Journal of Cellular Biochemistry 67:176–183 (1997)

Insulin-Like Growth Factor I Inhibits the Transcription of Collagenase 3 in Osteoblast Cultures

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Abstract Insulin-like growth factor (IGF) I is an autocrine regulator of bone remodeling which inhibits bone collagen degradation and interstitial collagenase 3 mRNA levels. The mechanism of this inhibitory effect on collagenase 3 expression is not known. We tested the effects of IGF I on collagenase 3 gene expression in cultures of osteoblast-enriched cells from 22 day fetal rat calvariae (Ob cells) to determine whether transcriptional or posttranscriptional mechanisms were involved in the regulation of the collagenase 3 gene. IGF I at 10–100 nM caused a dose-dependent decrease in collagenase mRNA and protein levels. IGF I did not modify the half-life of collagenase 3 mRNA in transcriptionally arrested Ob cells, whereas it decreased the levels of interstitial collagenase 3 heterogeneous nuclear RNA. In addition, IGF I decreased the rates of transcription of the collagenase gene and the activity of a 2.1 kilobase collagenase 3 promoter construct transiently transfected into Ob cells. In conclusion, IGF I decreases the expression of collagenase 3 mRNA by transcriptional mechanisms. J. Cell. Biochem. 67:176–183, 1997.

Key words: bone matrix; collagen; growth factors; interstitial collagenase; matrix metalloproteinases

Insulin-like growth factor (IGF) I is an autocrine regulator of osteoblastic function [Canalis et al., 1988a; Delany et al., 1994]. IGF I is well known for its growth-promoting properties in bone, but it also decreases bone collagen degradation [Hock et al., 1988; McCarthy et al., 1989]. This effect, critical in the maintenance of bone matrix, probably involves a decrease in the synthesis of interstitial collagenase by the osteoblast [Canalis et al., 1995]. Recently, IGF I was shown to decrease collagenase 3 transcript levels in osteoblast cultures without affecting the expression of tissue inhibitors of matrix metalloproteinases 1, 2, or 3 [Canalis et al., 1995]. Furthermore, IGF I and possibly IGF II play an autocrine role in the downregulation of collagenase 3 by the osteoblast [Delany et al., 1996].

Matrix metalloproteinases (MMPs) and their inhibitors are considered active participants in the degradation of osteoid. They are synthesized by osteoblasts and osteoclasts, although their expression varies with the cell line and culture conditions used [Delaisse et al., 1993; Matrisian and Hogan, 1990; Partridge et al., 1987; Rifas et al., 1994; Varghese et al., 1995]. Collagenases are MMPs that degrade collagen fibrils at neutral pH. There are three collagenases: collagenase 1, expressed by chondrocytes and stimulated human osteoblasts and fibroblasts; collagenase 2, expressed by neutrophils; and collagenase 3, expressed by rat osteoblasts, chondrocytes, and carcinoma of the breast cells [Freije et al., 1994; Mitchell et al., 1996; Partridge et al., 1987; Quinn et al., 1990; Rifas et al., 1994]. Collagenases 1, 2, and 3 degrade type I collagen with comparable collagenolytic efficiency [Knauper et al., 1996]. Since type I collagen is the major collagen present in bone, changes in interstitial collagenase expression are central to the regulation of skeletal collagen [McCarthy et al., 1988]. Although IGF I was shown to decrease collagenase 3 expression by the osteoblast, the mechanisms involved are unknown and could include transcriptional and posttranscriptional levels of regulation. Investigations on the mechanism of IGF I action on osteoblast gene expression are

Contract grant sponsor: National Institute of Diabetes and Digestive and Kidney Diseases; Contract grant number: DK 42424.

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Received 1 May 1997; accepted 18 June 1997

necessary to understand its role in bone remodeling.

In the present studies we examined the levels of regulation of IGF I on the expression of rat interstitial collagenase 3 in osteoblast-enriched cultures from 22 day fetal rat calvariae (Ob cells).

