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Regulation of Collagenase-3 by Bone Morphogenetic Protein-2 in Bone Cell Cultures*

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ABSTRACT

Bone morphogenetic protein-2 (BMP-2), a member of the transforming growth factor superfamily of peptides, induces ectopic bone formation *in vivo*. The actions of BMP-2 on osteoblastic cells include stimulation of collagen synthesis, but the role of BMP-2 on collagen degradation is not known. We examined whether BMP-2 affects the expression of collagenase-3, an enzyme that degrades type I collagen at neutral pH, and that of tissue inhibitors of matrix metalloproteinases (TIMPs) in primary osteoblast-enriched cells from 22-day-old fetal rat calvariae. BMP-2 suppressed collagenase messenger RNA (mRNA) and immunoreactive protein levels. BMP-2 did not affect collagenase mRNA stability, but it reduced collagenase heterogeneous nuclear RNA levels and decreased the rate of transcription of the collagenase gene. BMP-2 also stimulated TIMP 1 and TIMP 3 mRNA levels, but failed to alter TIMP 2 expression. In conclusion, our studies indicate that BMP-2 suppresses collagenase-3 gene transcription and stimulates TIMP 1 and TIMP 3 expression in osteoblasts. The regulation of collagenase and TIMPs by BMP-2 in osteoblasts may play a role in osteoinduction. (*Endocrinology* **138:** 1035–1040, 1997)

BONE MORPHOGENETIC proteins (BMPs) are present in cartilage and bone and were originally identified as stimulators of ectopic bone formation (1, 2). BMPs are classified as members of the transforming growth factor-β (TGFβ) superfamily due to their 30–40% sequence homology to members of the TGFβ family of polypeptides (3, 4). At least seven proteins, designated BMP-2 to BMP-8, are currently known to constitute the BMP subfamily of factors. BMP-2, a prototype of the BMP subfamily, has been widely studied for its actions on skeletal tissue. BMP-2 is known for inducing the differentiation of osteoblasts (5, 6) and for influencing the mineralization process (7). BMP-2 augments PTH-stimulated cAMP production and regulates the synthesis of collagen, alkaline phosphatase, and insulin-like growth factor I (IGF-I) in cells of the osteoblastic lineage (8–10). In addition, the temporal and spatial patterns of BMP-2 and -4 expression suggest a role in embryonic endochondral bone formation (11, 12).

Matrix metalloproteinases (MMPs) are a major group of proteinases that mediate the degradation of extracellular matrix components (13, 14). These proteinases are stored in the extracellular matrix in an inactive form, and their activities are regulated by specific activators and inhibitors. Collagenase-1 and -3 (MMP-1 and -13), 72- and 92-kDa gelatinases (MMP-2 and -9), and possibly other MMPs, are expressed by osteoblastic cells (15–19). Collagenase-1 and -3 can cleave intact collagen fibrils, whereas gelatinases degrade collagen fragments generated by prior collagenase digestion (20). Hence, collagenase-1 and -3 appear to be major regulators of collagen turnover and bone remodeling. Studies from this and other laboratories indicated that collagenase expression is regulated by various bone-remodeling agents, such as PTH and skeletal growth factors (15–18, 21–24). Bone cells also synthesize tissue inhibitors of matrix metalloproteinases (TIMPs) 1, 2, and 3, and their expression is regulated by various bone-remodeling agents (17, 18, 22, 23).

Growth factors known to stimulate bone collagen synthesis, such as IGFs, inhibit collagenase expression by osteoblasts (23). Consequently, they play a dual role in the maintenance of the bone collagen matrix, enhancing its synthesis and decreasing its degradation. In addition, there is an inverse correlation between the expression of the normal osteoblastic phenotype and the synthesis of collagenase (18, 23). As BMP-2 stimulates collagen synthesis and induces the expression of the osteoblastic phenotype, we postulated that it should regulate collagenase production in bone cells. This study was undertaken to examine the effects of BMP-2 on the synthesis of collagenase-3 and TIMPs 1, 2, and 3 in osteoblast-enriched (Ob) cells isolated from fetal rat calvariae.

