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Regulation of Rat Interstitial Collagenase Gene Expression in Growth Cartilage and Chondrocytes by Vitamin D_3, Interleukin-1β, and Okadaic Acid

R.M. Grumbles, L. Shao, J.J. Jeffrey, and D.S. Howell

Arthritis Research Laboratory, Miami V.A. Medical Center, and Department of Medicine, University of Medicine, Miami, Florida 33101 (R.M.G., L.S., D.S.H.); Department of Biochemistry, Albany Medical College, Albany, New York 12208 (J.J.J.)

Abstract The interstitial collagenase produced by the rat growth plate chondrocytes is the homologue of the human collagenase-3, or matrix metalloproteinase-13. This enzyme is responsible for the loss of collagen during hypertrophy of chondrocytes and for the degradation of transverse septa in long bone growth. Rachitic rats (42 days, male Sprague-Dawley) had an 8-fold higher level of collagenase mRNA in the hypertrophic versus proliferative zone of growth plate cartilage. Intramuscular injection of 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$; 1.0 μg/kg body weight) in rachitic rats increased collagenase mRNA another 1.5-fold in the hypertrophic zone. The regulation of collagenase gene by 1,25-$(OH)_2D_3$ and interleukin (IL)-1β in cultured proliferative chondrocytes was studied by means of steady-state mRNA and half-life determination of mRNA using the transcriptional inhibitor actinomycin D, and nuclear run-on transcription analyses. Treatment of cells with 1,25-$(OH)_2D_3$ (10^{-6} M) and IL-1β (2 ng/ml) increased collagenase mRNA 8- and 13-fold, respectively. Additionally, the collagenase mRNA half-life was increased by 1,25-$(OH)_2D_3$ and IL-1β. In the presence of a protein kinase C inhibitor, staurosporine, 1,25-$(OH)_2D_3$ induction of collagenase mRNA was blocked. Here the addition of phorbol 12-myrisate 13-acetate (PMA) to activate protein kinase C increased collagenase mRNA 10-fold. However, in the presence of staurosporine (50 nM), PMA induction was blocked, whereas IL-1β was not. IL-1β is known to activate several phosphorylation pathways. Okadaic acid (500 nM), a protein phosphatase inhibitor, increased the relative collagenase mRNA abundance 10-fold. The rate of the rat collagenase gene transcription in nuclei was increased with 1,25-$(OH)_2D_3$, IL-1β and okadaic acid. In separate experiments, the collagenase promoter was ligated to a reporter plasmid and the plasmid was transfected into chondrocytes. The results showed that 1,25-$(OH)_2D_3$, IL-1β, and PMA increased reporter activity 2.5-, 2.8-, and 3.27-fold, respectively. Thus, there are multiple nuclear and cytoplasmic mechanisms by which cartilage modulators regulate rat interstitial collagenase gene expression.

Key words: vitamin D_3, interleukin-1, collagenase-3 transcription, okadaic acid, hypertrophic chondrocyte

When rachitic rats are supplemented with typical dietary levels of vitamin D_3, cartilage growth function is rapidly restored [Atkin et al., 1985]. Initially, a cartilage scaffold is developed and is subsequently remodeled to allow for bone growth. For the rapid physiological resorption of the growth plate cartilage, matrix degradation, particularly through the action of collagenolytic enzymes, must occur [Howell and Dean, 1993]. A gene family of proteolytic enzymes, the matrix metalloproteinases, has been identified and described in the past several years [Nagase, 1994]. Collectively, this family of proteinases has the ability to degrade essentially all the macromolecular components of the extracellular matrix in mammals. Of this group of proteinases, the interstitial collagenases have been shown to be of particular significance in the degradation of type I, II, and III collagens [Welgus et al., 1983]. Interstitial collagenase has been shown to be required for the initial of the degradation of these collagens in the both physiological and pathological settings. These enzymes have been implicated in serving several important roles during blastocyst implantation [Werb et al., 1992], fetal development [Matrisian and Hogan, 1990], and normal adult physiological and pathological states [Matrisian, 1992]. Indeed, removal of the collagenase cleav-

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Address reprint requests to Dr. R.M. Grumbles, Division of Arthritis, Department of Medicine (D26), University of Miami School of Medicine, P.O. Box 016960, Miami, FL 33101.

age site in collagen type I in transgenic mice results in impaired tissue remodeling, including collagenous nodules in the uterine wall (Liu et al., 1995). In humans, two distinct genetic types of interstitial collagenase are expressed in mesenchymal cells: collagenases 1 and 3 (also known as human matrix metalloproteinases-1 and -13) (MMP-1 and -13) [Freije et al., 1994]. Collagenase-1 has been studied extensively, with respect to both tissue localization and mechanism of action. Interestingly, it is present at only low levels in chondrocytes, and kinetic studies have shown that it expresses extremely low activity against native type II collagen [Welgus et al., 1983], the predominant collagen of cartilage. On the other hand, the rodent analogue of collagenase-3 [Quinn et al., 1990] displays brisk activity against native type II collagen [Welgus et al., 1983], suggesting that this human enzyme could be particularly significant in the management of cartilage collagen degradation. Although the presence of collagenase-1 in human rheumatoid arthritis has been abundantly documented the role of human collagenase-3 in cartilage, and its regulation, is essentially unknown.

In contrast to the situation in humans, considerable evidence exists in the rat, however, that rat interstitial collagenase, which is highly homologous to human collagenase-3, is important for the regulated growth and differentiation of bone. First, collagenase activity has been localized to growth plates [Dean et al., 1985], and this activity increases when proliferative cells mature into hypertrophic cells [Dean et al., 1989]. Second, this collagenase has been detected in the rat growth plate and articular cartilage by immunohistochemistry [Blair et al., 1989]. Retinoic acid and transforming growth factor-β (TGF-β) also reduce collagenase mRNA abundance in chondrocytes [Ballock et al., 1994]. In rheumatoid arthritis and osteoarthritis, cytokines and other inflammatory mediators of the joint also contribute to destruction of cartilage through the induction of metalloproteinases such as collagenase, stromelysin, and gelatinase [Goldring, 1993].

The present study with rachitic animals was designed to define how rat interstitial collagenase gene expression is regulated by the principal active metabolite of vitamin D_3, 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$), an important regulator of growth cartilage processes [Atkin et al., 1985; Kato et al., 1990; Schwartz and Boyan, 1988]. Interleukin-1β (IL-1β) peptide is also hypothesized to mediate some actions of 1,25-$(OH)_2D_3$ in growth cartilage because IL-1β peptide increases fivefold in cartilage extracts after the addition of 1,25-$(OH)_2D_3$ [Howell et al., 1992]. Thus, does IL-1β peptide have a role(s) in growth cartilage that is unique to that of 1,25-$(OH)_2D_3$ in the regulation of collagenase? Furthermore, since phorbol myrisate acetate (PMA) and okadaic acid purportedly mimic some IL-1 effects in other cells [Guy et al., 1992], the effects of these modulators on rat interstitial collagenase gene expression are explored as well. The data presented here suggest that 1,25-$(OH)_2D_3$, IL-1β, PMA, and okadaic acid all can contribute to the activation of the rat interstitial collagenase gene. The results of our study, however, indicate the existence of both translational and post-translational effects of these chondrocyte-modifying molecules.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (40 g; 21 days) (Charles River Breeding Laboratories, Wilmington, MA) were fed a low-phosphate, vitamin D-deficient diet (U.S. Pharmacopeia Rachitogenic diet #12) to induce rickets [Dean et al., 1985]. At 42 days, animals were sacrificed with sodium pentobarbitol (10% in sterile water). 1,25-$[OH]_2D_3$-healing rachitic animals received a single intramuscular injection of 1,25-$(OH)_2D_3$ (kindly provided by I. Atkin, Ben Gurian University of the Negev, Beersheba, Israel), 1.0 μg/kg body weight in propylene glycol).