MATERIALS AND METHODS

Culture Technique

The culture method used was described in detail previously [McCarthy et al., 1988]. Parietal bones were obtained from 22-day-old fetal rats immediately after the mothers were sacrificed by blunt trauma to the nuchal area. This project was approved by the Institutional Animal Care and Use Committee of Saint Francis Hospital and Medical Center. Cells were obtained by five sequential digestions of the parietal bone using bacterial collagenase (CLS II; Worthington Biochemical, Freehold, NJ). Cell populations harvested from the third to the fifth digestions were cultured as a pool and were previously shown to have osteoblastic characteristics [McCarthy et al., 1988]. Ob cells were plated at a density of 8,000–12,000 cells/cm^2 and cultured in a humidified 5% CO_2 incubator at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (both from Summit Biotechnology, Fort Collins, CO). Cells were grown to confluence (about 50,000 cells/cm^2), except for transient transfection experiments, which were carried out in subconfluent cultures. All experiments were performed in primary cultures of Ob cells, except for the nuclear run-on assays, for which subconfluent cultures were treated with trypsin, harvested, subcultured, and grown to confluence. At confluence, the cells were rinsed and changed to serum-free conditions for 20–24 h, when they were again rinsed with serum-free medium, and exposed to test or control medium in the absence of serum for 2–24 h as indicated in the text and figure legends. IGF I (kindly provided by Eli Lilly Research Laboratories, Indianapolis, IN, and Genentech, South San Francisco, CA) was dissolved in 0.1 M acetic acid and diluted 1:1,000 in DMEM. 5,6 dichlorobenzimidazole riboside (DRB) (Sigma Chemical Co., St. Louis, MO) was dissolved in absolute ethanol and diluted in DMEM 1:200; control and test cultures contained an equal amount of alcohol. At the end of the incubation, the medium was harvested and stored at −80°C in the presence of 20 mM Tris buffer prior to Western blot analysis, the cell layer was extracted for RNA analysis and stored at −80°C, or the nuclei were extracted by Dounce homogenization.

Western Immunoblot Analysis

Medium samples were fractionated by polyacrylamide gel electrophoresis using denaturing nonreducing conditions and transferred onto Immobilon P membranes (Millipore, Bedford, MA). After blocking with 2% bovine serum albumin, the membranes were exposed to a 1:1,000 dilution of rabbit antiserum raised against rat collagenase 3 (kindly provided by J. Jeffrey, Albany, NY) [Jeffrey et al., 1990] followed by the addition of goat antirabbit IgG conjugated to horseradish peroxidase. The blots were washed and developed with a horseradish peroxidase chemiluminescent detection reagent (DuPont, Wilmington, DE), visualized by autoradiography on DuPont Reflection film employing Reflection intensifying screens, and analyzed by densitometry. Data shown are representative of four cultures.

Northern Blot Analysis

Total cellular RNA was isolated with guanidine thiocyanate, at acid pH, followed by a phenol-chloroform (Sigma) extraction [Chomczynski and Sacchi, 1987]. RNA was precipitated with isopropanol, resuspended, and reprecipitated with ethanol. The RNA recovered was quantitated by spectrophotometry, and equal amounts of RNA from control or test samples were loaded on a formaldehyde agarose gel following denaturation. The gel was stained with ethidium bromide to visualize ribosomal RNA, documenting equal RNA loading of the various experimental samples. The RNA was then blotted onto GeneScreen Plus charged nylon (DuPont). Restriction fragments containing a 2.6 kilobase (kb) rat collagenase 3 cDNA (kindly provided by C. Quinn, St. Louis, MO), and an 800 base pair (bp) rat glyceraldehyde-3 phosphate dehydrogenase (GAPD) cDNA (kindly provided by R. Wu, Ithaca, NY) were labeled with [α-^{32}P] deoxycytidine triphosphate (dCTP) and [α-^{32}P] deoxyadenosine triphosphate (dATP) (specific activity of 3,000 Ci/mmol; DuPont) using the random hexanucleotide primed second strand synthesis method [Quinn et al., 1990; Tso et al., 1985; Feinberg and Vogelstein, 1984]. Hybridizations were car-

ried out at 42°C for 16–72 h. Posthybridization washes were performed at 65°C in 1× saline sodium citrate (SSC). The bound radioactive material was visualized by autoradiography on Kodak X-AR5 film employing intensifying screens. Relative hybridization levels were determined by densitometry. Northern analyses shown are representative of three or more cultures.