Materials and Methods

Cell culture

Rat fetuses were removed from 22-day pregnant mothers and killed by blunt trauma to the nuchal area according to a protocol approved by the animal care and use committee of Saint Francis Hospital and Medical Center. Ob cells were isolated from the parietal bone of 22-day-old fetal rats as described previously (25). Cells were plated at a density of 8,000–10,000 cells/cm^2 onto plastic cell culture dishes (Corning Glass Works, Corning, NY) in DMEM (Summit Biotechnology, Fort Collins, CO) supplemented with nonessential amino acids, 100 μg/ml L-ascorbic acid, 20 mM HEPES (all from Life Technologies, Grand Island, NY), and 10% FBS (Summit Biotechnology) and cultured at 37 C in a CO_2 incubator. Except for nuclear run-off experiments, cells were grown to confluence, at which time culture medium was replaced with serum-free DMEM for 16–24 h. Cells were then incubated with serum-free medium in the presence and absence of BMP-2 for 1–24 h. For nuclear run-off

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assays, subconfluent cells were trypsinized, replated, and grown to confluence, at which time they were serum deprived and treated with BMP-2 for 2 h. BMP-2 (kindly provided by Genetics Institute, Cambridge, MA) was dissolved in water and 5,6-dichlorobenzimidazole riboside (DRB; Sigma Chemical Co., St. Louis, MO) was dissolved in ethanol and diluted in DMEM 1:200. Control cultures contained equal amounts of alcohol. Culture medium was collected for Western blot analysis, and Ob cells were harvested to isolate RNA or nuclei.

Northern blot analysis

Total RNA was isolated from Ob cells by the method of Chomczynski and Sacchi (26). Total RNA (5–15 μg/lane) was fractionated on a 1% agarose-formaldehyde gel (Life Technologies) containing 100 μg/ml ethidium bromide as previously described (27). Subsequent to electrophoresis, RNA was transferred onto a 0.2-μm Biotrans nylon membrane (ICN Biomedicals, Aurora, OH) by capillary action. The integrity and equal gel loading of RNA and the efficiency of transfer were assessed by visualizing the 28S and 18S ribosomal RNA (rRNA) bands under UV light. The RNA was cross-linked to the nylon membrane using CL-1000 UV cross-linker (UVP, San Gabriel, CA) and hybridized with ^{32}P-labeled complementary DNA (cDNA). The cDNA fragments were isolated by restriction endonuclease (New England Biolabs, Beverly, MA) digestion of plasmid clones containing a 2.6-kilobase (kb) rat interstitial collagenase-3 cDNA (kindly provided by Dr. Cheryl Quinn, St. Louis University School of Medicine, St. Louis, MO), a 0.83-kb murine TIMP 1 cDNA, a 0.7-kb murine TIMP 2 cDNA, a 0.75-kb murine TIMP 3 cDNA (all kindly provided by Dr. Dylan Edwards, University of Calgary Health Sciences Center, Calgary, Canada), and a 0.75-kb murine 18S rRNA cDNA (American Type Culture Collection, Rockville, MD) (28–31). All cDNAs were radiolabeled by the random hexanucleotide-primed second strand synthesis method using [α-^{32}P]deoxy (d)-ATP, [α-^{32}P]dCTP (3,000 Ci/mmol; DuPont, Wilmington, DE), Klenow fragment (New England Biolabs), and hexanucleotide primers (Boehringer Mannheim Biochemicals, Indianapolis, IN) (32). The hybridizations were performed as described at 42 C in the presence of 50% formamide (Sigma) using radiolabeled cDNAs (27). The final low stringency washes were performed at 60 C in SSC (0.15 M sodium chloride and 0.015 M sodium citrate, pH 7) for collagenase-3 and in TIMPs 1, 2, and 3, at 65 C in 0.1 × SSC for 18S rRNA. Autoradiography was performed by exposing the membrane to Kodak XAR film (Eastman Kodak, Rochester, NY) in the presence of DuPont Lightning Plus intensifying screens (DuPont, Wilmington, DE). The intensity of RNA bands was quantitated by densitometric scanning of the autoradiographs. For rehybridization of blots, the radiolabeled cDNAs were removed by boiling the nylon membrane in 0.1% SDS for 5–10 min.