Cell Culture

We used a previously described microdissection technique to remove specific zones of cartilage from the growth plate of rachitic animals [Dean et al., 1985, 1990]. Growth cartilage removed from proximal tibias of both legs and cut in cross-sectional pieces yielded a proximal half of proliferative zone cells and a half of hypertrophic zone cells. Chondrocytes isolated by digestion of proliferative zone cartilage with collagenase type 2 (Worthington Biochemicals, Freehold, NJ) (2 h) were grown in Eagle's minimum essential medium (MEM) (Life Technologies) supplemented with 10% fetal calf serum (FCS) (Hyclone Laboratories, Logan, UT), 50 μg/ml ascorbate, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a 5% CO_2–95% air atmosphere. Cells grown to

2 days postconfluence were treated with 0.5% charcoal-stripped fetal calf sera (CS-FCS) for 2 days before the initiation of experiments studying the regulation of collagenase gene expression. To maintain a chondrocyte morphology and high expression of collagen type II (not shown), the culture dishes were coated with a rat tail collagen preparation [Freshney, 1987]. Second-passage cells were used throughout these experiments.

Isolation and Analysis of RNA

Total RNA (tRNA) was isolated from growth cartilage by harvesting in the presence of 5 M guanidinium isothiocyanate, 50 mM Tris–HCl (pH 7.50), 1 mM EDTA, 0.05% sarkosyl, 10 mM β-mercaptoethanol, and 0.01% antifoam A (Sigma). The sample was homogenized in a Brinkman polytron and clarified by centrifugation for 15 min at 10,000 rpm in a SS-34 rotor at 4°C. The RNA was purified by centrifugation through a cesium–trifluoroacetic acid block gradient [Smale and Sasse, 1993]. Using aerosol-resistant pipettor tips, the RNA was dissolved in 200 μl of 20 mM Tris–HCl, pH 8.0, 1 mM EDTA and 0.2% sodium dodecyl sulfate (SDS). The sample was extracted once with buffered phenol and chloroform, the phases separated and the aqueous phase extracted twice with chloroform. The RNA was precipitated with ethanol and stored at −70°C. Chondrocyte RNA was isolated using an acidic-guanidinium method provided in a commercial kit, RNAZOL (Tel-Test, Friendswood, TX) [Chomczynski and Sacchi, 1987].

For Northern blot analysis, 20 μg of total tissue RNA was electrophoresed on agarose–formaldehyde gels and transferred to a BIOTRANS membrane (ICN Biomedicals, Costa Mesa, CA) by capillary elution [Fourney et al., 1988]. RNA integrity was confirmed by ethidium bromide staining of gels after electrophoresis and/or detection of the β-actin mRNA. The β-actin mRNA was detected by using a gel-purified PCR product (534 bp). The β-actin PCR oligonucleotides were ACT5 (GGGGCGCCCCAGGCACC) and ACT3 (CTTAATGTCACGCACGATTTC). Prehybridization (4 h) and hybridization (24 h) were performed at 42°C in 50% formamide, 5 × standard saline (1 × SSC = 0.15 M NaCl, 0.0125 M nacitrate, pH 7.0), 0.5% SDS, 5 μg/ml heat-denatured salmon sperm DNA, and 5 × Denhardt's reagent. The blot are washed two times at room temperature in 2 × SSC, 0.5% SDS, and then four times for 20 min at 60°C in 0.1 × SSC, 0.5% SDS, and finally subjected to autoradiography at −80°C. The relative amounts of radioactivity associated with the messenger RNA (mRNA) was estimated by densitometric scanning of the autoradiogram. The rat interstitial collagenase probe was a full-length complementary DNA (cDNA) clone [Quinn et al., 1990]. The DNA probes were first agarose gel electrophoresed, the correct size fragment cut out of the gel, and the DNA purified with the Elu-Quick DNA purification kit (Schleicher & Schuell, Keene, NH). The probes were ^{32}P-labeled (α-^{32}P dCTP; 3,000 Ci/mmol) by using the Prime-It RmT random priming kit (Stratagene, La Jolla, CA). In order to reduce radioactivity usage and to measure collagenase transcript level in minute cartilage samples, a relative reverse transcriptase-polymerase chain reaction (RT-PCR) method was used. A protocol similar to that one documenting accuracy and reproducibility of this RT-PCR method was used [Chen and Klebe, 1993; Magnuson et al., 1991]. For detection of collagenase transcript by RT-PCR two oligonucleotides were synthesized: COLL5 (TTGGCTTAGATGTGACTGGC) and COLL3 (AGAAGACCAGAAGGC). The product size was 310 bp and its DNA sequence confirmed by DNA sequence analysis (not shown). Rat elongation factor 1a PCR oligonucleotides were ELF5 (AGGTGATTATCCTGAACCATCC) and ELF3 (AGTGGAGGGTAGTCAGAGAAGC) generating 234-bp product. RNA (250 ng) was reverse-transcribed with Mo-MLV reverse transcriptase (Life Technologies) and random DNA sequence priming. We used VENT DNA polymerase (New England Biolabs, Beverly, MA) at 1 unit per 50 μl reaction volume to amplify the specific cDNA sequences. A thermal cycle profile was denaturing for 30 s at 94°C, annealing of the primers for 30 s at 55°C and extending for 45 s at 73°C; this was repeated 21 times. The PCR products were separated on 2% agarose gels containing either ethidium bromide or SYBR Green I (Molecular Probes, Eugene, OR) [Schneeberger et al., 1995]. The gel was viewed under ultraviolet (UV) light. The DNA was quantified by scanning a polaroid (type 665) picture of the gel.

Nuclear Run-off

Nuclei were isolated by gentle disruption of cells in 10 mM Tris, pH 7.4, 3 mM $MgCl_2$, 0.1 mM EDTA, and 0.5% NP-40 and the elongation reactions performed as described [Celano et al.,

1989]. Cells were placed onto ice, washed twice with PBS and once with Tris buffer (10 mM Tris, 10 mM NaCl, and 3 mM $MgCl_2$ pH 7.4) and 4 ml lysis buffer (0.5% v/v Nonidet P-40, 10 mM Tris, 10 mM NaCl, 3 mM $MgCl_2$) was added for 20 min. The cells were detached by dynamic shaking and the remaining whole cells and the nuclei collected by centrifugation. The pellet was washed once in lysis buffer and the pellet dissolved in storage buffer: 50 mM Tris, pH 8.3, 40% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 3,000 U/ml RNasin, and 0.6 mM PMSF. The nuclei were used immediately for elongation of transcripts with 10 μl of α-^{32}P UTP (3,000 Ci/mmol) following a described protocol [Celano et al., 1989]. After 30 min with shaking at 30°C, the 10 μl of 20 mM $CaCl_2$ and 10 μl of 10 mg/ml RNase-free DNase I were added. For detection of a specific transcript, labeled RNA was hybridized to a blot containing bluescript plasmid (Stratagene), β-actin PCR DNA fragment cloned in bluescript, and collagenase full-length cDNA [Quinn et al., 1990]. For binding, plasmids were linearized by restriction digestion and the DNA denatured by incubation with 0.2 M NaOH for 20 min at 23°C. The plasmids were neutralized with 10 vol of 20 × SSC and spotted onto BIOTRANS nylon membrane at 10 μg per spot. The DNA was cross-linked to the membrane with a UV transilluminator (302 nm) for 90 s. Labeled transcripts were hybridized to the membrane with the same number of counts per minute (cpm) of ^{32}P-labeled RNA in a solution of 0.5 M sodium phosphate, pH 7.2, 7% SDS for 18 h at 65°C. The filters were washed once in 250 mM sodium phosphate, pH 7.2, 1% SDS, and two times in 100 mM sodium phosphate pH 7.2, 1% SDS for 15 min at 65°C. The filter was exposed to X-ray film at −70°C. Autoradiographic results were scanned and analyzed.

DNA Transfection and Promoter Activity

A 2.1-kb rat genomic clone (Not/*Xho*I restriction fragment) (J.J. Jeffrey, unpublished data) was inserted into the expression vector, pSVCat, containing the chloramphenicol acetyltransferase gene downstream [Gorman et al., 1982]. Chondrocytes in their second passage were transfected at 90–100% confluence, using lipofectamine (Life Technologies). Chondrocytes were treated with 2% sera containing MEM overnight and then 48 h with sera-free medium. The cells were harvested and lysate assayed for the conversion of [^{3}H]chloramphenicol (NEN Research Products, Boston, MA) to the monoacetate form using a standard procedure [Ausubel et al., 1992]. Each lysate was corrected for the amount of protein in the cell extract. Each value is the average of duplicate or triplicate determinations. Protein assays were performed using a protein assay (Pierce) according to the manufacturer's instructions.