Reverse Transcription/Polymerase Chain Reaction (RT/PCR)

Collagenase heterogeneous nuclear RNA (hnRNA) was analyzed by RT/PCR using a sense primer 5′-CATTCAGCTATTCTGGCCAC-3′, spanning nucleotides 27–46 of exon 1 of the rat collagenase gene, and an antisense primer, 5′-AAAAGACCAGAACAACCAGC-3′, spanning nucleotides 61–80 of intron 1, to yield a 186 bp product [Buttice and Kurkinen, 1993; Delany et al., 1995; Quinn et al., 1990; Rajakumar and Quinn, 1996]. RNA samples were extracted as described for Northern analysis, treated with amplification grade DNase I according to the manufacturer's instructions (Life Technologies, Grand Island, NY) to remove potentially contaminating DNA. RNA (1 µg) was copied into cDNA using Moloney murine leukemia virus reverse transcriptase (Life Technologies) and the antisense primer according to the manufacturer's instructions, except that Taq polymerase buffer was used instead of reverse transcriptase buffer [Delany et al., 1995; Buttice and Kurkinen, 1993]. A DNA standard was synthesized by PCR amplification of pGL2-Basic plasmid DNA (Promega Corporation, Madison, WI) using the rat collagenase hnRNA primer set and low stringency annealing conditions, as described by Forstr [1994]. The newly synthesized collagenase 3 cDNA and 0.05 attomole of DNA standard were amplified by PCR using 24 cycles of 94°C for 1 min, 59°C for 1 min, and 72°C for 1 min in the presence of Taq polymerase (Life Technologies), 0.15 µmoles of sense and antisense interstitial collagenase primers, and 5 µCi [α-^{32}P]dCTP. PCR products were resolved on 6 or 8% polyacrylamide urea gels (Gel-Mix 6 or 8; Life Technologies) and visualized by autoradiography. The amplification protocol yielded products which were within the linear range for both the collagenase hnRNA and the standard. Data on hnRNA are representative of three cultures.

Nuclear Run-On Assay

To examine changes in the rate of transcription, we isolated nuclei by Dounce homogenization in a Tris buffer containing 0.5% Nonidet P-40 [Ausubel et al., 1993]. Nascent transcripts were labeled by incubation of nuclei in a reaction buffer containing 500 µM each of dATP, dCTP, and guanidine triphosphate, 150 units of RNAsin (Promega), and 250 µCi of [α-^{32}P] uridine triphosphate (UTP) (3,000 Ci/mmol; DuPont) [Ausubel et al., 1993]. Nuclear RNA was isolated by treatment with DNase I and proteinase K, followed by phenol-chloroform extraction and ethanol precipitation. Linearized plasmid DNA containing about 1 µg of cDNA was immobilized onto GeneScreen Plus by slot blotting according to the manufacturer's directions. The plasmid vector pGL2-Basic was used as a control for nonspecific hybridization, and a rat GAPD cDNA was used to estimate uniformity of counts applied to the membrane. Equal counts/minute of [^{32}P] RNA from each sample were hybridized to cDNAs using the conditions described for Northern analysis and were visualized by autoradiography. Cytoplasmic RNA was isolated from the same cells by treatment with proteinase K, followed by phenol-chloroform extraction, and analyzed for collagenase 3 mRNA by Northern analysis. The nuclear run-on assay was performed twice.

Transient Transfections and Reporter Gene Assays

To examine changes in the activity of the collagenase 3 promoter, Ob cells were cultured to approximately 80% confluence and transiently transfected with a chimeric construct of the rat collagenase 3 gene cloned into the luciferase expression vector pGL2-Basic [Delany et al., 1995]. A Not I/Xho I rat genomic DNA fragment containing a 2.1 kb fragment of the rat collagenase 3 promoter (kindly provided by J. Jeffrey) was transiently transfected into Ob cells by calcium phosphate/DNA coprecipitation, followed by glycerol shock as described [Chen and Okayama, 1987; Delany et al., 1995; Pash and Canalis, 1996]. The cytomegalovirus promoter driven β-galactosidase gene (pCMVβ-Gal; Clontech, Palo Alto, CA) was cotransfected to control for transfection efficiency. Cells were washed, serum-deprived, and exposed to control or test medium for up to 24 h. Cells were lysed in 1× Reporter Lysis Buffer (Promega), and luciferase activity was measured by inject-

ing luciferase assay reagent (Promega) into an aliquot of the cell lysate. Photons emitted were counted using an Optocomp luminometer (MGM Instruments, Hamden, CT) according to the manufacturer's instructions. β-galactosidase activity was measured by incubating cell lysates with the chemiluminescent substrate for β-galactosidase 3-(4-methoxyspirol[1,2-dioxetane-3,2′-tricyclo[3.3.1.1.^{3,7}]decan]- y)phenyl β-D-galactopyranoside (Galacton; Tropix, Bedford, MA). Luciferase activity was normalized to β-galactosidase activity.