Nuclear run-off assay

Nuclei were isolated from Ob cells by Dounce homogenization (Kontes Co., Vineland, NJ) in Tris buffer containing 0.5% Nonidet P-40 (33). Nascent transcripts were radiolabeled by incubation of nuclei at room temperature for 30 min in a reaction buffer containing 250 μCi (800 Ci/mmol) [α-^{32}P]UTP, 500 μM ATP, CTP, and GTP and 150 U RNasin (Promega Corp., Madison, WI). ^{32}P-Labeled RNA was isolated by treatment with deoxyribonuclease I and proteinase K, followed by phenol-chloroform extraction and ethanol precipitation using ammonium acetate. Linearized plasmid DNA containing 1 μg cDNA for collagenase or 18S rRNA, or vector DNA was immobilized onto a nylon membrane using a slot blot apparatus (Schleicher and Schuell, Keene, NH). Each nylon membrane with a panel of immobilized DNAs was hybridized with ^{32}P-labeled RNA (3 × 10^6 cpm/ml) from test or control samples at 42 C for 72 h, using conditions identical to those for Northern hybridization analysis, and washed in SSC-0.1% SDS at 45 C. Hybridization of nascent transcripts to different cDNAs was visualized by autoradiography and quantified by densitometry.

Reverse transcription-PCR

Collagenase-3 heterogeneous nuclear RNA (hnRNA) was analyzed by reverse transcription-PCR as previously described using a sense primer (5′-CATTCAGCTATTCTGGCCAC-3′) and an antisense primer (5′-AAAAGACCAGAACAACCAGC-3′) corresponding to nucleotides (nt) 29–48 of exon 1 and nt 61–80 of intron 1, respectively, to yield a 186-bp product (24). Briefly, 1 μg RNA was treated with deoxyribonuclease I (Life Technologies) and copied into cDNA using Moloney murine leukemia virus reverse transcriptase (Life Technologies) and the antisense primer (34). A DNA standard of 150 bp was synthesized by PCR amplification of pGL2-Basic plasmid DNA (Promega Corp., Madison, WI) using the rat collagenase hnRNA primer set and low stringency annealing conditions, as described by Forstr (35). The newly synthesized cDNA and 0.05 attomoles 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 μmol of sense and antisense primers; and 5 μCi [α-^{32}P]dCTP. PCR products were resolved on a 8% polyacrylamide gel and visualized by autoradiography. The amplification protocol yielded products that were within the linear range for collagenase hnRNA and the standard.

Western immunoblot analysis

Aliquots from the medium of different cultures were adjusted to a final concentration of 0.1% polyoxyethylene sorbitan monolaurate (Pierce Chemical Co., Rockford, IL), fractionated by PAGE under denaturing conditions, and transferred onto an Immobilon P membrane (Millipore Corp., Bedford, MA). After blocking with 2% BSA, the membrane was exposed to a 1:1000 dilution of rabbit antiserum raised against rat collagenase-3 (kindly provided by Dr. John J. Jeffrey, Albany Medical College, Albany, NY) (36), followed by the addition of goat antirabbit IgG conjugated to horseradish peroxidase. The blots were washed and developed with a horseradish peroxidase chemiluminescence detection reagent (DuPont). The chemiluminescent bands were visualized after exposure to DuPont Reflection film employing Reflection intensifying screens.

Statistical methods

Data on collagenase messenger RNA (mRNA) decay were analyzed by linear regression, and the slopes of the regression lines obtained for control and BMP-2-treated cells were compared for significant differences as described by Sokal and Rohlf (37). Statistical analysis of the difference between TIMP mRNA levels in control and BMP-2-treated cultures was performed using Student's *t* test.

Results

Suppression of collagenase mRNA and proteinase by BMP-2

Northern hybridization analysis of total RNA from confluent Ob cells exposed to BMP-2 at 3.3 nM for 1–24 h revealed a time-dependent suppression of approximately 2.9-kb collagenase mRNA (Fig. 1). BMP-2 decreased collagenase mRNA after 2 and 4 h by approximately 15% and 70%, respectively. A maximal inhibition of 80–90% was observed after 8–16 h of exposure to BMP-2, and the effect was sus-

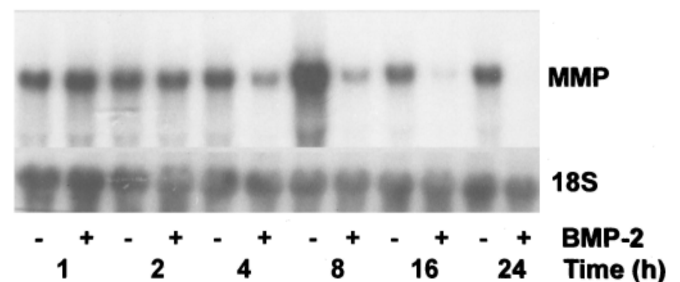

FIG. 1. Time-dependent regulation of collagenase mRNA by BMP-2. Northern blot analysis of total RNA (9 μg/lane), isolated from control (−) or 3.3 nM BMP-2-treated (+) Ob cells for 1–24 h, was performed using ^{32}P-labeled collagenase-3 and 18S rRNA cDNAs. The *upper panel* shows collagenase (MMP) mRNA, and the *lower panel* shows 18S rRNA levels from one of four independent cultures.

