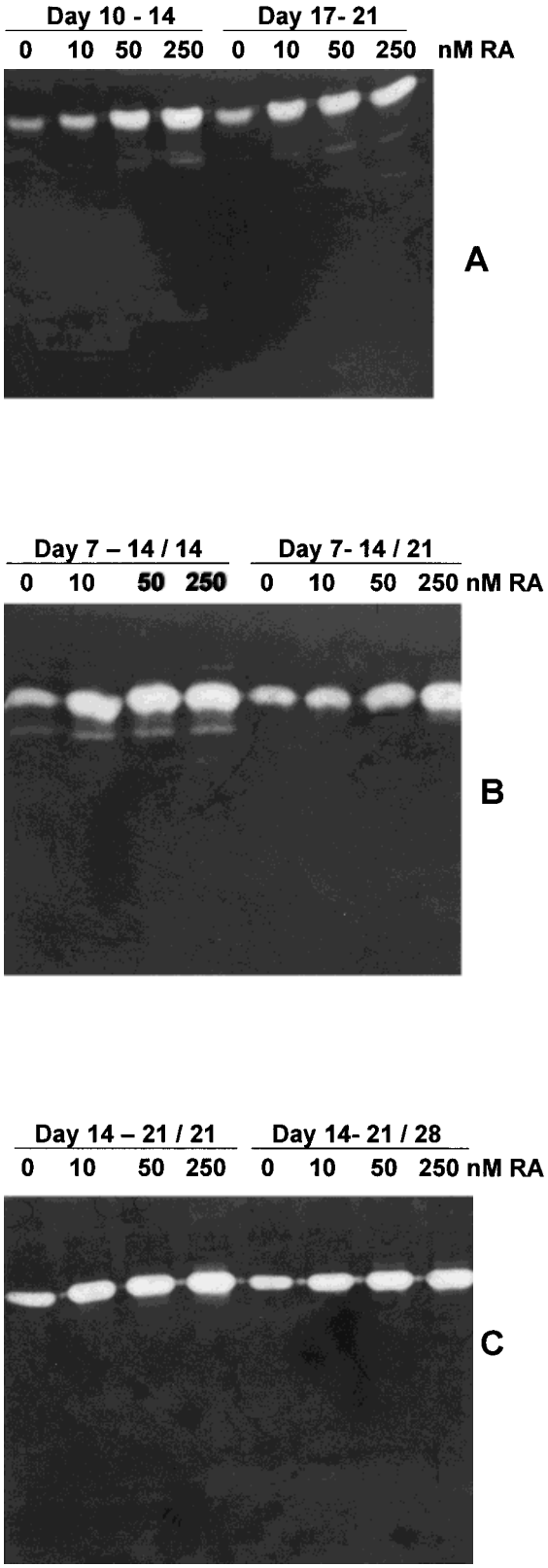

Fig. 1. Zymogram of RA-enhanced protease activities. **A:** On day 10 or 17, the cultures were treated with either 0, 10, 50, or 250 nM RA. After 4 days of treatment, culture media were harvested, and samples (25 µl) of the control or RA-treated (10, 50, and 250 nM) culture media were mixed with 25 µl of 2× nonreducing Laemmli buffer and then applied to the SDS-gelatin substrate gel for proteolytic activity assay, as described in Materials and Methods. Longer exposure to RA was also tested in both preconfluent and postconfluent cells. **B:** Cultures were treated from day 7–14 and the media collected on days 14 and 21. **C:** Cultures were treated from day 14–21 and the media collected on days 21 and 28. Note the progressive increase in protease activity after each treatment with the graded levels of RA.

the figure, RA caused a dose-dependent increase in this protease, enhancing its activity at levels as low as 10 nM. The protease was identified as a *metallo* proteinase since its activity was susceptible to both EDTA and 1,10-phenanthroline treatment but was resistant to PMSF and benzamidine treatment (data not shown). It also should be noted from Figure 1A that RA treatment caused a small increase in the level of an ~50 kDa proteinase activity. Other biochemical parameters such as cellular protein, alkaline phosphatase activity and proteoglycan content were also analyzed (Table I). RA caused an inhibition on protein and proteoglycan content while stimulating alkaline phosphatase activity. Longer treatment with RA also increased the levels of the ~70 kDa MMP in preconfluent cultures treated from day 7 to day 14 as well as postconfluent cultures treated from day 14 to day 21 (Fig. 1B,C).