Statistical Analysis

Data for mRNA decay in transcriptionally arrested cells are expressed as means ± SEM, and slopes were analyzed by the method of Sokal and Rohlf [1981]. Data for transfection experiments are expressed as means ± SEM, and statistical differences were determined by analysis of variance and post hoc examination by Dunnet's using a Crunch Statistical Package (Crunch Software Corp., Oakland, CA) [Wall, 1986].

RESULTS

Confirming prior studies, IGF I at 1–100 nM for 6 h decreased collagenase 3 steady-state transcripts in Ob cells by 50–70% (Fig. 1) [Canalis et al., 1995]. The concentration of interstitial collagenase in the culture medium of Ob cells is low and frequently below the level of detection by immunoassay [Canalis et al., 1995]. However, Western blot analysis of Ob culture medium, using an antirat collagenase 3 antibody, revealed an immunoreactive protein band that comigrated with a purified rat uterine procollagenase and was inhibited by treatment with IGF I at 100 nM for 6 or 24 h by approximately 50% (Fig. 2). The antibody detected an additional protein, of lower molecular weight, which has not been characterized and was not regulated by IGF I. This protein may represent active collagenase or a related metalloproteinase [Knauper et al., 1996]. To examine whether or not the effects of IGF I on interstitial collagenase 3 mRNA levels were due to changes in transcript stability, we exposed confluent cultures of Ob cells to control or IGF I containing medium for 60 min and then treated them with the RNA polymerase II inhibitor DRB in the absence or presence of IGF I at 100 nM for 2, 4, and 8 h [Zandomeni et al., 1983]. Northern blot analysis and densitometry showed that the half-life of collagenase 3 mRNA in transcriptionally arrested Ob cells was about 4 h, and it was not changed by treatment with IGF I (Fig. 3).

To determine whether IGF I modified the transcription of the interstitial collagenase 3 gene, we examined the effects of IGF I on collagenase hnRNA levels and rates of transcription. Treatment with IGF I at 100 nM for 2 and 6 h inhibited the levels of collagenase hnRNA by 50–60% as determined by RT-PCR (Fig. 4).

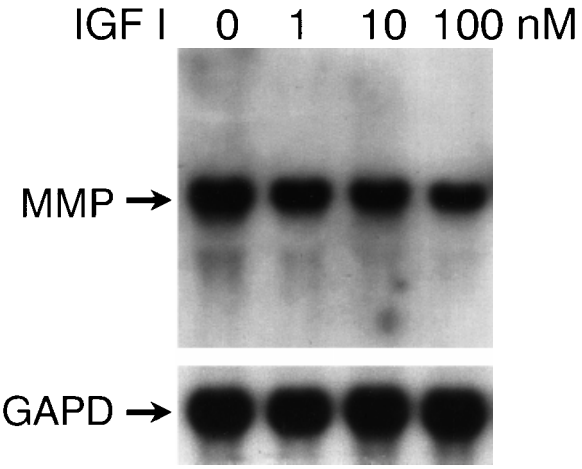

Fig. 1. Effect of insulin-like growth factor (IGF) I at 1–100 nM on collagenase 3 mRNA expression in confluent cultures of Ob cells treated for 6 h. Total RNA from control (0) or IGF-treated cultures (in nanomolar concentrations) was subjected to Northern blot analysis and hybridized with a ^{32}P-labeled collagenase 3 cDNA. The blot was stripped and hybridized with a ^{32}P-labeled rat GAPD cDNA to demonstrate equal RNA loading of the gel. Collagenase mRNA was visualized by autoradiography and is shown in the upper panel, while GAPD mRNA is shown below.