Statistical Analysis

For statistical evaluation of the results of mRNA hybridization or DNA level, a densitometry reading was taken and a paired Student's *t*-test or analysis of variance (ANOVA) used [Snedecor and Cochran, 1989]. The differences were considered statistically significant when $P < 0.05$.

RESULTS

Regulation of Rat Interstitial Collagenase mRNA in Rachitic Growth Cartilage

To verify the accuracy of the RT-PCR method used, tRNA was diluted in series and converted into cDNA. The cDNA was typically amplified with specific primers to the rat interstitial collagenase cDNA for 20–24 cycles and the products visualized after agarose gel electrophoresis with the SYBR Green I nucleic acid stain. Figure 1a shows that with successive dilutions of chondrocyte RNA, it is possible to detect a twofold difference with the RT-PCR method (see cycle lanes 20 and 22).

Rat interstitial collagenase mRNA was highest in the hypertrophic zone over a 0-, 4-, 24-, and 48-h time course after 1,25-$(OH)_2D_3$ treatment of rachitic animals (Fig. 1b). The addition of 1,25-$(OH)_2D_3$ increased mRNA in the lower hypertrophic zone, while in the proliferative zone mRNA decreased. However, at times later than 48 h, the collagenase mRNA level had returned to control levels (not shown). To demonstrate that comparable amounts of RNA were used in each reaction, an elongation factor transcript was also amplified, resulting in similar product yields (bottom row). A plot of the densitometric readings from the RT-PCR products (corrected for RNA variances by using the elongation factor 1a PCR primers) shows that the level of rat interstitial collagenase mRNA is eightfold higher in the hypertrophic zone versus proliferative zone (Fig. 1c). Thus as shown for collagenolytic activity [Dean et al., 1985] and immunolocaliza-

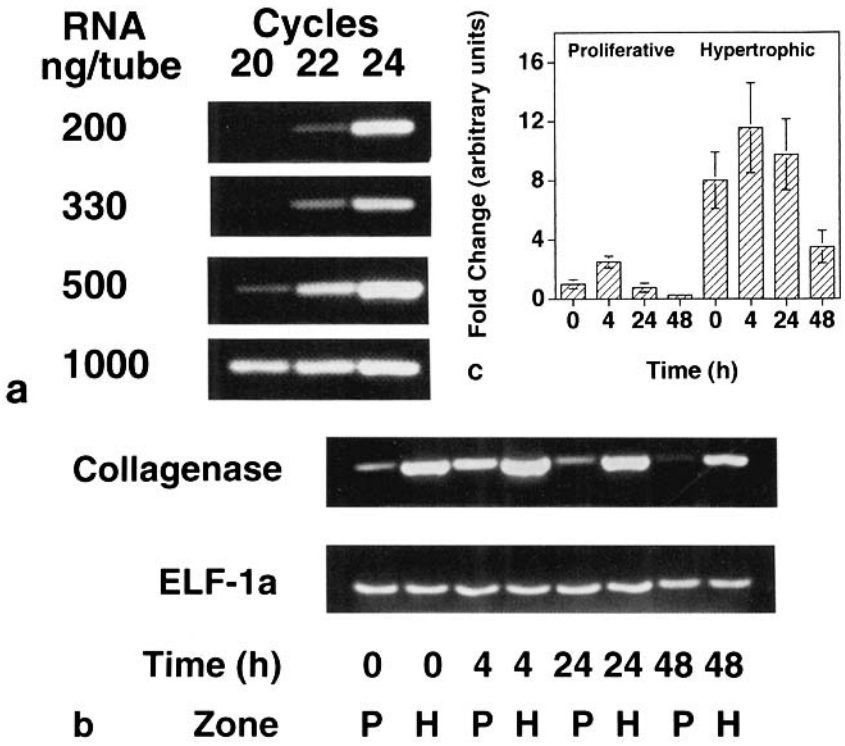


Fig. 1. Effects of 1,25-$(OH)_2D_3$ on the level of rat interstitial collagenase mRNA in rachitic growth cartilage. **a:** Quantification of collagenase mRNA by the RT-PCR method. Typical agarose gel showing serially diluted samples of total RNA. RNA content was kept level by adding tRNA to the dilutions. RNA was converted into cDNA and amplified with RT-PCR method. DNA was detected by staining the gel with SYBR Green I. **b:** Rats were given 1,25-$(OH)_2D_3$ and at 0, 4, 24, or 48 h, total RNA was isolated and the rat interstitial collagenase mRNA measured by RT-PCR. **c:** Specific changes in collagenase mRNA from the growth plate were determined by densitometric scanning, corrected for RNA differences by measurement of elongation factor 1a. Data represent mean (±SE) from four independent experiments. Rat interstitial collagenase mRNA level in the rachitic (time 0) proliferative cartilage is arbitrarily set to 1.0, the basal level for all subsequent RNA comparisons including that of the RNA from the hypertrophic cartilage. P, proliferative zone cartilage; H, hypertrophic zone cartilage.

tion [Blair et al., 1989], rat interstitial collagenase mRNA varies between the proliferative and lower hypertrophic zones of cartilage, and with the level of vitamin D_3 in the diet.

Regulation of Collagenase mRNA Level by 1,25-$(OH)_2D_3$ and IL-1β

In order to investigate further the regulation of rat interstitial collagenase gene by 1,25-$(OH)_2D_3$ and IL-1β, chondrocytes were isolated from the proliferative zone of rat rachitic growth cartilage. These chondrocytes grew rapidly in culture, providing enough cells for study in the second passage, as opposed to hypertrophic cells (not shown) and proliferative cells differentiate into hypertrophic ones during growth plate maturation [Howell and Dean, 1992]. Chondrocytes were exposed to different concentrations of 1,25-$(OH)_2D_3$ (0–10^{-6} M) and IL-1β (0.2–2.0 ng/ml) for 24 h; tRNA was isolated and blotted onto a nylon membrane for hybridization with the full-length cDNA clone. Northern blot analysis (Fig. 2a) shows that addition of 10^{-8} M 1,25-$(OH)_2D_3$ increased rat interstitial collagenase mRNA. This effect was dose dependent and at 10^{-6} M, relative mRNA abundance was eight-fold higher than the basal expression (Fig. 2b). The addition of interleukin-1β (IL-1β) also increased rat interstitial collagenase mRNA, and at the maximum dose (2.0 ng/ml) the yield was 13-fold higher than control levels. When 1,25-$(OH)_2D_3$ and IL-1β treatments were combined (lanes A, B), the level of collagenase mRNA was never above that of the IL-1β treatment alone.

Transcriptional and Post-Transcriptional Regulation of Rat Interstitial Collagenase by 1,25-$(OH)_2D_3$ and IL-1β

The increase in collagenase mRNA induced by 1,25-$(OH)_2D_3$ was protein synthesis dependent. When cycloheximide (10 μg/ml) was added with 1,25-$(OH)_2D_3$, collagenase mRNA was reduced

