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MMP-13 Is Induced During Chondrocyte Hypertrophy

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Abstract During development, mRNA for matrix metalloproteinase-13 (MMP-13) is found associated with cartilage undergoing hypertrophy, suggesting that this collagenase plays a role in cell enlargement and/or cartilage calcification. Using chondrocytes from prehypertrophic cartilage of chick embryo sternae, we have examined the relationship between MMP-13 expression and the transition to hypertrophy. When hypertrophy was induced by serum-free culture with ascorbate and bone morphogenetic protein-2 (BMP-2), MMP-13 mRNA levels paralleled those for type X collagen. Chondrocytes from the caudal, nonhypertrophying portion of chick sternae expressed neither type X collagen nor MMP-13, confirming that MMP-13 mRNA is a marker for hypertrophy. Zymography with conditioned medium yielded a proteinase band at 59 kDa, which was absent in nonhypertrophic chondrocytes. A polyclonal antibody raised against chick MMP-13 reacted with the 59-kDa protein, confirming that it is MMP-13. Although mRNA for MMP-13 peaked at days 4–5 of culture, only low levels of MMP-13 activity were present, and the activity increased gradually in parallel with later increases in MMP-2. These results suggest that MMP-13 is activated by MMP-2 during chondrocyte maturation, and that the combination of both proteinases is required to prepare cartilage matrix for subsequent calcification, before endochondral ossification. J. Cell. Biochem. 77:678–693, 2000.

Key words: collagenase-3; MMP; metalloproteinases; growth plate; MMP-2; mineralization

Endochondral bone formation is a process in which osteoblast-mediated bone formation occurs on a calcified cartilage scaffolding. Production of this calcified matrix is associated with several changes in the differentiation status of chondrocytes in the growth plate. These changes are characterized by marked alterations in gene expression: chondrocytes start expressing type X collagen and alkaline phosphatase and concomitantly decrease expression of type II, IX, and XI collagen mRNA. Associated with these events is an increase in chondrocyte size. Cell hypertrophy might be expected to require alterations in the extracellular matrix, including local degradation of matrix [Ballock et al., 1993]. Once fully differentiated hypertrophic chondrocytes are formed, matrix calcification is initiated. Studies of changes in matrix components have led to the proposal that establishment of a calcifiable matrix also requires degradation of some matrix molecules [Alini et al., 1992; Hunter 1991; Boskey et al., 1992; Alini et al., 1994].

Several studies have suggested that matrix metalloproteinases (MMPs) are associated with both chondrocyte hypertrophy [Dean et al., 1989] and with matrix vesicles implicated in cartilage matrix mineralization [Dean et al., 1992; Schmitz et al., 1996; Dean et al., 1996]. Investigations into chondrocyte production of MMPs have recently been stimulated by the

Abbreviations used: APMA, aminophenylmercuric acetate; Asc, ascorbate phosphate; BMP-2, bone morphogenetic protein-2; CMF-HBSS, calcium- and magnesium-free Hanks buffered saline solution; DMEM-hiG, Dulbecco's minimal essential medium with high glucose; FBS, fetal bovine serum; IL-1, interleukin-1; MMP, matrix metalloproteinase; MMP-13, collagenase-3; MMP-2; gelatinase A; TGF-β, transforming growth factor beta; TIMP, tissue inhibitor of metalloproteinases.

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discovery that MMP-13 is expressed both in osteoarthritic cartilage [Mitchell et al., 1996] and in hypertrophic and calcifying cartilage of mammalian growth plate [Mattot et al., 1995; Johansson et al., 1997; Wu et al., 1997]. MMP-13, also known as collagenase-3, cleaves type I collagen and was previously thought to be the rodent analogue of human interstitial collagenase (MMP-1). It is therefore also referred to as rodent interstitial collagenase. Although capable of cleaving in the triple helical region of a variety of collagens, human MMP-13 shows particularly potent activity towards type II collagen [Knäuper et al., 1996a], which is the major protein in chondrocyte matrix. MMP-13 can also degrade type X collagen and cartilage aggrecan [Mitchell et al., 1996; Knäuper et al., 1996b; Fosang et al., 1996a], and may therefore contribute to both collagen and proteoglycan degradation in the hypertrophying and calcifying chondrocyte matrix.