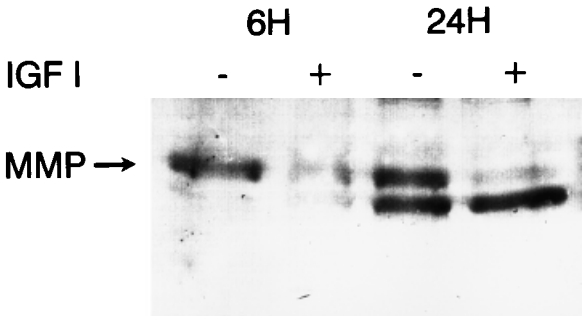

Fig. 2. Effect of insulin-like growth factor (IGF) I at 100 nM on collagenase 3 levels in the medium of confluent cultures of Ob cells treated for 6 or 24 h. Medium from control (−) or IGF I–treated (+) cultures was subjected to Western blot analysis, and collagenase was detected using an antirat collagenase antibody and a chemiluminescence detection system. The immunoreactive band (MMP) with the same mobility as purified rat uterine procollagenase is indicated by the arrow.

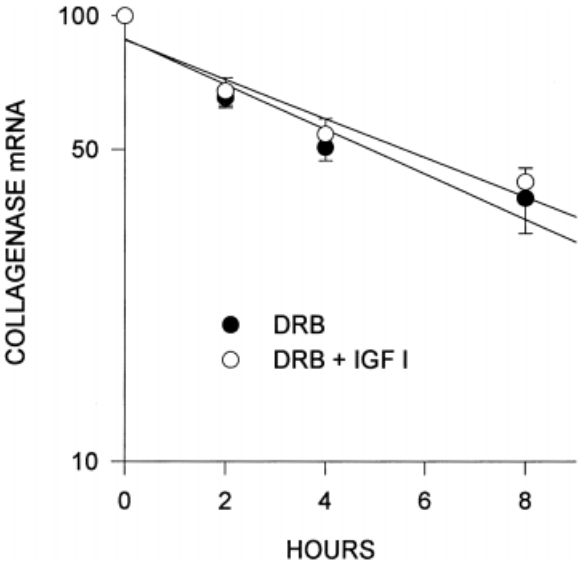

Fig. 3. Effect of insulin-like growth factor (IGF) I at 100 nM on collagenase 3 mRNA decay in transcriptionally arrested Ob cells. Confluent cultures of Ob cells were treated with IGF I 1 h before and 2, 4, or 8 h after the addition of 5,6-dichlorobenzimidazole riboside (DRB). RNA was subjected to Northern blot analysis and hybridized with an [α-^{32}P]-labeled collagenase 3 cDNA. Collagenase mRNA was visualized by autoradiography and quantitated by densitometry. Values are means ± SEM for three cultures. The slopes of the two lines are not statistically different.

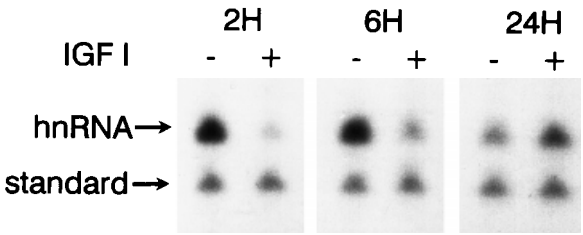

Fig. 4. Effect of insulin-like growth factor (IGF) I at 100 nM on collagenase 3 hnRNA levels in confluent cultures of Ob cells treated for 2, 6, or 24 h. Total RNA from control (−) and IGF I–treated (+) cultures was reverse-transcribed and amplified by polymerase chain reaction (PCR) in the presence of 5 µCi [α-^{32}P]dCTP using collagenase 3 exon 1– and intron 1–specific primers to generate a 186 base pair product. A DNA internal standard was coamplified to assess PCR efficiency. PCR products were visualized by autoradiography.

In addition, IGF I downregulated collagenase gene transcription by 50–70%, as determined by nuclear run-on assays performed on nuclei from Ob cells treated for 2 or 6 h (Fig. 5). To confirm transcriptional downregulation of the collagenase 3 gene, we transiently transfected Ob cells with a construct containing a 2.1 kb fragment of the rat collagenase 3 promoter driving the expression of a luciferase reporter gene.