TABLE I. Effect of Retinoic Acid on Cell Protein, Alkaline Phosphatase Activity, and Proteoglycan Content of Primary Cultures of Growth Plate Chondrocytes†

	Treated day 10–14 harvested day 14		
	Protein (μg/dish)	AP (nmol/min/dish)	PG (μg/dish)
Control	449.2 ± 19	42.4 ± 5	8.1 ± 0.3
10 nM	370.2 ± 16*	53.5 ± 2	2.8 ± 0.3***
50 nM	373.2 ± 6*	65.5 ± 10	4.1 ± 0.4***
100 nM	364.0 ± 25*	116.4 ± 14**	2.8 ± 0.2***
250 nM	291.0 ± 5***	102.6 ± 20*	3.4 ± 0.4***
	Treated day 17–21, harvested day 21		
	Protein (μg/dish)	AP (nmol/dish)	PG (μg/dish)
Control	578.3 ± 21	24.4 ± 2.2	10.9 ± 0.5
10 nM	468.7 ± 19**	44.7 ± 6.9*	6.6 ± 0.6***
50 nM	460.1 ± 34*	40.8 ± 4.8*	5.2 ± 0.5***
100 nM	420.1 ± 14***	45.4 ± 6.0*	5.1 ± 0.4***
250 nM	390.3 ± 15***	61.7 ± 11*	5.7 ± 0.3***

†Chondrocytes were treated with RA at the indicated concentrations and cells were harvested on day 14 or day 21 and analyzed for protein, alkaline phosphatase activity, and proteoglycan content as described by Wu et al. [1997]. Values are the mean ± S.E.M. of four samples.
*Significance of differences between treated and control cultures, $P < 0.05$.
**Significance of differences between treated and control cultures, $P < 0.01$.
***Significance of differences between treated and control cultures, $P < 0.001$.

Identification as Avian MMP-2 (Gelatinase A, Type IV Collagenase)

To establish the identity of this metalloproteinase, we purified the enzyme (see Methods). The proteolytic activity was found to have an affinity for gelatin, being recovered mainly from the gelatin-bound fraction (Fig. 2). Its size, sensitivity, and specificity indicated that this metalloproteinase was probably avian MMP-2. This was confirmed by N-terminal sequencing of the purified metalloproteinase. The N-terminal 20 amino acid sequence of this protein (APSPI

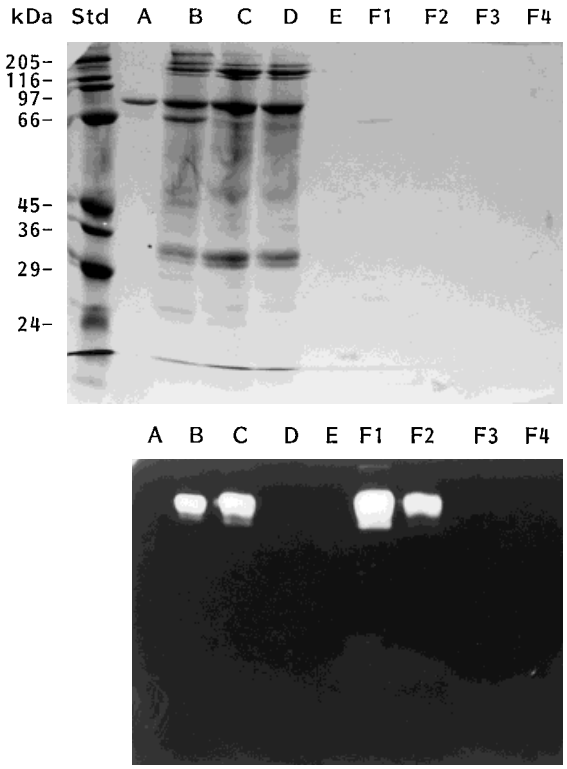

Fig. 2. Purification of the RA-enhanced metalloproteinase. Media (serum-free HL-1) from the chondrocyte cultures treated with 50 nM RA were harvested, pooled, concentrated, and subjected to the purification procedure described in Materials Methods. Profiles of the proteins present in of each fraction were analyzed by SDS-PAGE with Coomassie blue staining using 60 μl of each sample (**upper panel**) and by the gelatinase assay using 10 μl of each sample (**lower panel**). **Lane A:** Fresh HL-1 medium. **Lane B:** Flowthrough from the DEAE column. **Lane C:** Reconstitute of the precipitate from 70% saturation $(NH_4)_2SO_4$ treatment of the flowthrough from the DEAE column. **Lane D:** Flowthrough from the gelatin-Sepharose column. **Lane E:** Non-eluting wash from the gelatin-Sepharose column. **Lanes F1–F4:** Sequential fractions of the adsorbed protein eluted from the gelatin-Sepharose column using the 7.5% DMSO buffer. Note that fresh HL-1 medium did not have any gelatinase activity and that the proteinase activity adsorbed with high affinity and specificity to the gelatin-Sepharose column.

