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Skeletal Bone Morphogenetic Proteins Suppress the Expression of Collagenase-3 by Rat Osteoblasts*

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ABSTRACT

Bone morphogenetic proteins (BMPs) are secreted by skeletal cells, induce the differentiation of mesenchymal cells into cells of the osteoblastic lineage, and increase their differentiated function. BMPs also decrease collagenase-3 expression by the osteoblast. We tested the autocrine role of BMPs on collagenase-3 expression in osteoblast-enriched cells from fetal rat calvariae (Ob cells) by examining the effects of noggin, a specific inhibitor of BMP binding and function. Although collagenase-3 transcript expression declined in untreated Ob cells in culture over a 24-h period, BMP-2, -4, and -6 decreased collagenase-3 messenger RNA levels in cells treated for 2–24 h. The addition of noggin prevented the decrease of collagenase-3 transcripts in control cultures, opposed the inhibitory actions of BMP-2, and increased the levels of the protease in the culture medium. Noggin did not alter the decay of collagenase-3 messenger RNA in transcriptionally arrested cells, and it increased the levels of collagenase-3 heterogeneous nuclear RNA in Ob cells. In conclusion, noggin enhances the synthesis of collagenase-3 in osteoblasts, supporting the notion that BMPs act as autocrine suppressors of collagenase-3 in skeletal cells, an effect that may contribute to the maintenance of the bone matrix. (*Endocrinology* **140:** 562–567, 1999)

BONE MORPHOGENETIC proteins (BMPs) are a family of polypeptides originally described for their ability to induce endochondral bone formation (1, 2). In addition, BMPs have important effects on bone cell differentiation and bone remodeling. BMPs seem to be responsible for the ability of osteoblastic cells to form mineralizing nodules in culture, an event considered to be representative of bone formation (3). BMPs induce the differentiation of mesenchymal cells into cells of the osteoblastic lineage and enhance the expression of the differentiated function of the osteoblast (3, 4). BMPs increase alkaline phosphatase activity and collagen synthesis, and decrease collagenase-3 expression by the osteoblast, whereas they increase the expression of tissue inhibitor of matrix metalloproteinases (MMPs) 1 and 3 (4–6). Consequently, BMPs not only increase the bone matrix, but they have the capability of preventing its degradation and may play a role in the maintenance of bone mass. Osteoblastic cells express BMP-2, -4, and -6, and these BMPs have similar activities and may act as autocrine regulators of selective osteoblastic functions (3, 7).

MMPs are a family of related proteolytic enzymes, including collagenases, gelatinases, and stromelysins (8–10). Collagenases cleave fibrillar collagen at neutral pH and are considered important in matrix remodeling. Three collagenases have been described: collagenase-1, secreted by human fibroblasts, osteoblasts, and chondrocytes; collagenase-2, secreted by human neutrophils; and collagenase-3, secreted by human breast carcinoma cells, human chondrocytes, and rat osteoblasts (9, 11–15). Type II collagen is preferentially hydrolyzed by collagenase-3, whereas collagenases 1, 2, and 3 degrade fibrillar type I collagen with similar efficiency (16). Human and rat osteosarcoma cells express collagenase-1 and -3, respectively, but unstimulated normal human and rat osteoblasts secrete only limited amounts of collagenase (11–15). The levels of collagenase secreted by the osteoblast are dependent on the effects of stimulatory and inhibitory cytokines present in the bone microenvironment. Of these cytokines, insulin-like growth factor (IGF) I and IGF-II, transforming growth factor (TGF) β1, and BMP-2 have been shown to inhibit collagenase-3 transcript and protein levels in rat osteoblast cultures (6, 17, 18). We, therefore, postulated that a reason for the unstimulated normal osteoblast to underexpress collagenase is the secretion of IGFs, TGF β, and BMPs. Recently, we showed that IGF-I and IGF-II are autocrine inhibitors of collagenase-3 expression by the osteoblast (19). We postulated that BMPs may play a similar role and contribute to the autocrine down-regulation of collagenase-3 expression by the osteoblast.

In the present study, we examined the actions of BMP-2, -4, and -6 on collagenase-3 transcripts in cultures of osteoblast-enriched cells from 22-day fetal rat calvariae (Ob cells). We also determined the role of the locally produced BMPs on collagenase-3 synthesis by testing the actions of noggin, a glycoprotein that binds BMPs specifically and blocks their biological activities (20, 21).