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tained for up to 24 h. Collagenase mRNA levels in control cultures were higher after 8 h, although this did not preclude the inhibitory effect of BMP-2. The effect of BMP-2 on collagenase expression was dose dependent and was observed at concentrations as low as 0.03 nM (Fig. 2).

Western blot analysis was performed to determine whether the levels of procollagenase were affected by BMP-2. After exposure of Ob cells to BMP-2 at 3.3 nM for 24 h, the amount of immunoreactive procollagenase in the culture medium was decreased by 70% (Fig. 3). Procollagenase was identified by comigration with a purified rat procollagenase standard. The antibody cross-reacted with an unidentified protein migrating with a lower mol wt, although its levels did not change in the presence of BMP-2 (Fig. 3). Colloidal gold staining of the blot indicated that equal amounts of protein were present in control and treated cultures (not shown).

Transcriptional regulation of the collagenase gene by BMP-2

To determine whether BMP-2 caused a change in collagenase mRNA stability, the rate of collagenase mRNA degradation was tested in control and BMP-2-treated cultures. Ob cells were exposed to control or BMP-2-containing medium 4 h before the addition of DRB, a RNA polymerase II inhibitor (38). Collagenase mRNA levels were determined immediately before the addition of DRB and 4–24 h after exposure to DRB (Fig. 4). The half-life of collagenase mRNA was approximately 6 h, and collagenase mRNA decay occurred at similar rates in both control and BMP-2-treated cultures, indicating the lack of a BMP-2 effect on mRNA destabilization.

Because transcriptional regulation frequently leads to changes in hnRNA synthesis, the effect of BMP-2 on collagenase hnRNA levels was tested. Treatment of Ob cells with BMP-2 at 3.3 nM for 2–8 h decreased collagenase hnRNA by 50–80%, suggesting transcriptional down-regulation (Fig. 5). Coamplification of an exogenous DNA standard, designed to use the same set of primers, revealed uniform PCR efficiency. The absence of DNA contamination in the RNA samples was indicated by the lack of signal when reverse transcriptase was omitted. To confirm the transcriptional suppression of

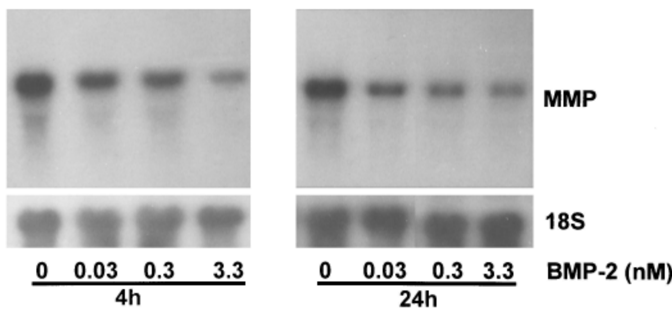

FIG. 2. Dose-dependent changes in collagenase mRNA by BMP-2. Northern blot analysis of total RNA (6 μg/lane) isolated from Ob cells exposed to 0–3.3 nM BMP-2 for 4 h and 24 h, was performed by using ^{32}P-labeled collagenase-3 and 18S rRNA cDNAs. The *upper panel* shows collagenase mRNA (MMP), and the *lower panel* shows 18S rRNA levels from one of two independent cultures.

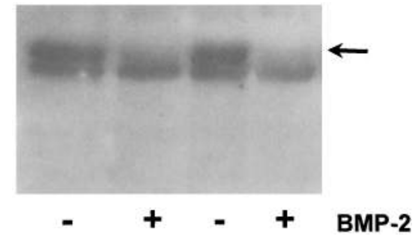

FIG. 3. Inhibition of immunoreactive collagenase secretion by BMP-2. Western blot analysis was performed using equal amounts of culture medium from two independent cultures of Ob cells treated with (+) or without (−) BMP-2 at 3.3 nM for 24 h. Collagenase-3 (*arrow*) was detected using a rabbit antirat collagenase antibody and a horseradish peroxidase chemiluminescence detection system.