IKFPG DSTPK TDKEL) was identical to that deduced from chicken fibroblast MMP-2 cDNA [Aimes et al., 1994]. As is the case for other MMPs, the purified avian MMP-2 was found complexed with an ~24 kDa protein (Fig. 2, top panel, lane F1) presumed to be TIMP-2.

Time Course of RA Elevation of MMP-2 Levels

Timed studies indicated that 50 nM RA increased the level of MMP-2 substantially within 16 h; it became clearly obvious after 24 h of treatment (Fig. 3). The elevation of MMP-2 in the RA-treated chondrocytes was dependent on de novo protein synthesis; cycloheximide treatment of the chondrocytes inhibited MMP-2 expression in both the control and RA-treated cells (Fig. 4). The elevation in MMP-2 required the consistent presence of RA; 12 h of RA pretreatment failed to increase the level of MMP-2 (data not shown).

Further Modulation of MMP-2 Levels in GP Chondrocytes

It has been reported that the phorbol ester, phorbol myristate acetate (PMA), decreases MMP-2 and its mRNA levels in human fibroblasts and some tumorigenic cell lines [Brown et al., 1990]. Accordingly, we examined the effect of PMA on MMP-2 levels in the chondrocytes (Fig. 4). PMA treatment had little effect on MMP-2 levels in either the control or RA-treated cells; however, it increased the levels of both a higher and lower MW gelatinase. The high MW MMP is probably MMP-9, based on its 92 kDa size.

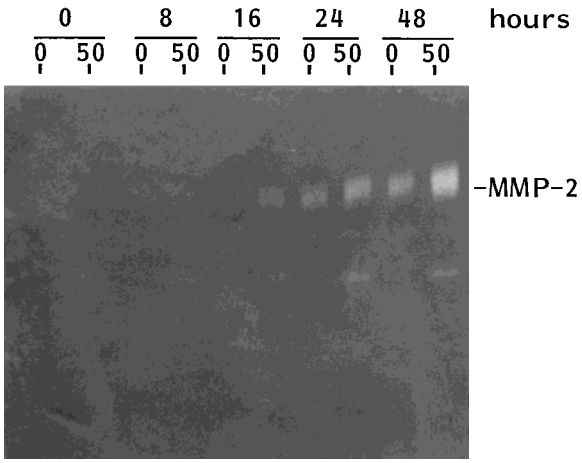

Fig. 3. Time course in the elevation of MMP-2 levels by RA. Culture media, harvested at 8, 16, 24, and 48 h after treatment of the chondrocytes with RA, were analyzed for MMP-2 activity using the SDS-gelatin substrate gel. At each time point, samples of media (50 µl) from either the control (no RA) or the 50 nM RA-treated cultures were assayed. RA stimulation of MMP-2 synthesis can be seen first at 16 h and increased progressively out to 48 h after RA treatment. Low levels of MMP-2 activity can be seen in the control media after 24 and 48 h of culture. A weak, low MW RA-induced gelatinase activity is evident as early as 8 h after treatment.

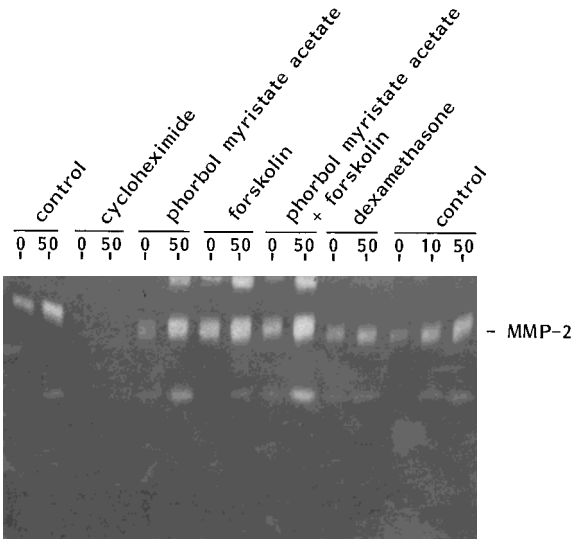

Fig. 4. Modulation of RA enhancement of MMP-2 levels by cycloheximide, phorbol myristate acetate (PMA), forskolin, and dexamethasone. Chondrocyte cultures in HL-1 media were treated with RA (50 nM) on day 10, alone or in combination with cycloheximide (2.5 µg/ml), PMA (100 ng/ml), forskolin (10 µM), PMA + forskolin (levels as above), or dexamethasone (1 µM). Media were harvested 48 h after treatment, and 50 µl samples were analyzed for MMP-2 levels using the SDS-gelatin substrate gels. Note that cycloheximide totally blocked MMP-2 formation in the presence or absence of RA, whereas dexamethasone had no effect. Note that forskolin alone increased MMP-2 levels and that PMA + forskolin potentiated the RA-dependent enhancement of MMP-2. Note as well that forskolin, alone or in combination with RA, also induced the expression of a high MW gelatinase, whereas PMA in combination with RA increased the levels of both the high and low MW gelatinases in these cultures.