Materials and Methods

Culture technique

The culture method used to obtain Ob cells was described in detail previously (22). Parietal bones were obtained from 22-day-old fetal rats immediately after the mothers were killed by blunt trauma to the nuchal

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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 Corp., Freehold, NJ). Cell populations harvested from the third to the fifth digestion were cultured as a pool, and about 80% or more of these cells were previously shown to display osteoblastic characteristics (22, 23). Ob cells were plated at a density of 8,000–12,000 cells/cm^2 and were cultured in a humidified 5% CO_2 incubator at 37 C until reaching confluence (about 50,000 cells/cm^2). Cells were cultured in DMEM supplemented with nonessential amino acids, 100 μg/ml L-ascorbic acid, penicillin, streptomycin, and 20 mM HEPES, pH 7.0 (all from Life Technologies, Grand Island, NY), and 10% FBS (Summit Biotechnology, Fort Collins, CO). Ob cells were grown to confluence, transferred to serum-free medium for 20–24 h, and transferred to freshly prepared test or control solutions, in the absence of serum, for 2–24 h, as indicated in the text and legends. Recombinant human BMP-2 was added directly to the culture medium, and BMP-4 and BMP-6 (all BMPs a gift from Genetics Institute, Cambridge, MA) were dissolved in 0.1% trifluoracetic acid and diluted 1:8000 and 1:4000, respectively, in DMEM. Recombinant human noggin (a gift from Regeneron Pharmaceuticals, Inc., Tarrytown, NY) was dissolved in 50 mM sodium citrate, 150 mM sodium chloride, 1 mM magnesium acetate, and 20% glycerol, and diluted 1:10,000, or greater, in DMEM. 5,6-Dichlorobenzimidazole riboside (DRB; Sigma Chemical Co., St. Louis, MO) was dissolved in ethanol and diluted 1:200 in DMEM. An equal amount of solvent was added to control cultures. In experiments performed to quantitate protease levels, the medium was harvested in the presence of 0.1% polyoxyethylene sorbitan monolaurate (Pierce, Rockford, IL) and stored at −80 C, before Western immunoblot analysis. Independent experiments were performed to quantitate changes in messenger RNA (mRNA) levels; and, at the end of the incubation period, the cell layer was extracted and stored at −80 C.

Northern blot analysis

Total cellular RNA from approximately 1×10^6 cells was isolated by RNeasy kit, per manufacturer's instructions (Qiagen, Chatsworth, CA). The RNA recovered was quantitated by spectrophotometry, and equal amounts (10–15 μg) of RNA from control or test samples obtained from parallel cultures of the same experiment were loaded on a formaldehyde-agarose gel after denaturation. The gel was stained with ethidium bromide to visualize RNA standards and ribosomal RNA, before and after transfer, documenting equal RNA loading of the samples. The RNA was blotted onto Gene Screen Plus charged nylon (DuPont, Wilmington, DE). Restriction fragments containing a 2.6-kb interstitial collagenase-3 complementary DNA (cDNA) (kindly provided by C. Quinn, St. Louis, MO) and a 750-bp murine 18S ribosomal RNA cDNA (American Type Culture Collection, Rockville, MD) were labeled with [α-^{32}P]-deoxycytidine triphosphate (dCTP) and [α-^{32}P]-deoxy-ATP (specific activity of 3,000 Ci/mmol; DuPont), using the random hexanucleotide primed second strand synthesis method (14, 24). Hybridizations were carried out at 42 C for 16–72 h. Posthybridization washes were performed in 1 × saline sodium citrate at 65 C for collagenase-3 and in 0.1 × saline sodium citrate at 65 C for 18S ribosomal RNA. The bound radioactive materials were visualized by autoradiography on Kodak X-AR5 Biomax (Eastman Kodak Co., Rochester, NY) or DuPont Reflection film (DuPont) employing intensifying screens. Relative hybridization levels were determined by densitometry. Northern analyses shown are representative of three or more cultures.

Western immunoblot analysis

Medium samples were fractionated by PAGE, using denaturing, nonreducing conditions, and were transferred onto Immobilon P membranes (Millipore Corp., Bedford, MA) (25). After blocking with 2% BSA, the membranes were exposed to a 1:1000 dilution of rabbit antiserum raised against rat collagenase-3 (kindly provided by J. Jeffrey, Albany, NY), previously characterized for specificity and immunoreactivity, followed by the addition of goat antirabbit IgG conjugated to horseradish peroxidase (26). The blots were washed and developed with a horseradish peroxidase chemiluminescence detection reagent (DuPont), visualized by autoradiography on DuPont Reflection film employing Reflection intensifying screens, and analyzed by densitometry. Data shown are representative of three or more cultures.