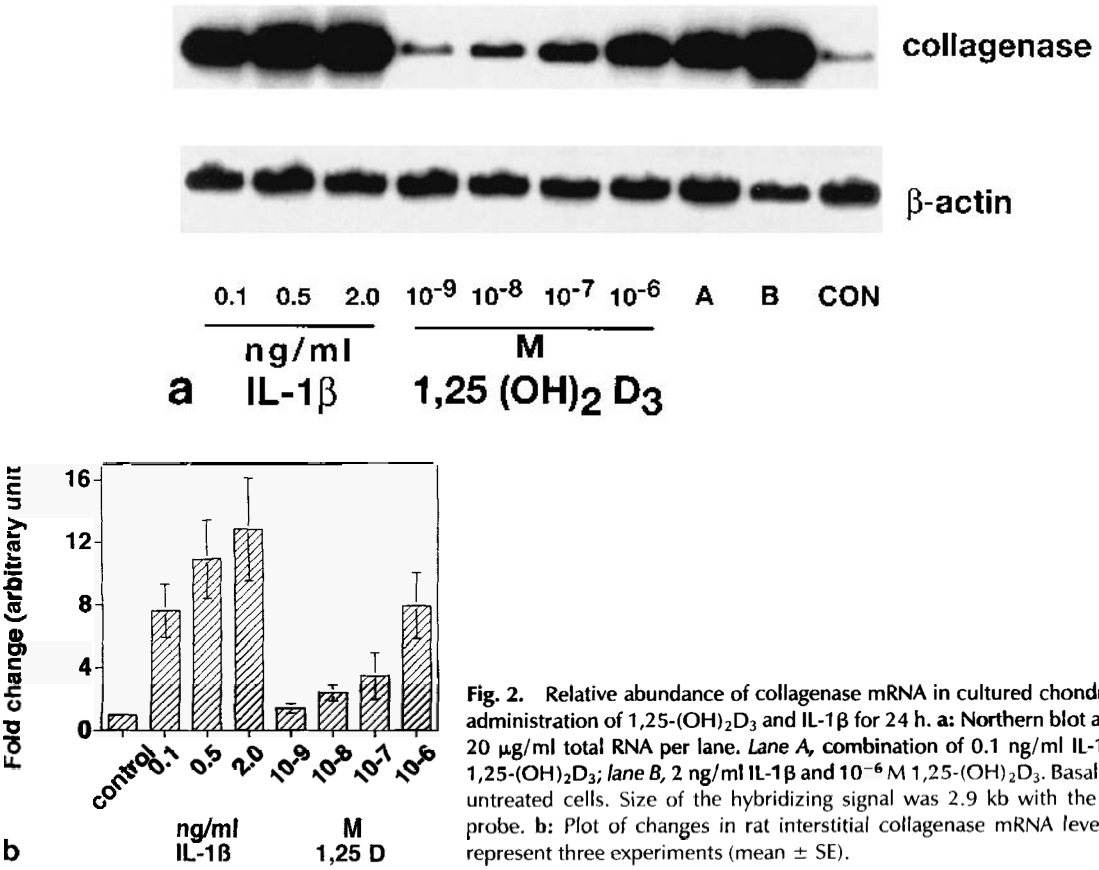


Fig. 2. Relative abundance of collagenase mRNA in cultured chondrocytes after administration of 1,25-$(OH)_2D_3$ and IL-1β for 24 h. **a:** Northern blot analysis used 20 μg/ml total RNA per lane. *Lane A,* combination of 0.1 ng/ml IL-1β and 10^{-9} 1,25-$(OH)_2D_3$; *lane B,* 2 ng/ml IL-1β and 10^{-6} M 1,25-$(OH)_2D_3$. Basal is RNA from untreated cells. Size of the hybridizing signal was 2.9 kb with the collagenase probe. **b:** Plot of changes in rat interstitial collagenase mRNA levels. The data represent three experiments (mean ± SE).

to 12% ($P < 0.05$) of that when 1,25-$(OH)_2D_3$ was used alone (not shown). Thus, de novo protein synthesis was needed for induction of collagenase mRNA. To determine if induction of collagenase expression by 1,25-$(OH)_2D_3$ and IL-1β occurs via changes in transcription, nuclei were isolated from chondrocytes and the rate of transcription measured in vitro. Based on preliminary experiments that determined nuclear transcript run-off levels, confluent chondrocytes were treated with 10^{-6} M 1,25-$(OH)_2D_3$ for 24 h or 2 ng/ml IL-1β for 4 h and were gently disrupted for nuclei isolation; and nascent RNA transcript synthesis continued in the presence of ^{32}P-UTP. Figure 3a shows that the collagenase transcript level in these cells was higher (3.7 ± 0.9-fold; mean ± SE; n = 3) than that in control chondrocytes. For IL-1β, the average (±SE) increase in collagenase nuclear RNA was 9 ± 2.3-fold (mean ± SE; n = 3) that of control nuclei (Fig. 3b). The transcription rate for β-actin did not change over this time period.

Additionally, an active post-transcriptional mechanism may influence the final abundance of collagenase mRNA in rat chondrocytes. This was explored by treating cells with 1,25-$(OH)_2D_3$ or IL-1β and actinomycin D (5 μg/ml) for 30 h. In the presence of this transcriptional blocker alone, rat interstitial collagenase mRNA declined relatively rapidly with a half-life of 4.3 h (Fig. 4). However, with 1,25-$(OH)_2D_3$ treatment as well, the rat interstitial collagenase mRNA half-life increased to approximately 5.2 h (Fig. 4). Similarly, collagenase mRNA half-life studies show that there is a difference in collagenase mRNA level turnover in control cells versus those treated with IL-1β, as IL-1β prolonged mRNA half-life to 6.8 h (Fig. 4). These data also suggest that 1,25-$(OH)_2D_3$ and IL-1β are regulating the mRNA presence by a post-transcriptional mechanism.

Role of Protein Kinase C in the Regulation of Rat Interstitial Collagenase mRNA Level

Part of the regulation of chondrocytes by 1,25-$(OH)_2D_3$, as well as IL-1β, is likely to include a protein kinase C-transducing cascade [Slater et al., 1995]. Here, staurosporine, an inhibitor of protein kinase C [Tamaoki, 1991], caused no changes in morphology or cell death of chondro-

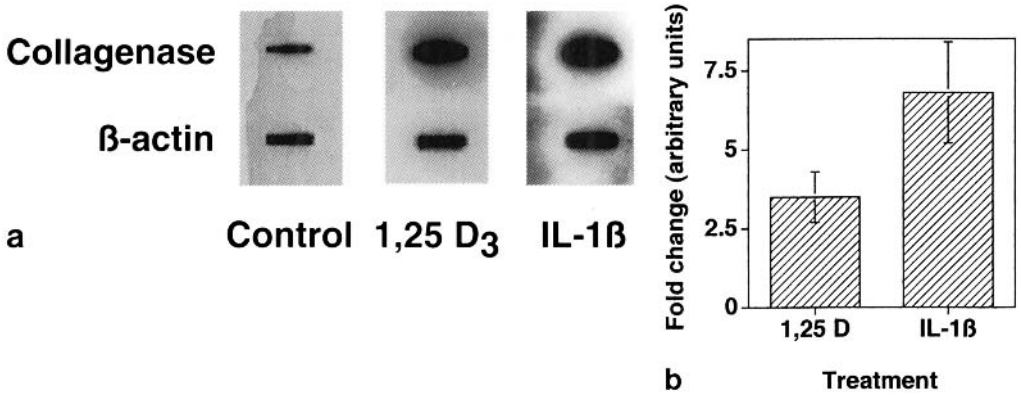


Fig. 3. **a:** Nuclear run-on transcription from isolated nuclei treated with 1,25-$(OH)_2D_3$ or IL-1β versus control rates of transcription. Specific transcription was determined with hybridization of ^{32}P-labeled transcript RNA to immobilized full-length collagenase cDNA. Normalized rates of transcription between treatments were assessed by the presence of β-actin plasmid. **b:** Plot of the changes in collagenase transcript levels with isolated nuclei. Treatments were plotted against the basal rate of transcription in 0.5% fetal calf serum (FCS) alone, and this value was arbitrarily set at 1.0. Data from three separate experiments (mean ± SE).