In contrast, forskolin treatment alone or in combination with PMA increased MMP-2 levels in the absence of RA. In combination with RA, forskolin or forskolin + PMA further increased MMP-2 levels of the treated cells. The highest level of MMP-2 was seen in cultures treated with RA in combination with forskolin and PMA. Notice that forskolin, in combination with RA, also dramatically increased the levels of the higher MW but had no effect on the lower MW gelatinase. On the other hand, dexamethasone neither increased nor inhibited RA enhancement of MMP-2 levels.

Elevation of MMP-2 mRNA Levels by RA

To document that RA induction of MMP-2 affected mRNA as well, we used RT-PCR to compare levels of MMP-2 transcripts before and after RA treatment. We found that treatment with RA at levels of 10, 50, 250, and 1000 nM caused dramatic increases in the levels of MMP-2 mRNA, whereas mRNA levels of the internal control, glyceraldehyde 3-phosphate dehydrogenase (G3PDH) remained nearly constant (Fig. 5). RA treatment enhanced the expression of MMP-2 mRNA as early as 4–6 h after the cultures received RA, and transcript levels remained elevated at least 24 h (Fig. 6). The increase in MMP-2 mRNA was dependent on de novo protein synthesis, since cycloheximide treatment abolished the RA-induced increase in MMP-2 mRNA expression (Fig. 7).

DISCUSSION

Despite the fact that there is an increase in the expression and activation of MMP-2 in a number of physiological and pathological conditions (tissue remodeling, angiogenesis, tumor invasion, and metastasis), the regulation of MMP-2 gene expression remains to be elucidated. In this study, we report for the first time that the level of MMP-2 can be regulated by RA treatment using our avian GP chondrocyte culture system. Few cytokines, hormones, or growth factors have been shown to affect the expression of the MMP-2 gene in cell culture. Hence, this avian GP chondrocyte culture system provided a good model to study the regulation of the MMP-2 gene.

In fibroblasts, transforming growth factor β (TGFβ) has been shown to increase the levels of MMP-2 and its mRNA [Overall et al., 1991]. Previous investigations have shown that RA exerts some of its effects through stimulating expression of various TGFβ isoforms. For example, when cultured mouse keratinocytes were

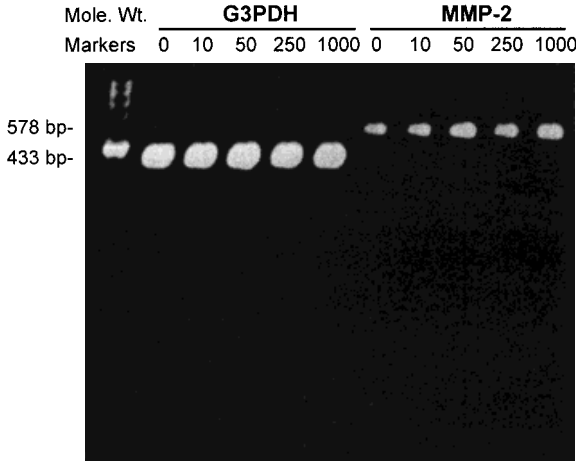

Fig. 5. RA enhances the expression of MMP-2 mRNA levels in growth plate chondrocytes. Chondrocyte cultures were treated with RA at 0, 10, 50, 250, and 1,000 nM for 24 h on day 10; then total RNA was isolated from the cell layer using acidic guanidinium thiocyanate followed by extraction with phenol:chloroform: isoamyl alcohol (100:10:0.2). Total RNA was precipitated from the aqueous phase by addition of 2.5 volumes of ethanol and then reverse-transcribed using random hexamers as primers. Aliquots of each cDNA sample containing an amount equivalent to 0.5 µg of total RNA were amplified using the polymerase chain reaction Perkin-Elmer EZ rTth PCR kit (for more details, see Materials and Methods). Note that the internal standard, glyceraldehyde 3-phosphate dehydrogenase (G3PDH) mRNA (433 bp) remained at constant levels, whereas MMP-2 mRNA (578 bp) levels increased dramatically after >50 nM RA treatment.