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-3 gene, and an antisense primer, 5′-AAAAGACCAGAACAACCAGC-3′, spanning nucleotides 61–80 of intron 1, to yield a 186-bp product (14, 27). RNA was extracted as described for Northern analysis, and samples were treated with amplification-grade DNase I, according to manufacturer's instructions (Life Technologies), 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 manufacturer's instructions, except that *Taq* polymerase buffer was used instead of reverse transcriptase buffer (28). A DNA standard was synthesized by PCR amplification of plasmid DNA pGL2-Basic (Promega Corp. Corporation, Madison, WI) using the rat collagenase hnRNA primer set and low-stringency annealing conditions, as described by Forstr (29). The cDNA and 0.05 attomole DNA standard were amplified by PCR using 24 cycles at 94 C for 1 min, 59 C for 2 min, and 72 C for 1 min in the presence of *Taq* polymerase (Life Technologies), 0.15 μmol of sense and antisense primers, and 5 μCi [α-^{32}P]-dCTP. PCR products were resolved on an 8% polyacrylamide gel (Gel-Mix 8, Life Technologies), containing 100 mM Tris-borate/1 mM EDTA, and visualized by autoradiography. The amplification protocol yielded products that were within the linear range for both the collagenase hnRNA and the standard. Data on hnRNA are representative of three cultures.

Statistical analysis

Data are expressed as means ± SEM, and statistical differences were determined by ANOVA and *post hoc* examination by Ryan-Einot-Gabriel-Welch F test using a Crunch Statistical Package (Crunch Software Corp., Oakland, CA) (30). Data for mRNA decay in transcriptionally arrested cells are expressed as means ± SEM, and slopes were analyzed by the method of Sokal and Rohlf (31).

Results

Northern blot analysis of total RNA from Ob cells revealed a rat collagenase-3 transcript of 2.9 kb (Fig. 1). Confirming prior observations, a decrease in collagenase-3 was noted in confluent serum-deprived control cultures 24 h after the medium was changed. Continuous treatment of Ob cells with BMP-2, -4, or -6 at 100 ng/ml caused a decrease in collagenase steady-state transcripts. This decrease initially occurred after

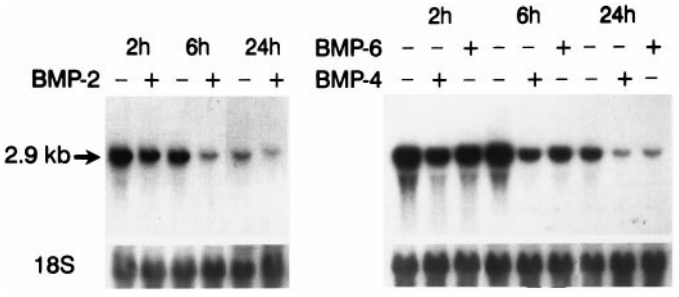

FIG. 1. Effect of BMP-2 (*left*), -4, and -6 (*right*), at 100 ng/ml, on collagenase-3 mRNA levels in cultures of Ob cells treated for 2, 6, or 24 h. Total RNA from control (−) or BMP-treated (+) cultures was subjected to Northern blot analysis and was hybridized with an [α-^{32}P]-labeled collagenase-3 cDNA. The blot was stripped and rehybridized with an [α-^{32}P]-labeled 18S cDNA. Collagenase transcripts of 2.9 kb were visualized by autoradiography and are shown in the *upper panel*, whereas 18S ribosomal RNA is shown *below*.

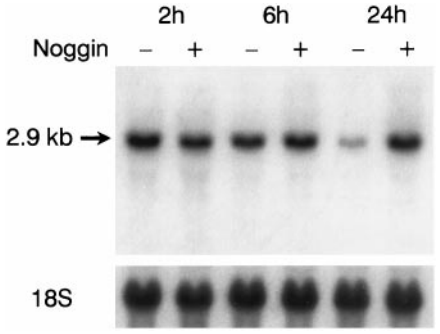

FIG. 2. Effect of noggin, at 100 ng/ml, on collagenase-3 mRNA levels in cultures of Ob cells treated for 2, 6, and 24 h. Total RNA from control (−) or noggin-treated (+) cultures was subjected to Northern blot analysis and hybridized with an [α-^{32}P]-labeled collagenase-3 cDNA. The blot was stripped and rehybridized with an [α-^{32}P]-labeled 18S cDNA. Collagenase transcripts of 2.9 kb were visualized by autoradiography and are shown in the *upper panel*, whereas 18S ribosomal RNA is shown *below*.