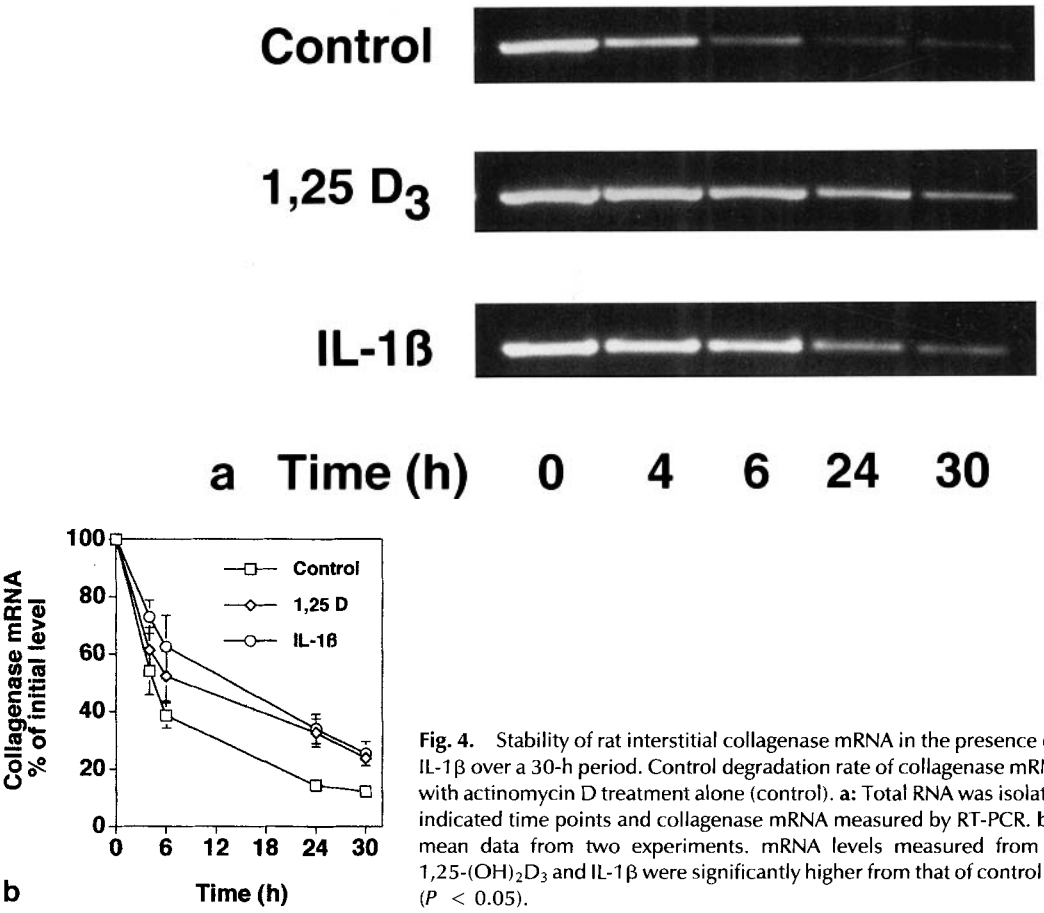


Fig. 4. Stability of rat interstitial collagenase mRNA in the presence of 1,25-$(OH)_2D_3$ or IL-1β over a 30-h period. Control degradation rate of collagenase mRNA was determined with actinomycin D treatment alone (control). **a:** Total RNA was isolated from cells at the indicated time points and collagenase mRNA measured by RT-PCR. **b:** Results represent mean data from two experiments. mRNA levels measured from cells treated with 1,25-$(OH)_2D_3$ and IL-1β were significantly higher from that of control cells at 24 and 30 h ($P < 0.05$).

cytes over the 24-h study period (not shown). Staurosporine also had little effect on the basal rat interstitial collagenase mRNA level. However, 1,25-$(OH)_2D_3$ induction of collagenase mRNA was reduced in the presence of staurosporine (Fig. 5a). Thus, for 1,25-$(OH)_2D_3$ plus 500 μM staurosporine, mRNA levels were reduced by an average of 78 ± 11% ($P < 0.05$, n = 3 experiments). Staurosporine did not prevent the induction of collagenase by IL-1β.

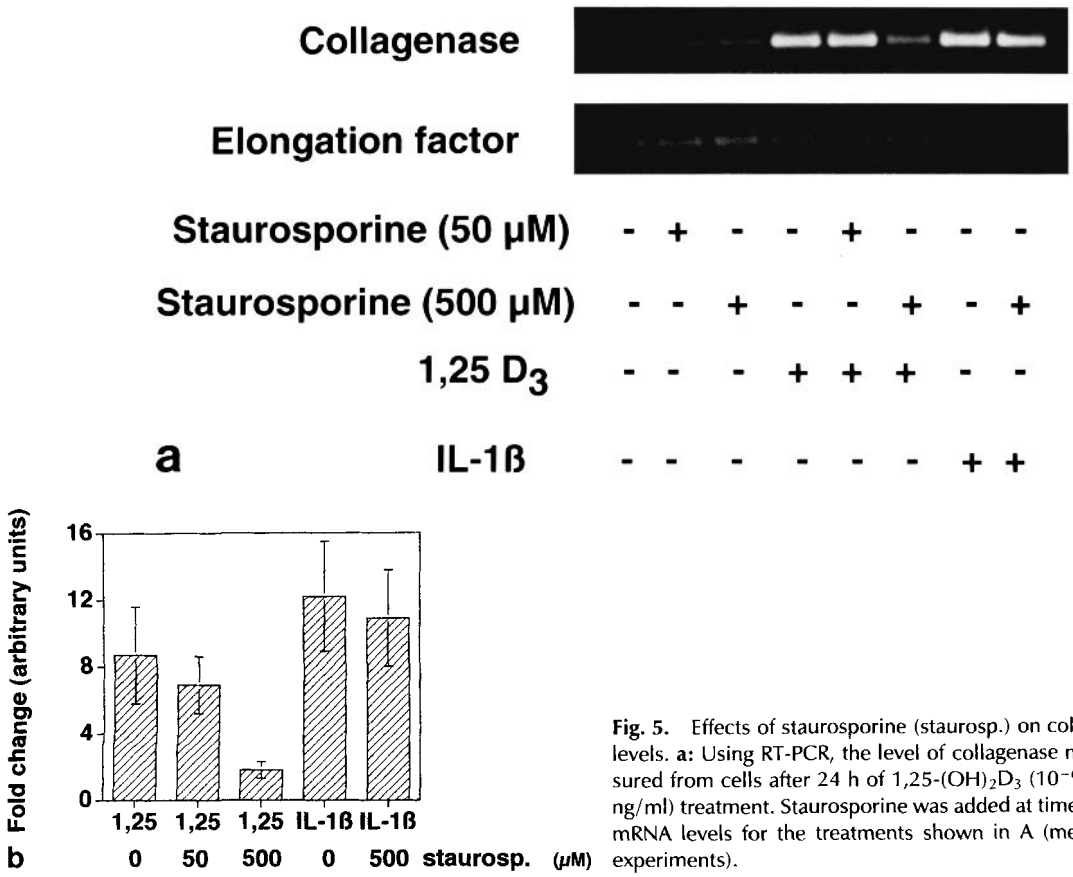


Fig. 5. Effects of staurosporine (staurosp.) on collagenase mRNA levels. **a:** Using RT-PCR, the level of collagenase mRNA was measured from cells after 24 h of 1,25-$(OH)_2D_3$ (10^{-6} M) or IL-1β (2 ng/ml) treatment. Staurosporine was added at time 0 h. **b:** Relative mRNA levels for the treatments shown in A (mean ± SE, n = 3 experiments).

PMA, a protein kinase C agonist, mimics some actions of IL-1β and was added as a comparison to the IL-1β induction. Less mRNA was induced with PMA than with IL-1β (Fig. 6a). Again, IL-1β increased rat interstitial collagenase mRNA relative abundance. In the presence of H-7, a different blocker of protein kinase C activity, PMA-induction of collagenase was inhibited and IL-1β induction was not. To determine whether protein synthesis was required for the PMA- and IL-1β-mediated regulation of collagenase mRNA, cells were exposed to cycloheximide, an inhibitor of peptide elongation. No cytotoxicity to cycloheximide was observed over the 24-h study period. When cycloheximide was present at the time of addition of IL-1β or PMA, final collagenase mRNA levels (arbitrary units with controls levels equal to 1.0) were 0.5 ± 0.1 and 0.9 ± 1.0, respectively (Fig. 6b).