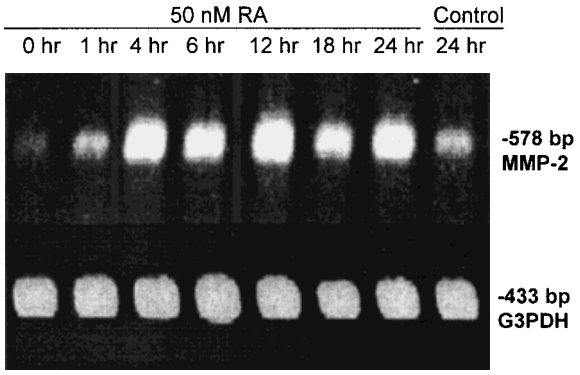

Fig. 6. Effect of length of RA treatment on stimulation of MMP-2 mRNA levels. Chondrocyte cultures were treated with 50 nM RA at various times (0, 1, 4, 6, 12, 18, and 24 h) and then total RNA was isolated from the cell layer. RT-PCR analysis was performed as described in Fig. 5. Note that as early as 4–6 h after RA treatment MMP-2 mRNA expression was significantly enhanced by RA.

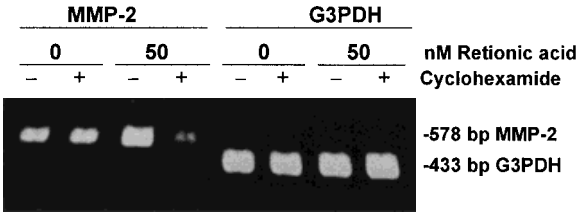

Fig. 7. Increase in MMP-2 mRNA levels after RA treatment was dependent on de novo protein synthesis. Chondrocyte cultures were treated with RA (50 nM) on day 10, alone or in combination with cycloheximide (2.5 µg/ml). Total RNA was isolated 24 h after treatment, and each sample was subjected to RT-PCR analysis as described in the caption to Fig. 5. Note that cycloheximide treatment abolished the increase in MMP-2 mRNA levels caused by RA treatment. This indicates that de novo protein synthesis is required for the RA-dependent increase in MMP-2 mRNA levels.

treated with RA, potent upregulation of TGF-β2 gene expression occurred [Glick et al., 1989]. However, in GP chondrocytes, it is unlikely that RA increased the level of MMP-2 through stimulation of expression of TGFβ. First, previous study has shown that RA treatment does not cause appreciable change in the expression of several TGFβ isoforms in cultured GP chondrocytes [Ballock et al., 1994]. Second, we did not find appreciable increase of MMP-2 levels in GP chondrocytes treated with TGFβ using zymography assays (data not shown).

The elevation of MMP-2 levels in cultured chondrocytes by RA treatment did not result from an overall increase in cellular protein. When day 10 or day 17 growth plate chondrocytes were treated with RA, there was no increase in cellular protein; in fact, it was somewhat inhibitory (Table I). Further, time-course studies indicate that elevation of MMP-2 levels in the culture medium is a relatively early response to RA treatment. An increase in MMP-2 activity could be detected within 16 h of treatment.

Recombinant MMP-2 has been shown to cleave the interglobular domain of the core protein of cartilage aggrecan at Asn341–Phe342 [Flannery et al., 1992; Fosang et al., 1992]. Thus, the drastic reduction in proteoglycan levels in growth plate cultures in the presence of RA [Wu et al., 1997] may well be due to both decreased synthesis of proteoglycan and an increase in the breakdown of proteoglycan resulting from the increased production of MMP-2 induced by RA. In fact, the increase in MMP-2 levels upon RA treatment was accompanied by an upregulation of MMP-2 mRNA levels. As little as 10 nM RA significantly increased the level of MMP-2 mRNA. Despite the semiquantitative nature of RT-PCR, a major increase in MMP-2 mRNA levels upon RA treatment was clearly detected. The fact that G3PDH mRNA levels were not significantly affected indicates that the effects of RA on MMP-2 mRNA levels are specific in these cells. Further, the finding that the increase in MMP-2 mRNA induced by RA was abolished by treatment with cycloheximide (which is known to inhibit protein synthesis) indicates that de novo synthesis of some proteinaceous mediator(s) is required for the RA effect on MMP-2 levels.

The mechanism of RA regulation of MMP-2 levels in GP chondrocytes invites speculation. Only one potential binding site has been identified in the gene structure of human MMP-2 for a nuclear transcription factor [Huhtala et al., 1990]. This is an AP-2 site. No RA-responsive *cis*-element has been found in either human or avian MMP-2 genomic DNA. Since AP-2 is a RA-inducible transcription factor [Williams and Tjian, 1991], it is possible that RA may stimulate MMP-2 expression through upregulation of AP-2. Since the transcriptional activity of AP-2 can be enhanced by the activities of both protein kinases C and A [Imagawa et al., 1987], it is not surprising that PMA treatment failed to suppress the upregulation of MMP-2 levels by RA and that forskolin treatment alone increased the MMP-2 level in this culture system. However, the possible involvement of AP-2 in the RA-dependent upregulation of MMP-2 level in GP chondrocytes obviously needs careful study.