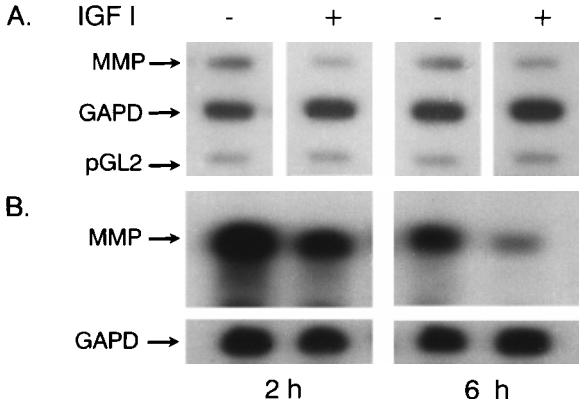

Fig. 5. Effect of insulin-like growth factor (IGF) I on collagenase 3 transcription rates in confluent cultures of Ob cells treated for 2 or 6 h. **A:** Nascent transcripts from control (−) and IGF I–treated (+) cultures were labeled in vitro with [^{32}P]-UTP, and the labeled RNA was hybridized to immobilized cDNA for interstitial collagenase 3. Hybridization to GAPD cDNA demonstrates uniform loading of ^{32}P-labeled RNA. pGL2-Basic vector DNA was used as a control for nonspecific hybridization. **B:** Cytoplasmic RNA from control (−) and IGF I–treated (+) cultures was subjected to Northern blot analysis and hybridized with a ^{32}P-labeled collagenase 3 cDNA. The blot was stripped and hybridized with a ^{32}P-labeled rat GAPD cDNA to demonstrate equal RNA loading of the gel.

Treatment of transfected cells with IGF I at 100 nM for 6 h (not shown) or 16 h (Fig. 6) inhibited luciferase activity by 20–50%. The inhibitory effect was dose-dependent and observed at IGF I doses as low as 10 nM (Fig. 6).

DISCUSSION

Recent studies demonstrated that IGF I inhibits collagen degradation and collagenase 3 expression in skeletal tissue [McCarthy et al., 1989; Canalis et al., 1995]. The present investigation was undertaken to determine possible mechanisms involved in the effect of IGF I on collagenase 3 expression. IGF I decreased collagenase steady-state transcripts and protease levels in osteoblast cultures. IGF I did not modify the half-life of interstitial collagenase mRNA in transcriptionally arrested Ob cells and reduced the levels of collagenase hnRNA and the rate of collagenase transcription, indicating that it acts by transcriptional mechanisms. Although changes in hnRNA frequently match changes in the rate of transcription, hnRNA levels may also reflect alterations in RNA processing [Buttice and Kurkinen, 1993]. IGF I also inhibited the activity of a 2.1 kb rat collagenase 3 promoter construct, suggesting that IGF I–responsive regions may be within

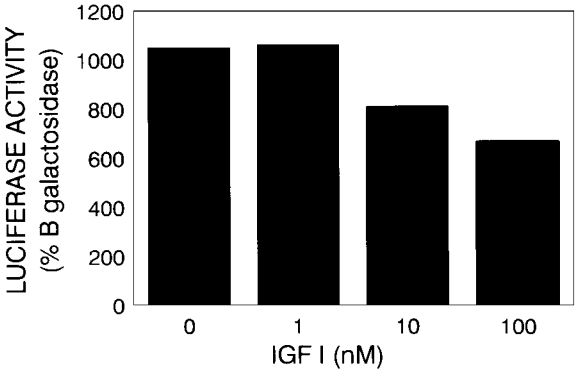

Fig. 6. Effect of insulin-like growth factor (IGF) I on collagenase 3 promoter activity in transiently transfected Ob cells. A 2.1 kb fragment of the collagenase 3 promoter was used to drive the expression of the luciferase gene in the promoterless reporter plasmid pGL2-Basic. Ob cells were transiently cotransfected with the collagenase promoter–luciferase plasmid and a plasmid containing the cytomegalovirus promoter driving the expression of the β-galactosidase gene. Transfected cells were treated for 16 h with control medium (0) or with medium containing IGF I at 1–100 nM. Luciferase activity was normalized to β-galactosidase activity to control for differences in transfection efficiency. The results are representative of six cultures. Bars are means of corrected luciferase activity; SEMs were 1–6% of the mean. Values for IGF I at 10 and 100 nM are significantly different from control, $P < 0.05$.