Role of Protein Phosphatases and Tyrosine Kinase in Rat Interstitial Collagenase mRNA Levels

It is possible that the 1,25-$(OH)_2D_3$ and IL-1β effects on rat interstitial collagenase depend on reversible phosphorylation of proteins, independent of the cascade initiated through protein kinase C. Many of the most common serine/threonine protein phosphatases are widely expressed in tissues and okadaic acid is a specific inhibitor of these phosphatases [Haystead et al., 1989]. Cells at confluence were given okadaic acid (0–200 nM). Figure 7a shows that in the absence of okadaic acid there was a low-level expression of collagenase mRNA. As okadaic acid concentration increased, the relative abundance of collagenase mRNA was enhanced reaching a 10- ± 1.8-fold change at 200 nM (Fig. 7c, mean ± SE, n = 3 experiments). This okadaic acid effect was evident within 3 h (Fig. 7b). The effects of okadaic acid were blocked by cycloheximide, suggesting that regulation by okadaic acid requires active protein synthesis. Part of the change in collagenase mRNA is likely the result in gene activation. Nuclei were isolated from control cells. They were incubated with 200 nM okadaic acid for 4 h. The ^{32}P-labeled nuclear RNA was analyzed for collagenase and β-actin sequences at the time of nuclei isolation. Figure 7 shows that the collagenase RNA transcript

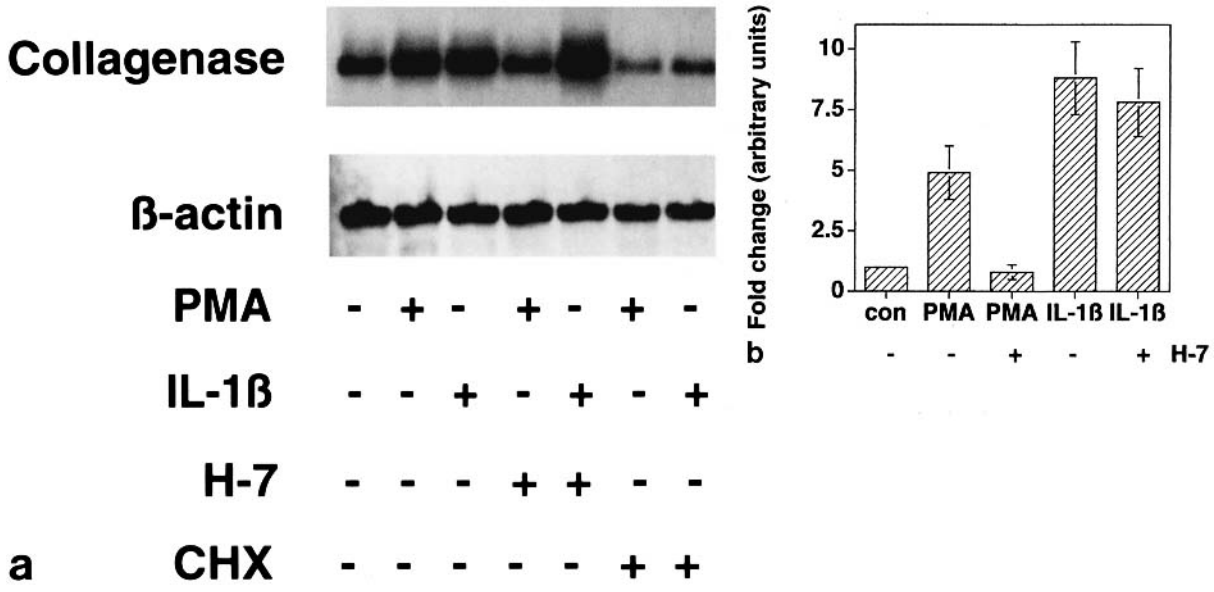


Fig. 6. Effects of PMA, IL-1β, H-7, and cycloheximide (CHX) on rat interstitial collagenase gene regulation. **a:** Northern hybridization analysis of the level of collagenase mRNA measured from cells after 24 h of PMA (25 μM) or IL-1β (2 ng/ml) treatment. H-7 (1 μM) or cycloheximide (10 μg/ml) was added at time 0 h. **b:** Relative mRNA levels for the treatments shown in **a** (mean ± SE, n = 3 experiments).

was 4.9 ± 1.1-fold (mean; n = 3) above basal transcript level, but there was no change in β-actin levels. Thus, okadaic regulation of collagenase includes a transcriptional mechanism.

Confluent chondrocytes were exposed to different concentrations of okadaic acid both with and without 1,25-$(OH)_2D_3$. A combination of the two effectors yielded a greater rat interstitial collagenase mRNA abundance than the separate addition of the two (Fig. 8). By 24 h, the combined level was 24-fold greater collagenase mRNA relative abundance versus the 10 of okadaic acid and the 8 of 1,25-$(OH)_2D_3$, respectively (Fig. 8). By contrast, we were unable to find any cooperative regulation of okadaic with IL-1β (not shown).

Throughout this study, chondrocytes were cultured on collagen-coated plates. Substrate-coated plates can elevate collagenase-1 mRNA in keratinocytes via a tyrosine kinase-mediated response [Sudbeck et al., 1994]. In this study, rat interstitial collagenase mRNA was always expressed at a low level. To ascertain whether the underlying mechanism of this elevated collagenase mRNA involved a tyrosine kinase, sodium orthovanadate (0–100 nM) was added to the medium. This effective phosphatase inhibitor elevates the number of phosphorylated proteins in other cell types. Here, the inclusion of sodium orthovanadate increased the rat interstitial collagenase mRNA levels by 6.2-fold (Fig. 9a). When genistein (5 or 50 μM), a specific tyrosine kinase inhibitor [Akiyama et al., 1987], was added to cells at the time of 1,25-$(OH)_2D_3$ treatment, there was a significant reduction in the relative collagenase mRNA abundance with a final 50% (±12%; $P < 0.05$) at 50 μM (Fig. 9b,c). Tyrosine kinase(s) therefore mediate some of the observed changes in collagenase mRNA in chondrocytes.

Transfection With 2.1-kb Rat Interstitial Collagenase Promoter

We further analyzed transcriptional regulation by performing transient transfection assays with a 2.1-kb 5′-flanking sequence of the rat interstitial collagenase gene. A reporter plasmid containing the gene encoding the chloroamphenicol acetyltransferase was transfected into proliferative zone chondrocytes. 1,25-$(OH)_2D_3$ treatment increased promoter activity 2.5-fold above the expression level of transfected cells not treated with agonists (Fig. 10). Likewise, IL-1β and phorbol ester increased promoter activity 2.8- and 3.0-fold, respectively. Thus, in this comparative transcriptional assay with both treated and nontreated cells, rat interstitial collagenase

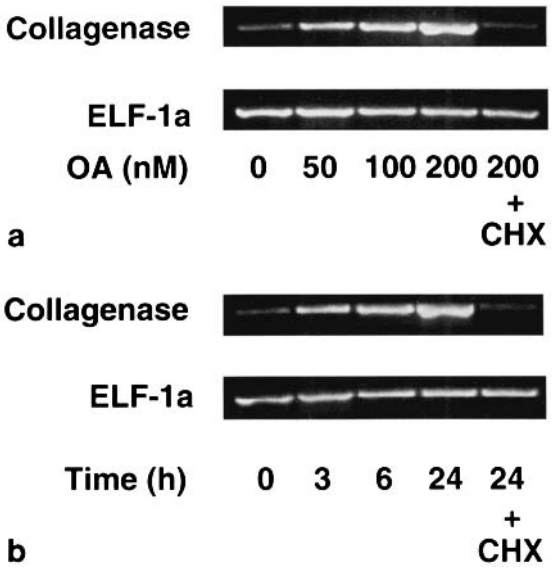
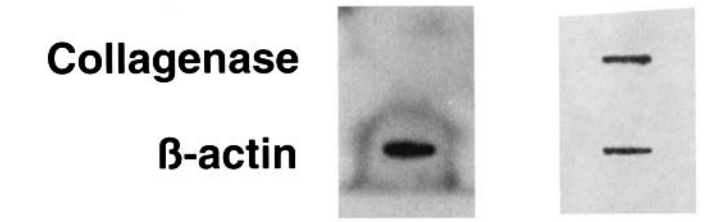


Fig. 7. Okadaic acid regulation of rat interstitial collagenase mRNA levels and on transcription. Dose (**a**) and time (**b**)-dependent induction of collagenase mRNA by okadaic acid. Okadaic acid regulation of collagenase at the transcriptional level (**c**). Cells were treated with okadaic acid before the nuclei were isolated (4 h). +, −, addition or absence of respective factor(s). Data are the average of three separate experiments (mean ± SE). +, −, addition or absence of the respective factor(s).