Upregulation of MMP-2 by RA in GP chondrocytes also has implications in the differentiation and calcification of GP chondrocytes during endochondral ossification. The level of RA in serum is ~50 nM, the majority of which is bound to serum album [Smith et al., 1978]. RA has been identified as a key inducer that governs skeletal patterning and development [Eichele, 1989; Tabin, 1991; Helms et al., 1996; Thaller and Eichele, 1987]. A gradient of RA is known to occur across the developing chick limb bud and may be responsible for the normal pattern of digit formation [Eichele, 1989]. Alteration of this RA gradient at different time points during limb bud maturation results in marked perturbation in the development and organization of the extremity [Bryant and Gardiner, 1992].

Another clue to the function of retinoids in developing cartilage comes from clinical reports of skeletal toxicity in hypervitaminosis A (i.e., prolonged exposure to elevated levels of vitamin A). Arrest of bone growth from premature closure of the growth plate is a recognized manifestation of chronic vitamin A intoxication [Peace, 1962]. This premature closure is due to precocious matrix calcification and vascular invasion without proper proliferation of growth plate chondrocytes [Thomas et al., 1960]. In contrast, in vitamin A deficiency (hypovitaminosis A), the growth plate has an ill-defined, immature proliferative zone with few mitotic figures [Wolbach and Hegsted, 1952; Howell and Thompson, 1967] as well as a narrow, undercalcified maturation-hypertrophic zone. In addi-

tion, there is an abundant proteoglycan-rich extracellular matrix and reduced vascular invasion of the hypertrophic zone [Howell and Thompson, 1967; Havivi and Wolf, 1967].

In the noncalcifying zone of GP cartilage, there is a minimal supply of blood due to the relative avascularity of the tissue and the abundance of high MW proteoglycans that provide a strong diffusion barrier, especially to protein-bound factors such as RA. However, in the metaphyseal region of the GP where calcification and substantial tissue remodeling occur, the abundant supply of blood from the ingrowing blood vessels would provide adequate levels of RA to induce MMP-2 synthesis. Thus, the increase in MMP-2 expression that we have observed upon exposure of GP chondrocytes to RA could readily explain the breakdown of the cartilage matrix and the invasion of incoming metaphyseal blood vessels during endochondral ossification.

One final point concerns the effect of RA on the expression of tissue inhibitors of MMPs. If the effects of RA just described are of physiological significance in the terminal GP, then it should be expected that RA would reciprocally regulate TIMP and MMP levels. This has in fact been observed in rat bone cell populations [Overall, 1995], where RA increases expression of MMPs, as well as in noncalcifying tissues such as skin and synovial fibroblasts where RA inhibits expression of MMPs [Bigg and Cawston, 1996]. The question of whether RA downregulates TIMP levels in the GP is currently being explored.

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REFERENCES

Aimes RT, Quigley JP (1995): Gelatinase A is an interstitial collagenase. J Biol Chem 270:5872–5876.

Aimes RT, French DL, Quigley JP (1994): Cloning of a 72 kDa matrix metalloproteinase (gelatinase) from chicken embryo fibroblasts using gene family PCR: Expression of the gelatinase increases upon malignant transformation. Biochem J 300:729–736.

Alini M, Matsui Y, Dodge GR, Poole AR (1992): The extracellular matrix of cartilage in the growth plate before and during calcification: Changes in composition and degradation of type II collagen. Calcif Tissue Int 50:327–335.

Alini M, Carey, Hirata S, Grynpas MD, Pidoux I, Poole AR (1994): Cellular and matrix changes before and at the time of calcification in the growth plate studied in vitro: Arrest of type X collagen synthesis and net loss of collagen when calcification is initiated. J Bone Miner Res 9:1077–1087.

Ballock RT, Heydemann A, Wakefield LM, Flanders KC, Roberts AB, Sporn MB (1994): Inhibition of the chondrocyte phenotype by retinoic acid involves upregulation of metalloproteinase genes independent of TGF-β. J Cell Physiol 159:340–346.

Bigg HF, Cawston TE (1996): Effect of retinoic acid in combination with platelet-derived growth factor-BB or transforming growth factor-beta on tissue inhibitor of metalloproteinases and collagenase secretion from human skin and synovial fibroblasts. J Cell Physiol 166:84–93.

Brighton CT (1978): Structure and function of the growth plate. Clin Orthop Relat Res 136:22–32.

Brown CC, Hembry RM, Reynolds JJ (1989): Immunolocalization of metalloproteinases and their inhibitor in the rabbit growth plate. J Bone Joint Surg [Am] 71:580–593.

Brown PD, Levy AT, Margulies IMM, Liotta LA, Stetler-Stevenson WG (1990): Independent expression and cellular processing of Mr 72,000 type IV collagenase and interstitial collagenase in human tumorigenic cell lines. Cancer Res 50:6184–6191.