this promoter fragment. There is limited information about specific elements responsible for the effects of IGF I on various genes. IGF I upregulates the elastin gene through an Sp1 site, but the elements involved in the collagenase 3 response have not been defined [Jensen et al., 1995]. Collagenase 3 degrades type I collagen at neutral pH, and type I collagen is the predominant collagen synthesized by bone; therefore, it is probable that the transcriptional downregulation of collagenase 3 by IGF I is mechanistically important in the inhibitory effects of IGF I on bone collagen degradation.

IGF I has modest mitogenic activity and increases type I collagen expression and decreases collagenase synthesis in Ob cells [Canalis et al., 1995; McCarthy et al., 1989]. This is in contrast to the actions of growth factors with potent mitogenic activity for bone cells, since they do not stimulate osteoblastic collagen synthesis and enhance collagenase expression [Hock and Canalis, 1994; Varghese et al., 1995, 1996). Platelet-derived growth factor (PDGF) and fibroblast growth factor increase collagenase expression in Ob cells [Varghese et al., 1995, 1996]. PDGF acts by transcriptional and posttranscriptional mechanisms, and the gene elements involved are currently under study. While PDGF increases bone resorption, osteoclast number, and collagenase expression, these functions are not always correlated [Hock and Canalis, 1994; Varghese et al., 1996]. For instance, IGF I inhibits collagenase expression but enhances the recruitment of osteoclasts, an effect that may be due to an increase in interleukin-6 expression and that may result in increased bone resorption [Mochizuki et al., 1992; Slootweg et al., 1992]. More similar are the effects of IGF I and TGF β1 on skeletal tissue. TGF β1, like IGF I, stimulates bone matrix apposition rates and inhibits collagenase 3 expression by the osteoblast [Hock et al., 1990; Rydziel et al., 1997]. Similarly, bone morphogenetic protein-2 (BMP-2), a member of the TGF β family of peptides, inhibits collagenase 3 synthesis in osteoblasts [Varghese and Canalis, 1997]. However, TGF β1 acts on collagenase 3 expression both at the transcriptional and posttranscriptional level, whereas BMP-2 and IGF I act only transcriptionally. This would suggest that various growth factors regulate collagenase 3 expression in osteoblasts by different mechanisms.

In addition to a role in bone matrix degradation, collagenase regulates the availability of bone growth factors. For example, localized matrix degradation may release growth factors sequestered in the matrix, which may stimulate or inhibit osteoblastic function or may activate or be chemotactic for osteoclasts [Canalis et al., 1988b, 1991]. Collagenase may also regulate the availability or activity of growth factors by acting on their binding proteins. Six IGF binding proteins (IGFBP) are known to be expressed by skeletal cells, and their degradation can be regulated by proteases, including calcium-dependent serine proteases and metalloproteinases [Cheung et al., 1994; Fowlkes et al., 1995; Nam et al., 1996; Rechler, 1993]. Collagenase has the ability to degrade IGFBP-5, and IGF I is known to stabilize this binding protein [Conover et al., 1993; Dong and Canalis, 1995]. This effect may be due to the inhibitory actions of IGF I on collagenase transcription. Since IGFBP-5 enhances the effects of IGF I on bone cell growth, its stabilization by IGF I may prove critical to the regulation of bone formation.

In conclusion, the present studies demonstrate that IGF I decreases collagenase 3 expression by transcriptional mechanisms. This effect may prove central to the actions of IGF I on

bone collagen and on the maintenance of bone matrix integrity.

ACKNOWLEDGMENTS

The authors thank Dr. C. Quinn for the rat interstitial collagenase cDNA clone, Dr. R. Wu for the rat GAPD cDNA clone, Dr. J. Jeffrey for the rat collagenase 3 promoter fragment and rat collagenase antibodies, and Eli Lilly Research Laboratories and Genentech for IGF I. The authors thank Cathy Boucher, Deena Durant, and Susan O'Lone for technical assistance and Margaret Nagle for secretarial help. A.M.D. was the recipient of National Research Service Award DK 09038 from the NIDDK.

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