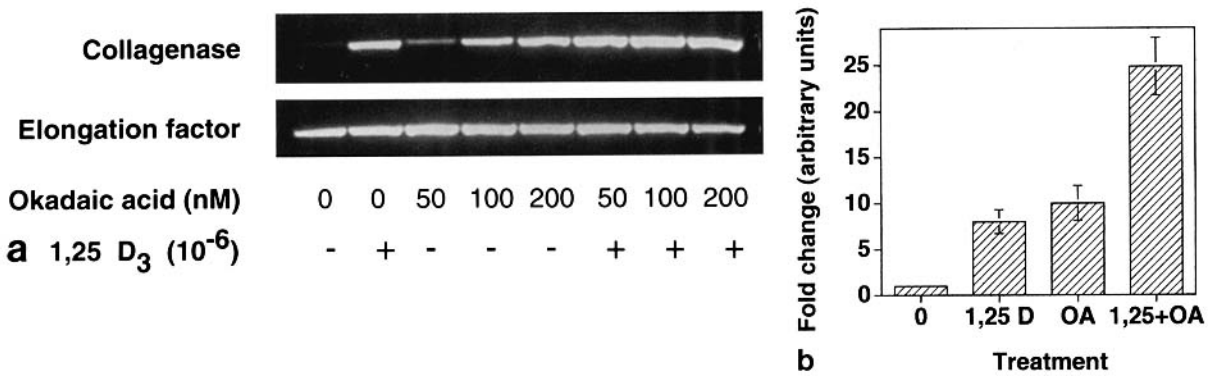


Fig. 8. Effects of combined treatment with okadaic acid (OA) and 1,25-$(OH)_2D_3$ (1,25-D) on the regulation of collagenase mRNA in rat chondrocytes. **a:** Cells were treated for 24 h with the indicated concentrations of okadaic acid and/or 1,25-$(OH)_2D_3$ and the total RNA (24 h). The amount of collagenase mRNA was measured with RT-PCR and DNA was detected by staining an agarose gel with SYBR Green I. **b:** Plot of relative collagenase mRNA level (mean ± SE). +, −, addition or absence of respective factor(s).

promoter activity is increased by 1,25-$(OH)_2D_3$, PMA, and IL-1β.

DISCUSSION

The present study shows that rat interstitial collagenase mRNA was expressed at higher levels in the hypertrophic versus the proliferative zone of rachitic cartilage, and that exogenous 1,25-$(OH)_2D_3$ stimulated this effect in the hypertrophic zone. These data support the enhancements of collagenolytic activity measured in rachitic rats using functional assays [Dean et al., 1985, 1989]. Evidence from the Northern hybridization, nuclear run-on, and mRNA half-life stud-

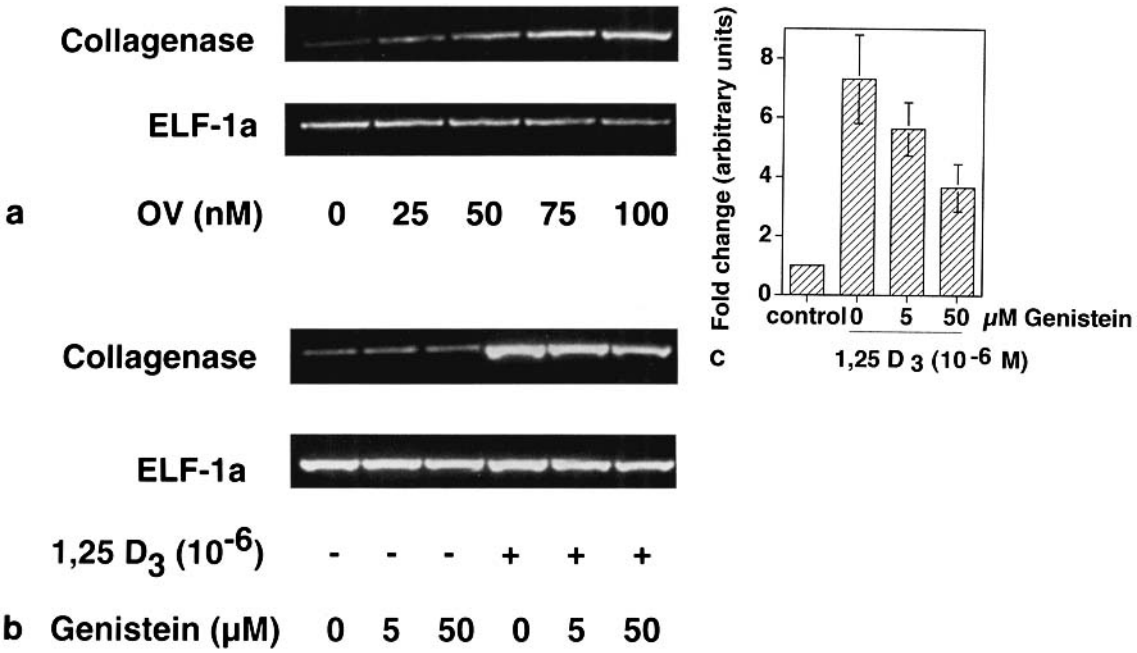


Fig. 9. Role of a tyrosine kinase in the regulation of collagenase mRNA. **a:** Orthovanadate regulation of collagenase mRNA levels. Cells were equilibrated for 2 days with 0.5% CS-FCS in MEM. Chondrocytes were exposed to increasing concentrations of sodium orthovanadate for 24 h at 37°C. The cells were harvested for total RNA and a RT-PCR method used to measure collagenase relative mRNA abundance. Elongation factor 1a mRNA levels was determined to verify equal amounts of RNA in the samples. The picture is a SYBR I stain of an agarose gel. **b:** Effect of genistein on the regulation by 1,25-$(OH)_2D_3$ on collagenase mRNA levels. The cells were then grown for 2 days in 0.5% CS-FCS. 1,25-$(OH)_2D_3$ added in the presence or absence of genistein for 24 h. Total RNA was isolated and a RT-PCR method used to measure collagenase relative mRNA abundance. **c:** Plot of relative collagenase mRNA level (mean ± SE).

ies in chondrocytes also show that 1,25-$(OH)_2D_3$ regulates this gene. As a steroid, it is typical for 1,25-$(OH)_2D_3$ to bind to a soluble receptor, which in turn binds directly to a unique sequence element to regulate gene expression [MacDonald et al., 1994]. Here the level of collagenase mRNA was dependent on protein synthesis and, at least partially, on that of a protein kinase C, since staurosporine, a protein kinase C inhibitor [Tamaoki, 1991], prevented 1,25-$(OH)_2D_3$ actions on the rat interstitial collagenase gene. These results strongly suggest that 1,25-$(OH)_2D_3$ is an important molecule in the generation of second-messenger signals [Slater et al., 1995]. Thus, these nongenomic processes have a bearing on collagenase gene expression in chondrocytes.

The importance of transcriptional regulation of the collagenase gene in chondrocytes was demonstrated by the enhanced levels of the collagenase transcript after 24 h of treatment with 1,25-$(OH)_2D_3$ (Fig. 10). These nuclear run-on assays measure the amount of RNA polymerase occupied on the collagenase gene using a crude nuclear fraction. The introduction of a reporter plasmid into the cells with the rat interstitial collagenase promoter also confirms this type of regulation as 1,25-$(OH)_2D_3$ increased chloramphenicol acetyltransferase activity 2.5-fold over the basal level (Fig. 10). However, additional molecular events are important in collagenase gene regulation. The half-life study involving actinomycin D and 1,25-$(OH)_2D_3$ showed increased stability of collagenase mRNA and, conceivably, post-transcriptional regulation of this gene.

Tropoelastin expression in fetal bovine chondrocytes derived from the ear is also regulated post-transcriptionally by 1,25-$(OH)_2D_3$ [Pierce et al., 1992]. However, the tropoelastin regulation occurs via an increase in the mRNA turnover by 1,25-$(OH)_2D_3$. Since 1,25-$(OH)_2D_3$ is the active metabolite in regulating hypertrophy [Kato et al., 1990] and vascular invasion into rachitic cartilage [Atkin et al., 1985], the data here suggest that collagenase may serve a critical role in the remodeling of the growth cartilage matrix during long bone growth.