Bryant SV, Gardiner DM (1992): Retinoic acid, local cell-cell interactions, and pattern formation in vertebrate limbs. Dev Biol 152:1–25.

Buckwalter JA, Mower D, Ungar R, Schaeffer J, Ginsberg B (1986): Morphometric analysis of chondrocyte hypertrophy. J Bone Joint Surg [Am] 68:243–255.

Chen JM, Aimes RT, Ward GR, Younglieb GL, Quigley JP (1991): Isolation and characterization of a 70-kDa metalloproteinase (gelatinase) that is elevated in Rous sarcoma virus–transformed chicken embryo fibroblasts. J Biol Chem 266:5113–5121.

Chomczynski P, Sacchi N (1987): Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159.

Collier IE, Wilhelm SM, Eisen AZ, Marmer BL, Grant GA, Seltzer JL, Kronberger A, He C, Bauer EA, Goldberg GI (1988): H-*ras* oncogene–transformed bronchial epithelial cells (TBE-1) secrete a single metalloproteinase capable of degrading basement membrane collagen. J Biol Chem 263:6579–6587.

Dean DD, Muniz OE, Berman I, Pita JC, Carreno MR, Woessner JF Jr, Howell DS (1985): Localization of collagenase in the growth plate of rachitic rats. J Clin Invest 76:716–722.

Eichele G (1989): Retinoids and vertebrate limb pattern formation. Trends Genet 5:246–251.

Farquharson C, Whitehead CC, Loveridge N (1994): Alterations in glycosaminoglycan concentration and sulfation during chondrocyte maturation. Calcif Tissue Int 54:296–303.

Flannery CR, Lark MW, Sandy DD (1992): Identification of a stromelysin cleavage site within the interglobular domain of human aggrecan. J Biol Chem 267:1008–1014.

Fosang AJ, Neame PJ, Last K, Hardingham TE, Murphy G, Hamilton JA (1992): The interglobular domain of cartilage aggrecan is cleaved by PUMP, gelatinases and cathepsin B. J Biol Chem 267:19740–19474.

Glick AB, Flanders KC, Danielpour D, Yuspa SH, Sporn MB (1989): Retinoic acid induces transforming growth factor-beta 2 in cultured keratinocytes and mouse epidermis. Cell Regul 1:87–97.

Havivi E, Wolf G (1967): Vitamin A, sulfation and bone growth in the chick. J Nutr 92:467–473.

Helms JA, Kim CH, Eichele G, Thaller C (1996): Retinoic acid signaling is required during early chick limb development. Development 122:1385–1394.

Howell DS, Dean DD (1992): The biology, chemistry and biochemistry of the mammalian growth plate. In Coe FL, Favus MJ (eds): "Disorders of Bone and Mineral Metabolism." New York: Raven Press Ltd, pp. 313–353.

Howell JM, Thompson JN (1967): Lesions associated with the development of ataxia in vitamin A–deficient chicks. Br J Nutr 21:741–754.

Hughes CE, Caterson B, Fosang AJ, Roughley PJ, Mort MS (1995): Monoclonal antibodies that specifically recognize neoepitope sequences generated by 'aggrecanase' and matrix metalloproteinase cleavage of aggrecan: Application to catabolism in situ and in vitro. Biochem J 305:799–804.

Huhtala P, Chow LT, Tryggvason K (1990): Structure of the human type IV collagenase gene. J Biol Chem 265:11077–11082.

Hunziker EB (1994): Mechanism of longitudinal bone growth and its regulation by growth plate chondrocytes. Microsc Res Tech 28:505–519.

Imagawa M, Chiu R, Karin M (1987): Transcription factor AP-2 mediates induction by two different signal transduction pathways: Protein kinase C and cAMP. Cell 51:251–260.

Kleiner DE, Stetler-Stevenson WG (1994): Quantitative zymography: Detection of picogram quantities of gelatinases. Anal Biochem 218:325–329.

Linsenmayer TF, Chen Q, Gibney E, Gordon MK, Marchant JK, Mayne R, Schmid TM (1991): Collagen types IX and X in the developing chick tibiotarsus: Analysis of mRNAs and proteins. Development 111:191–196.

Liotta LA, Steeg PS, Stetler-Stevenson WG (1991): Tumor metastasis and angiogenesis: An imbalance of positive and negative regulation. Cell 64:327–336.

Matrisian LM (1992): The matrix-dregrading metalloproteinases. Bioessays 14:455–463.

McCachren SS (1991): Expression of metalloproteinases and metalloproteinase inhibitor in human arthritic synovium. Arthritis Rheum 34:1085–1093.

Okada Y, Nagase H, Harris ED Jr (1986): A metalloproteinase from human rheumatoid synovial fibroblasts that digests connective tissue matrix components. Purification and characterization. J Biol Chem 261:14245–14255.