Most studies of chondrocyte MMPs have focused on cells from articular cartilage and the role of MMPs in the development of osteoarthritis [Billinghurst et al., 1997; Shlopov et al., 1997]. Articular chondrocytes express a variety of proteinases, including MMP-1 (interstitial collagenase) [Cawston et al., 1998], MMP-2 (gelatinase A) [Wardale and Duance, 1996], MMP-3 (stromelysin-1) [Wilhelm et al., 1993], MMP-8 (neutrophil collagenase) [Cole et al., 1996], and aggrecanase, a recently characterized aggrecan-cleaving enzyme that is a member of the ADAM family of proteinases [Hughes et al., 1998; Arner et al., 1999]. However, MMP-13 expression is low in normal articular cartilage and high only in osteoarthritic cartilage [Billinghurst et al., 1997; Moldovan et al., 1997; Shlopov et al., 1997]. Less information is available concerning MMP production by growth plate chondrocytes. Immunohistochemistry and in situ hybridization studies have localized "interstitial collagenase" to hypertrophic chondrocytes in both developing mouse embryos and rat long bones [Blair et al., 1989; Mattot et al., 1995]. Ballock et al. [1993] reported that pre-hypertrophic rat epiphyseal chondrocytes expressed low levels of stromelysin-1 (MMP-3) and "rat collagenase," and noted that both stromelysin and the collagenase mRNAs were markedly elevated in cultured chondrocytes that had undergone hypertrophy. Because the rodent collagenase studied in these reports is presumably the enzyme now known as MMP-13, this proteinase is implicated in the transition to chondrocyte hypertrophy. MMP-2 has also been found at high levels in growth plate [Wardale and Duance, 1996; Kawashima-Ohya et al., 1998], but the role of this proteinase during chondrocyte hypertrophy has been unclear. Retinoic acid, which promotes hypertrophy, increased MMP-2 expression [Nie et al., 1998]; however, parathyroid hormone (PTH)–related protein, which inhibits hypertrophy, has also been reported to increase MMP-2 levels [Kawashima-Ohya et al., 1998].

In the studies presented here, we demonstrate that MMP-13 gene expression in cultured prehypertrophic chick chondrocytes is a hypertrophy-related event. Using a recently cloned cDNA for chick MMP-13 (GenBank Accession number AF070478), we show that MMP-13 mRNA is induced by the same modulators that induce hypertrophy, and is expressed coordinately with type X collagen mRNA. However, studies examining the time course of MMP-13 activity demonstrate that the highest levels of MMP-13 activity are not seen until later stages of culture, implying that most enzyme activity is regulated by a nontranscriptional mechanism. In addition, we report parallel studies with MMP-2, showing that high levels of mRNA and activity are found in nonhypertrophic chondrocytes, but that MMP-2 expression decreases at early stages of hypertrophy. Furthermore, increases in MMP-2 mRNA and activity in late hypertrophic chondrocytes are coordinated with elevated levels of MMP-13 activity.

MATERIALS AND METHODS

Monolayer Cultures

Cells were isolated from the caudal and cephalic one-thirds of day 15 avian embryo by digestion at 37°C for 3 h with collagenase and trypsin as described previously [Leboy et al., 1997]. Primary cells were plated at a density of 3 sternae /100 mm tissue culture plate in Dulbecco's Modified Eagle's medium with high glucose (DMEM-hiG), supplemented with 10% NuSerum IV (Collaborative Biomed., Bedford, MA) and 100 U/ml penicillin/streptomycin. Primary cultures were maintained for 5 days at 37°C in 5% CO_2 and the floating cells were replated at a plating density of 4×10^4 cells/cm^2 in 10% NuSerum DMEM-hiG containing 4 U/ml hyaluronidase. On the following day, cul-

tures were supplemented with 0.15 mM ascorbate phosphate (WAKO Chem., Richmond, VA) and/or 30ng/ml BMP-2 (Genetics Institute, Cambridge, MA) to promote maturation [Leboy et al., 1997]. Forty-eight hours before harvesting, chondrocytes were switched to serum-free DMEM-hiG containing 1 mM cysteine, 10 pM triiodothyronine (Sigma Chemical Co., St. Louis, MO), and 60 ng/ml insulin (Sigma), with bone morphogenetic protein-2 (BMP-2) and/or ascorbate phosphate as required.

Alginate Bead Cultures

Type X collagen-positive, hypertrophic chondrocytes were isolated from the cephalic core of day 17 avian embryonic sternum [D'Angelo and Pacifici, 1997] and digested with trypsin and collagenase in calcium- and magnesium-free Hanks buffered saline solution for 3–4 h at 37°C. The cell suspensions were filtered through a Nitex filter, counted, and resuspended at a density of 5×10^6 cells/ml in alginate/0.15 M NaCl, pH 7.2 [Keltone LVCR™, Kelco, NJ). Bead cultures were prepared by extruding the alginate/chondrocyte suspension dropwise through an 18-gauge needle into 102 mM $CaCl_2$, resulting in beads containing approximately 1×10^5 chondrocytes/bead. Beads were rinsed with 0.15 M NaCl, placed into 35-mm Petri dishes and covered with 2 ml serum-free DMEM-hiG containing 50 U penicillin/streptomycin, 2 mM L-glutamine, 1 mM cysteine, and 1 mM sodium pyruvate.