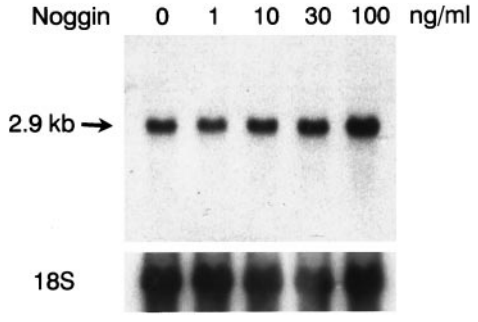

FIG. 3. Effect of noggin, at 1–100 ng/ml, on collagenase-3 mRNA levels in cultures of Ob cells treated for 24 h. Total RNA from control (0) or noggin-treated cultures was subjected to Northern blot analysis and was hybridized with an [α-^{32}P]-labeled collagenase-3 cDNA. The blot was stripped and rehybridized with an [α-^{32}P]-labeled 18S cDNA. Collagenase transcripts of 2.9 kb were visualized by autoradiography and are shown in the *upper panel*, whereas 18S ribosomal RNA is shown *below*.

2 h of exposure to BMP-2, -4, or -6, and the effect was maximal after 24 h when the three BMPs tested decreased collagenase-3 transcripts by 70–90%, compared with control cultures, so that the effect was more pronounced than the time-dependent decline (Fig. 1). This time-dependent decrease in collagenase mRNA levels was prevented by noggin at 100 ng/ml (Fig. 2). The effect of noggin was dose-dependent, and exposure of Ob cells to noggin at 30–100 ng/ml for 24 h increased collagenase transcripts, in relationship to the corresponding timed control, by (mean ± SEM) 2.4 ± 0.6 (n = 3) to 4.1 ± 0.4 (n = 14; $P < 0.05$)-fold (Fig. 3). Noggin, at 10 ng/ml for 24 h, had an inconsistent stimulatory effect of 1.5 ± 0.3 (n = 3)-fold ($P > 0.05$). The inhibitory effect of BMP-2 at 10 ng/ml on collagenase-3 transcript levels was prevented by noggin at 100 ng/ml (Fig. 4). Noggin, at 100 ng/ml for 24 h, increased the levels of immunoreactive collagenase-3 in the culture medium of Ob cells by (mean ± SEM; n = 3) 2.4 ± 0.5-fold ($P < 0.05$), as determined by Western blot analysis (Fig. 5). Collagenase was identified by comigration with a purified rat procollagenase-3 standard (kindly provided by J. Jeffrey).

To determine whether the effects of noggin on collagenase-3 mRNA levels were caused by changes in transcript stability, Ob cells were exposed to control or noggin-containing medium for 1 h and then treated with the RNA polymerase II inhibitor DRB at 75 μM, in the presence or absence of noggin for 2, 4, and 8 h (32). The half-life of collagenase-3 mRNA in transcriptionally-arrested Ob cells was approximately 4 h in control and test cultures (Fig. 6). Slope analysis indicated that noggin did not change the stability of collagenase mRNA. Analogous to the changes in collagenase mRNA, there was a decline in the levels of collagenase-3 hnRNA, 24 h after the serum-free medium of confluent Ob cell cultures was changed. Noggin maintained the expression of collagenase-3 hnRNA in Ob cells, as determined by RT/PCR, so that when the effect of noggin was compared with control cultures at 24 h, it increased hnRNA (mean ± SEM; n = 3) by 3.6 ± 0.4-fold ($P < 0.05$) (Fig. 7). Coamplification of an exogenous DNA standard, designed to use the same set of primers, revealed uniform PCR efficiency, and omission of the RT step resulted in no signal, proving lack of DNA contamination.