Oshima O, Leboy PS, McDonald SA, Tuan RA, Shapiro IM (1989): Developmental expression of genes in chick growth cartilage detected by in situ hybridization. Calcif Tissue Int 45:182–192.

Overall CM (1995): Repression of tissue inhibitor of matrix metalloproteinase expression by all-trans-retinoic acid in rat bone cell populations: Comparison with transforming growth factor-beta 1. J Cell Physiol 164:17–25.

Overall CM, Wrana JL, Sodek J (1991): Independent regulation of collagenase, 72-kDa progelatinase, and metalloendoproteinase inhibitor expression in human fibroblasts by transforming growth factor-β. J Biol Chem 266:14064–14071.

Peace CN (1962): Focal retardation and arrestment of growth of bones due to vitamin A intoxification. JAMA 182:980–985.

Pnabrieres T, Piechaczyk M, Rainer B, Dani CH, Fort PH, Riaad S, Marty L, Imbach JL, Jeanteur PH, Blanchard JM (1983): Complete nucleotide sequence of the messenger RNA coding for chicken muscle glyceraldehyde 3-phosphate dehydrogenase. Biochim Biophys Res Commun 118:767–773.

Poole AR (1991): The growth plate: Cellular physiology, cartilage assembly, and mineralization. In Hall B, Newman S (eds): "Cartilage: Molecular Aspects." Boca Raton, FL: CRC Press Inc, pp. 179–211.

Pyke C, Ralfkiaer E, Huhtala P, Hurskainen T, Dano K, Tryggvason K (1992): Localization of messenger RNA for Mr 72,000 and 92,000 type IV collagenases in human skin cancers by in situ hybridization. Cancer Res 52:1336–1341.

Reddi AH, Hascall VC, Hascall GK (1978): Changes in proteoglycan types during matrix-induced cartilage and bone development. J Biol Chem 253:2429–2436.

Schmid TM, Linsenmayer TF (1985): Immunohistochemical localization of short chain cartilage collagen (type X) in avian tissues. J Cell Biol 100:598–605.

Shinomura T, Kimata K (1992): Proteoglycan-Lb, a small dermatan sulfate proteoglycan expressed in embryonic chick epiphyseal cartilage, is structurally related to osteoinductive factor. J Biol Chem 267:1265–1270.

Sires UL, Schmid TM, Fliszar CJ, Wang Z-Q, Gluck SL, Welgus HG (1995): Complete degradation of type X collagen requires the combined action of interstitial collagenase and osteoclast-derived cathepsin B. J Clin Invest 95:2089–2095.

Smith JE, Milch PO, Muto Y, Goodman DS (1978): The plasma transport and metabolism of retinoic acid in the rat. Biochem J 132:821–827.

Stetler-Stevenson WG (1990): Type IV collagenases in tumor invasion and metastasis. Cancer Metastasis Rev 9:289–303.

Tabin CJ (1991): Retinoids, homeoboxes, and growth factors: Toward molecular models for limb development. Cell 66:199–217.

Thaller C, Eichele G (1987): Identification and spatial distribution of retinoids in the developing chick limb bud. Nature 327:625–628.

Thomas L, McCluskey RT, Potter JL, Weissmann G (1960): Comparison of the effects of papain and vitamin A on cartilage I. The effects in rabbits. J Exp Med 111:705–718.

Wilhelm SM, Shao Z-H, Housley T, Sperack PK, Baumann AP, Gunja-Smith Z, Woessner JF (1993) Matrix metalloproteinase-3 (stromelysin-1). J Biol Chem 268:21906–21913.

Williams T, Tjian R (1991): Analysis of the DNA-binding and activation properties of the human transcription factor AP-2. Science 251:1067–1071.

Woessner JF (1991): Matrix metalloproteinases and their inhibitors in connective tissue remodeling. FASEB J 5: 2145–2154.

Wolbach SB, Hegsted DM (1952): Vitamin A deficiency in the chick. Arch Pathol 54:548–563.

Woolley DE, Crossley MJ, Evanson JM (1977): Collagenase at sites of cartilage erosion in the rheumatoid joint. Arthritis Rheum 20:1231–1239.

Wu LNY, Genge BR, Ishikawa Y, Wuthier RE (1992): Modulation of cultured chicken growth plate chondrocytes by transforming growth factor-β_1 and basic fibroblast growth factor. J Cell Biochem 49:181–198.

Wu LNY, Ishikawa Y, Nie D, Genge BR, Wuthier RE (1997): Retinoic acid stimulates matrix calcification and initiates type I collagen synthesis in primary cultures of avian weight-bearing growth plate chondrocytes. J Cell Biochem 65:209–230.