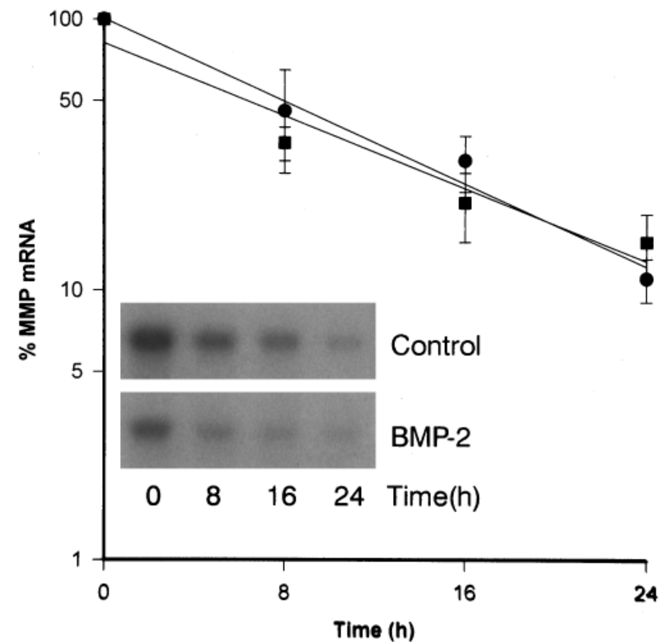

FIG. 4. Effect of BMP-2 on collagenase (MMP) mRNA stability. Ob cells were exposed to control or 3.3 nM BMP-2-containing medium for 4 h before the addition of DRB at 75 μM. Total RNA (7 μg/lane), obtained 0–24 h after DRB addition, was analyzed by Northern hybridization analysis with ^{32}P-labeled collagenase-3 cDNA. Collagenase mRNA was visualized by autoradiography and quantitated by densitometry. Data from control (*closed circles*) and BMP-2-treated (*closed squares*) cells are the mean ± SEM for five independent cultures and are expressed as a percentage of collagenase mRNA (% MMP mRNA) levels at the time of the addition of DRB. The *inset* shows a representative experiment revealing collagenase mRNA after the addition of DRB in control and BMP-2-treated cultures.

the collagenase gene by BMP-2, a nuclear run-off assay was performed. The transcriptional rate of the collagenase gene in Ob cells was decreased by 60% after exposure to BMP-2 at 0.3 nM for 2 h (Fig. 6).

Effect of BMP-2 on TIMP expression

Bone cells express approximately 0.9 kb TIMP 1, 3.5 and 1 kb TIMP 2, and 4.5 and 2.5 kb TIMP 3 mRNAs. Treatment of Ob cells with BMP-2 at 3.3 nM caused changes in the expression of TIMP 1 and TIMP 3, but did not alter the expression of TIMP 2 (Figs. 7–9 and Table 1). BMP-2 caused

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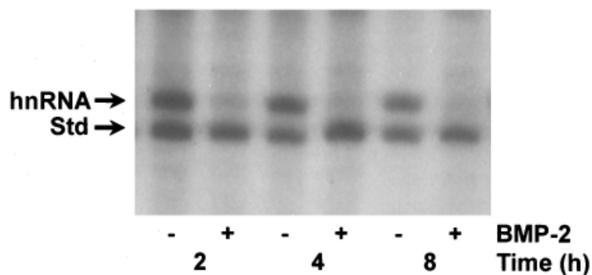

FIG. 5. Inhibition of collagenase hnRNA levels by BMP-2. Total RNA (1 μg) from control cultures (−) and cultures treated with 3.3 nM BMP-2 (+) for 2–8 h was reverse transcribed and amplified by PCR in the presence of 5 μCi [α-^{32}P]dCTP using collagenase exon 1- and intron 1-specific primers to generate a 186-bp product (hnRNA). An exogenous standard DNA (Std), designed to use the same set of primers, was coamplified with each reaction to assess PCR efficiency. PCR products from one of three independent cultures, visualized by autoradiography, are shown.