At least part of the IL-1β regulation rat interstitial collagenase was through a separate pro-

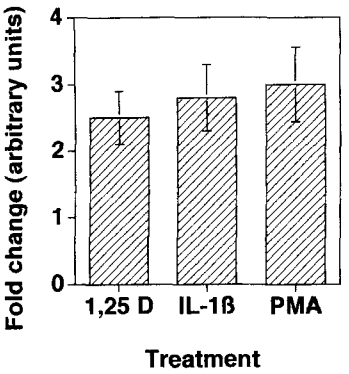


Fig. 10. Regulation of the activity of a CAT expression vector in response to 1,25-$(OH)_2D_3$, IL-1β, and PMA. Chondrocytes were transfected with 10 μg of plasmid DNA and left overnight in lipofectamine reagent. Next day, the cells were washed and incubated in low serum medium for 24 h, at which time agonist additions were made. +, −, addition or absence of respective factor(s). Data (mean ± SE) presented with the control (untreated) cells having an arbitrary activity of 1.0.

tein kinase pathway from that of 1,25-$(OH)_2D_3$, as staurosporine did not block IL-1β elevation of collagenase mRNA (Fig. 5). Cells treated with H-7, a protein kinase C inhibitor [Hidaka et al., 1984], blocked only the PMA increase in collagenase mRNA levels, and not that of IL-1β (Fig. 6). Differential regulation of interstitial collagenase-1 by phorbol esters and IL-1β has also been observed in human fibroblasts [Yang and Kurkinen, 1994]. In human synoviocytes, phorbol ester induction of prostaglandin production was blocked by staurosporine but increased in the presence of IL-1β [Taylor et al., 1990]. However, in keratinocytes, staurosporine alone induced interstitial collagenase [Lyons et al., 1993]. Thus, the phorbol ester and IL-1β effects, as well as the associated protein kinase C activities, appear to be mediated in a cell type and gene-specific manner.

Recent research on IL-1β regulation of collagenase-1 emphasizes the greater importance of post-transcriptional processes in rabbit synovial fibroblasts [Vincent et al., 1994]. The present data are consistent with transcriptional and post-transcriptional regulation of rat interstitial collagenase. Using half-life studies and chemical inhibition of new RNA synthesis, IL-1β significantly increased rat interstitial collagenase mRNA after 30 h (Fig. 4). However, the nuclear run-on data showed a six-fold change in the rate of transcription with IL-1β (Fig. 2). With Northern hybridization analysis, the ultimate change in relative collagenase mRNA abundance was 13-fold (Fig. 2). It is difficult to compare the relative contributions of transcriptional and post-transcriptional mechanisms, as one of these methods uses isolated nuclei, whereas the others measure relative cytoplasmic mRNA levels in intact cells in the presence of actinomycin D. Thus, future studies must address which means of regulation is more important in cartilage, and for determining the different mRNA levels in proliferative and hypertrophic cells.

In this study, we describe fluctuations in the level and transcription of rat interstitial collagenase gene as a function of okadaic acid, genistein, and sodium orthovanadate (Figs. 7–9). Others have shown that activation of protein kinase A induces the rat interstitial collagenase gene in a tumor cell line [Scott et al., 1992]. The importance of including okadaic acid is that its mode of action appears independent of that for protein kinase C and protein kinase A, as it is also an inhibitor of protein phosphatases PP1 and PP2 [Haystead et al., 1989], yet it increases phosphorylation of numerous proteins [Guy et al., 1992]. Here, okadaic acid was a potent activator of collagenase gene expression (Fig. 7), and this effect was further enhanced with 1,25-$(OH)_2D_3$ (Fig. 8). However, other types of phosphorylation mechanisms have a role in determining final collagenase mRNA levels. The data for genistein and orthovanadate imply that one or more tyrosine kinase associated events participate in regulation of rat interstitial collagenase mRNA abundance.

Thus, chondrocyte-specific rat interstitial collagenase expression appears to be coordinated through distinct biochemical pathways indicating more than one type of signaling event involving second-messenger-regulated protein kinases and phosphatases. Typically a tyrosine kinase mechanism involves protein phosphorylation events dominated through associations of adapter proteins that contain SH2 and SH3 domains. A downstream consequence of these phosphorylation-induced associations is elevated mitogen-activated protein (MAP) kinase activities, which can directly phosphorylate and activate numerous transcription factors [Marshall, 1995]. It is unlikely that, as a steroid, a 1,25-$(OH)_2D_3$ nongenomic mechanism would involve such adapters or MAP kinase activities. Protein kinase C activity is activated by Ca^{2+}/phospholipid-dependent mechanisms [Sanz et al., 1994], which might explain the 1,25-$(OH)_2D_3$

nongenomic response, as Ca^{2+}/phospholipid metabolism is elevated in several cell types by steroids [Doolan and Harvey, 1996; Khoury et al., 1995; Schwartz and Boyan, 1988]. It is possible that IL-1β operates differently from that of a typical protein kinase C cascade pathway in the regulation interstitial collagenase. For instance, a number of serine/threonine phosphorylation events occur in cells treated with IL-1 [Bird and Saklatvala, 1990; Guy et al., 1991]. At least two mitogen-activated protein (MAP) kinases have been characterized in fibroblasts activated with IL-1 [Freshney et al., 1994]. Although several growth factors and oncogenes control MAP kinase activities, not all operate through typical MAP kinase pathways or are restricted by cell-specific mechanisms. For instance, IL-1β can activate a stress-activated kinase through a mechanism independent from those that activate typical MAP kinase activities [Bird et al., 1994]. We have yet to identify a steroid response element in the proximal portion of the rat interstitial collagenase gene that responds to vitamin D_3 (not shown); here we emphasize the role that phospoproteins have in mediating 1,25-$(OH)_2D_3$ regulation of collagenase. A chondrocyte may have a mosaic of options that can be activated by perturbation of protein phosphorylation resulting in collagenase mRNA elevation. The particular pathway induced by 1,25-$(OH)_2D_3$ or IL-1β may initially be separate ones and may have eventual separate transcription factor targets or, as for fos/jun activation by protein kinases in other cell types, involve a cross-talk mechanism targeting a common transcriptional factor [Franklin and Kraft, 1995; Treisman, 1995]. However, as the extent of activities for IL-1β or 1,25-$(OH)_2D_3$ phosphorylation events in these chondrocytes has yet to be determined, we suspect that the IL-1β versus 1,25-$(OH)_2D_3$-specific differences in activation of key kinases will parallel rat interstitial collagenase mRNA increases.

The requirement for new protein synthesis in experiments with okadaic acid suggests that okadaic acid acts indirectly on rat interstitial collagenase gene transcription by inducing the synthesis of one or more proteins (Fig. 7). Okadaic acid is known to increase c-*jun* gene expression, which is part of the AP-1 transcriptional complex [Guy et al., 1992]. Regulation of a number of metalloproteinases occurs, at least in part, via an AP-1 mechanism [Buttice and Kurkinen, 1994]. Promoter activity is regulated by interactions between competing and synergistic *trans*-acting factors binding at multiple DNA elements [Miner and Yamamoto, 1991]. Putatively, the role of multiple elements in the regulation of collagenase might explain how both 1,25-$(OH)_2D_3$ and okadaic acid cooperatively increased collagenase mRNA above that of either factor alone (Fig. 8).

In summary, this study demonstrates that both 1,25-$(OH)_2D_3$ and IL-1β modulate rat interstitial collagenase gene expression in cartilage. Clearly, transcriptional mechanisms are involved, but post-transcriptional regulation seems to occur as well. One hypothesis to link hormone and biomechanical abnormalities that result in long-term pathological disorders is the induction of transcriptional regulatory proteins. Collagenase uniquely cleaves collagen at a neutral pH. Thus, stringent control of collagenase expression via cell-type-specific transcriptional activators is likely to be a crucial step in maintaining tissue function. Improper induction of this gene will result in extracellular matrix loss, compromise of cartilage function, and abnormal growth. More precise analysis involving mutational alterations in the 5′ regulatory DNA region are required to better define which sequences are used by these modulators as well determine whether differential expression of collagenase between proliferative and hypertrophic chondrocytes can be defined through activation at a DNA element. Eventually, this will establish the role of such elements in regulating collagenase gene expression in cartilage. Thus, fundamental understanding of the molecular mechanisms underlying collagenase gene regulation in this rachitic growth plate model will be applicable to other pathological situations.

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