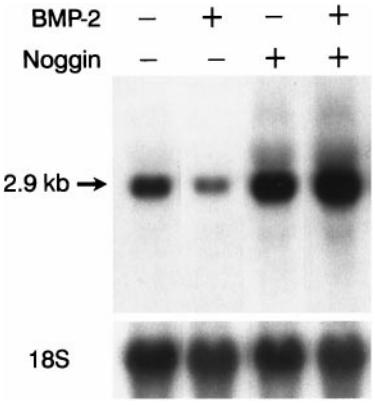

FIG. 4. Effect of BMP-2, at 10 ng/ml, in the presence or absence of noggin at 100 ng/ml, on collagenase-3 mRNA levels in cultures of Ob cells treated for 24 h. Total RNA from control or BMP-2 and noggin-treated cultures was subjected to Northern blot analysis and was hybridized with an [α-^{32}P]-labeled collagenase-3 cDNA. The blot was stripped and rehybridized with an [α-^{32}P]-labeled 18S cDNA. Collagenase transcripts of 2.9 kb were visualized by autoradiography and are shown in the *upper panel*, whereas 18S ribosomal RNA is shown *below*.

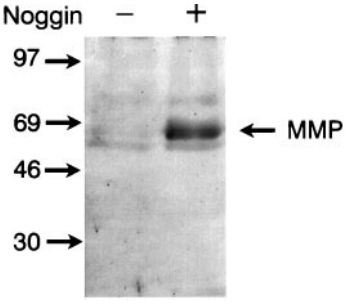

FIG. 5. Effect of noggin, at 100 ng/ml, on procollagenase-3 secretion in Ob cell cultures treated for 24 h. Western blot analysis was performed using equal amounts of culture medium from control (−) or noggin-treated (+) cultures. Procollagenase (MMP) was detected using rabbit antirat collagenase antibody and a horseradish peroxidase chemiluminescence-detection system.

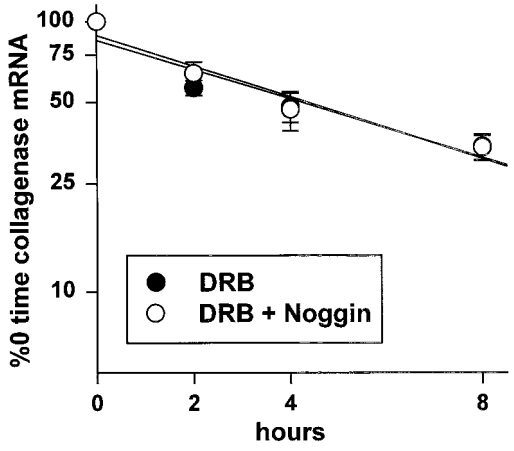

FIG. 6. Effect of noggin, at 100 ng/ml, on collagenase-3 mRNA decay in Ob cell cultures. Confluent cultures of Ob cells were serum-deprived and exposed to control or noggin-containing medium for 1 h before the addition of DRB at 75 μM. Total RNA, obtained 0–8 h after DRB or noggin and DRB addition, was 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. Data from DRB (*closed circles*) and DRB+ noggin-treated (*open circles*) cells are expressed as means ± SEM for three cultures and as percent of mRNA levels present before the addition of DRB.

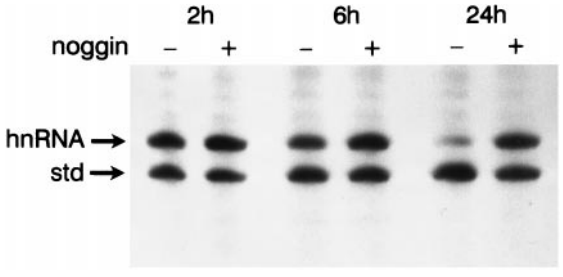

FIG. 7. Effect of noggin, at 100 ng/ml, on collagenase-3 hnRNA levels in cultures of Ob cells treated for 2, 6, or 24 h. Total RNA from control (–) and noggin-treated (+) cultures was reverse-transcribed and amplified by PCR in the presence of 5 μCi [α-^{32}P]-dCTP using collagenase exon 1 and intron 1 specific primers to generate a 186-bp product. An exogenous DNA standard (std), designed to use the same primers, was coamplified with each reaction to assess PCR efficiency. PCR products were visualized by autoradiography.