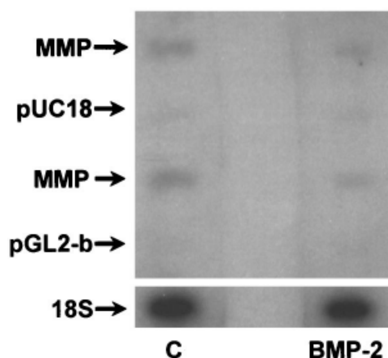

FIG. 6. Inhibition of collagenase gene transcription by BMP-2. A nuclear run-off assay was performed using nuclei isolated from Ob cells exposed to control (C) or to BMP-2 at 0.3 mM for 2 h. Nascent transcripts were radiolabeled with [α-^{32}P]UTP and hybridized to immobilized cDNAs for collagenase-3 (MMP) in duplicate, 18S rRNA, and vector DNAs pUC 18 and pGL2-Basic (pGL2-b).

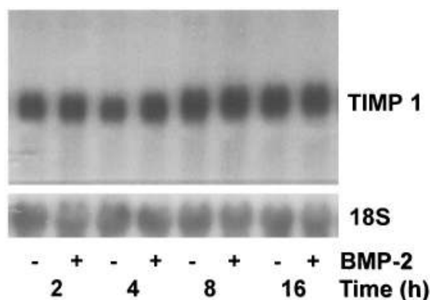

FIG. 7. Effect of BMP-2 on TIMP 1 mRNA. Northern blot analysis of total RNA (9 μg/lane) isolated from Ob cells cultured in the presence (+) or absence (−) of BMP-2 at 3.3 nM for 2–16 h was performed using ^{32}P-labeled cDNAs for TIMP 1 and 18S rRNA. The *upper panel* shows TIMP 1 mRNA, and the *lower panel* shows 18S rRNA from one of four independent cultures.

a modest stimulation of TIMP 1 mRNA levels after 4–8 h and increased the levels of TIMP 3 mRNAs by approximately 2-fold after 4–16 h. TIMP 3 mRNA levels, unlike those of TIMP 1 and TIMP 2, remained slightly elevated after 24 h in the presence of BMP-2 (data not shown).

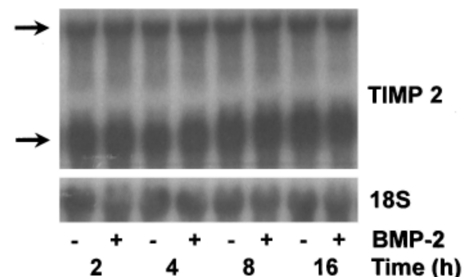

FIG. 8. Effect of BMP-2 on TIMP 2 mRNAs. Northern blot analysis of total RNA (9 μg/lane) isolated from Ob cells cultured in the presence (+) or absence (−) of BMP-2 at 3.3 nM for 2–16 h was performed using ^{32}P-labeled cDNAs for TIMP 2 and 18S rRNA. The *upper panel* shows TIMP 2 mRNAs (*arrows*), and the *lower panel* shows 18S rRNA from one of four independent cultures.

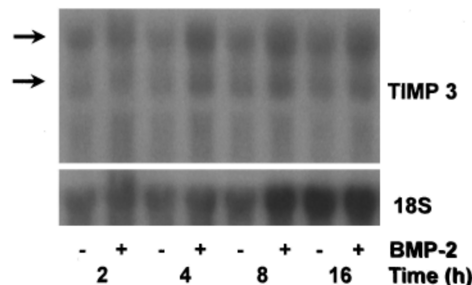

FIG. 9. Effect of BMP-2 on TIMP 3 mRNAs. Northern blot analysis of total RNA (7 μg/lane) isolated from Ob cells cultured in the presence (+) or absence (−) of BMP-2 at 3.3 nM for 2–16 h was performed using ^{32}P-labeled cDNAs for TIMP 3 and 18S rRNA. The *upper panel* shows TIMP 3 mRNAs (*arrows*), and the *lower panel* shows 18S rRNA from one of three independent cultures.

TABLE 1. Regulation of TIMP expression by BMP-2

Time (h)	TIMP 1	TIMP 2	TIMP 3
2	1.1 ± 0.1	1.1 ± 0.2	1.4 ± 0.2
4	1.8 ± 0.2[a]	1.2 ± 0.2	1.9 ± 0.2[a]
8	1.4 ± 0.1[a]	1.1 ± 0.1	1.9 ± 0.3[a]
16	1.2 ± 0.1	0.9 ± 0.1	1.7 ± 0.3

The levels of TIMP 1 mRNA, 1-kb TIMP 2 mRNA, and 3.5-kb TIMP 3 mRNA in three or four independent cultures were quantified by densitometry, and the data show mean relative changes ± SEM in TIMP mRNAs in cultures exposed to BMP-2 for 2–16 h *vs.* control values.

[a] Significantly different from control, $P < 0.05$.

Discussion

This study demonstrates that BMP-2 inhibits collagenase-3 mRNA and proteinase levels in osteoblastic cells. BMP-2 suppresses collagenase mRNA levels by up to 90%, and it is effective at concentrations of 0.03 nM. An increase in collagenase mRNA levels was noted after 8 h in control cultures. We speculate that this increase may be caused by the secretion of a collagenase stimulatory agent(s) into the culture medium, and this agent did not preclude the effect of BMP-2, suggesting that BMP-2 may prevent the action of collagenase stimulators. Consistent with this idea, BMP-2 prevented the increase in collagenase mRNA by retinoic acid, a stimulator of collagenase in osteoblasts (21) (Varghese, S., and E. Canalis, unpublished observations). The inhibition of collagenase mRNA by BMP-2 led to a decrease in the secretion of

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immunoreactive collagenase. The anticollagenase antibody cross-reacted with another protein that did not comigrate with purified procollagenase. The identity of this protein is currently unknown, and it might be active collagenase or a protease related to collagenase-3. Rat procollagenase-3 is an approximately 58-kDa proenzyme that is converted into an approximately 48-kDa active form (39). The levels of the cross-reacting protein remained unaltered in control and BMP-2-treated cultures, suggesting that this protein does not represent a processed form of procollagenase-3. BMP-2 also stimulated TIMP 1 and TIMP 3 expression, but did not alter TIMP 2 expression. The actions of BMP-2 on collagenase and TIMPs suggest that BMP-2 has the potential to inhibit collagen degradation.

BMP-2 did not alter the stability of collagenase mRNA and decreased the levels of collagenase hnRNA and the rate of transcription, indicating transcriptional down-regulation of the collagenase gene. Although hnRNA analysis does not distinguish between transcriptional regulation and changes in the processing of primary transcripts, a nuclear run-off assay confirmed transcriptional down-regulation.

Collagenase expression is stimulated by growth factors, such as basic fibroblast growth factor and platelet-derived growth factor BB, and inhibited by IGFs and TGFβ1 (22–24, 40). The inhibitory effect of BMP-2 on collagenase-3 expression is similar to that observed with TGFβ1 and IGFs, and these factors suppress collagenase expression after 2–4 h. The inhibitory effect of IGFs on collagenase expression is not apparent after 24 h, whereas those of BMP-2 and TGFβ1 persist after this time period, suggesting different mechanisms of action. TGFβ1 inhibits collagenase-3 transcription and destabilizes collagenase transcripts. In contrast, BMP-2 does not modify the stability of collagenase 3 mRNA, suggesting that the molecular actions of BMP-2 and TGFβ1 differ. The effects of BMP-2 and TGFβ1 on the regulation of TIMPs in Ob cells are similar, except that BMP-2, unlike TGFβ1, does not inhibit TIMP 2 expression (31, 40) (Canalis, E., unpublished observations). The regulation of TIMPs by BMP-2 differs from that of IGF-I and IGF-II because IGFs do not modify their expression in osteoblasts (23).

Inducers of bone formation may increase collagen content by stimulating collagen synthesis or by decreasing its degradation. In contrast, various bone-resorbing agents, such as PTH, inhibit collagen synthesis and stimulate collagen degradation and collagenase production (15, 16, 41). Thus, it appears that bone formation is associated with inhibition of collagenase expression, whereas bone resorption is associated with stimulation of collagenase synthesis. The precise roles of BMP-2 and other BMPs in osteoinduction and bone remodeling remain to be defined. The regulation of collagenase and TIMPs by BMP-2 reported in this study and the previously documented actions of BMP-2 on collagen synthesis may play a role in the osteoinduction caused by BMP-2.

In conclusion, BMP-2 suppresses the expression of collagenase-3 in osteoblastic cells by transcriptional mechanisms. BMP-2 also stimulates the expression of TIMP 1 and TIMP 3, but does not alter the expression of TIMP 2. We postulate that BMP-2 may reduce collagen degradation by modulating the synthesis of collagenase-3 and TIMPs in bone cells, and that these effects may in part mediate the effects of BMP-2 on bone formation.

Acknowledgments

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