Discussion

Our studies confirm that confluent Ob cells display a decline in collagenase-3 expression, with time in culture (19). After a 24-h period of serum deprivation, this decline occurs over the next 6–24 h, and it seems to parallel the accumulation of factors in culture, such as IGFs, that down-regulate the protease, because removal of the culture medium results in a prompt up-regulation of collagenase-3 (19). The present investigation was undertaken to determine whether BMPs play an autocrine role in the down-regulation of collagenase-3 expression by rat osteoblasts. BMP-2, -4, and -6 decreased collagenase-3 transcripts in Ob cells; and the specific inhibitor of BMP binding, noggin, blocked the effect of BMP-2 on collagenase transcript levels and maintained the expression of collagenase mRNA, hnRNA, and protease levels in osteoblast cultures (20, 21). Noggin did not alter collagenase mRNA stability in transcriptionally-arrested Ob cells, and it increased collagenase hnRNA levels in Ob cells. These results suggest that noggin increases collagenase-3 transcription by rat osteoblasts, an effect that is in accordance with the inhibition of collagenase transcription by BMP (6).

Although it is possible that noggin has a direct effect on the osteoblast, a more likely explanation is that the increase in collagenase-3 expression by noggin is caused by its ability to bind and inactivate BMPs (20, 21). This would support the concept that BMPs act as autocrine down-regulators of collagenase-3 transcription in osteoblasts. The notion that noggin acts indirectly is based on the lack of known receptors for noggin and its ability to block BMP-2 actions on collagenase expression, as shown in the present study. Therefore, noggin would not act directly on osteoblasts but simply would bind BMPs available to the cells. Recent studies from our laboratory have demonstrated that primary Ob cell cultures do not express detectable noggin. However, BMP-2, -4, and -6 induce noggin mRNA and protein levels in osteoblastic cultures, a mechanism that would limit overexposure of osteoblastic cells to BMPs (33). The newly synthesized noggin, under the influence of BMPs, appears in the extracellular matrix and culture medium, where it would be available to bind BMPs. The physiological function of noggin was recently substantiated in mice with disruption of the noggin gene, which manifest a lethal phenotype, with neurological and skeletal abnormalities attributed to tissue overexposure to BMPs (34).

The synthesis of collagenase-1 and -3 by human and rat osteoblasts, respectively, is regulated by systemic hormones and by cytokines present in the bone microenvironment. Consequently, the apparent constitutive level of collagenase expression by the osteoblast is in fine balance and depends on the exposure of the osteoblast to factors that stimulate, and factors that inhibit, collagenase synthesis (12, 17, 18, 35). BMPs have modest mitogenic activity and increase collagen expression while decreasing collagenase synthesis in Ob cells (4–6). This is in contrast to the actions of growth factors with potent mitogenic activity for bone cells, such as platelet-derived growth factor and fibroblast growth factor, because they do not stimulate osteoblastic collagen synthesis and enhance collagenase expression by the osteoblast (12, 36–38). Similar to the effects of BMPs on skeletal tissue are those of IGF-I and IGF-II and TGF β1. These factors stimulate bone collagen and inhibit collagenase-3 expression by the osteoblast (4–6, 17, 18, 39, 40). IGF-I inhibits collagenase-3 expression at the transcriptional level. Although BMP-2 is a member of the TGF β family of peptides, TGF β1 inhibits collagenase-3 expression at the transcriptional and posttranscriptional levels, whereas BMP-2 acts only transcriptionally (6, 18). This would suggest that various growth factors regulate collagenase-3 expression in osteoblasts by different mechanisms. Recently, we demonstrated that IGF-I and IGF-II are autocrine down-regulators of collagenase-3 in Ob cells (19). Therefore, accumulation of IGF-I, IGF-II, BMPs, and possibly TGF β, in the bone microenvironment may result in decreased collagenase synthesis by the osteoblast. In addition to the presence of skeletal cytokines, the state of osteoblastic maturation may influence the level of collagenase expression (13, 41). Although noggin could prevent the induction of immature cells into more differentiated cells,

this is probably not the case in our studies, because the cultures were confluent and examined at a stage that cells clearly express markers of the differentiated osteoblast (22). Furthermore, the effect of noggin on collagenase-3 expression was observed after a relatively short-term exposure of Ob cells to noggin.

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 (42). Collagenase may also regulate the availability or activity of growth factors by acting on their binding proteins. For example, six IGF binding proteins are known to be expressed by skeletal cells, and their degradation can be regulated by proteases, including calcium-dependent serine proteases and metalloproteinases (43–46). Collagenase-3 has the ability to fragment IGF-binding protein-5, and the fragments of this binding protein may have unique effects in bone (45).

In conclusion, the present studies demonstrate that BMPs decrease rat collagenase-3 expression in osteoblasts and that noggin enhances or supports the expression of this protease. This suggests that BMPs act as autocrine down-regulators of collagenase, and this effect probably plays a role in the maintenance of the bone collagen matrix and bone mass.

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