Gelatin and β-Casein Zymography

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed on conditioned media isolated at different days of culture from chondrocytes in monolayer or alginate bead serum-free cultures. All conditioned media represented 48-h samples, and was stored at −70°C until use. Preliminary experiments indicated that neither trypsin nor APMA activation was required. Eight percent polyacrylamide gels containing 0.1% gelatin (Sigma) or 10% polyacrylamide gels with 0.1% β-casein (Sigma) loaded with 3–20 μl medium/well were run for 1.5–2 h at 18 V/cm under nonreducing conditions. SDS was removed by 30-min incubation in 1% Triton X-100, and the gel-immobilized enzymes were permitted to react overnight at 37°C in 100 mM Tris (pH 8.0)/10 mM $CaCl_2$ with or without 20 mM EDTA to identify metalloproteinases. Enzyme activity was localized by staining gels with Coomasie Blue and destaining in 7% acetic acid/25% methanol. The relative density of cleared bands was determined using a Molecular Dynamics Densitometer with Image Quant software (Molecular Dynamics, Sunnyvale, CA).

Northern Analysis of Total mRNA

Total RNA from monolayer cultures was prepared, run on agarose gels, and Northern blotted as described previously [Leboy et al., 1997]. To ensure equal loading of RNA, blots were hybridized with probe for constitutively expressed glyceraldehyde-3-phosphate dehydrogenase; however, expression of this mRNA was found to be suppressed by ascorbate and therefore unsuitable for normalization. All blots were therefore stained with methylene blue to verify equal loading. Levels of specific mRNAs were detected by hybridizing the blots with ^{32}P-labeled probes prepared by either random priming (MMP-13 and MMP-2) or riboprobe synthesis (type II and X collagen). A 2-kb chick MMP-2 cDNA [Aimes et al., 1994] subcloned into Bluescript SK+ (Stratagene, La Jolla, CA) was provided by Dr. James P. Quigley, SUNY Stony Brook. The 450-bp MMP-13 probe has been described previously [Lei et al., 1999], as have the 183-bp type X collagen and 800-bp type II collagen probes [Leboy et al., 1989]. Quantitation of probe hybridization was performed with a Molecular Dynamics FluorImager.

Peptide Antibody Production

Polyclonal antibody was raised by Cocalico Biologicals, PA against a synthetic peptide sequence (Bio-Synthesis, Inc., Lewisville, TX) corresponding to putative amino acids 268–282 of the avian MMP-13 sequence. The peptide was chosen by selecting amino acid sequences deduced from the cDNA [Lei et al., 1999] for those with high antigenicity, specificity for MMP-13 but not other MMPs, and the ability to cross react with MMP-13 from mammalian sources. Antibody titer was determined by enzyme-linked immunosorbent assay against synthetic peptide antigen, the IgG fraction was precipitated with ammonium sulfate, and the antibody was finally affinity-purified by passage over a Sulfo-Link column (Pierce Chem., Rockford, IL) containing the peptide antigen.

Western Blot Analysis

Total protein in conditioned media was determined by the BCA-enhanced protocol kit (Pierce Chem., Rockford IL). Conditioned media was concentrated 10–20-fold using a Centricon 30MW ultrafiltration system (Millipore, Bedford, MA). Samples containing equal protein were loaded in Laemmli sample buffer without β-mercaptoethanol and subjected to SDS polyacrylamide gel electrophoresis on 4–20% Tris-glycine gradient gels or 8–16% Tris-glycine gradient gels (Novex, San Diego, CA). Samples were transferred to Protran nitrocellulose (Schleicher & Schuell, Keene, NH), and were blocked for 2 h at room temperature in 3% nonfat dried milk dissolved in phosphate-buffered saline (PBS). Blots were incubated with primary antibody raised against avian MMP-13 (as described above) or avian MMP-2 (a gift from Dr. James Quigley) in 1% nonfat dried milk in PBS, and bands of immunoreactivity were visualized by the Renaissance Western Blot Chemiluminescence reagent (NEN, Boston, MA). The same antibodies were also tested with purified rat MMP-13 (a gift from Dr. John Jeffrey). Blots were exposed to X-Omat Blue film (NEN), and stained protein was quantitated using a Molecular Dynamics ImageQuant program and densitometer.

Immunohistochemistry

Embryonic day 18 chick sternae were frozen in liquid nitrogen and embedded in OCT mounting medium. Transverse sections (6 μm) were cut, placed in a humidified chamber, and blocked without fixation in 3% normal goat serum in PBS for 1 h at room temperature. Samples were then reacted with primary antibody overnight at 4°C, washed with serum/PBS, and reacted with rhodamine-conjugated secondary antibody. After washing, the tissue was mounted with Fluormount and viewed using a Fluoview confocal laser scanning system (Olympus America, Melville, NY) with an excitation filter of 488 nm. Images were captured with a 585IF filter in place, and all regions of the tissue were imaged using the same instrument settings.

Substrate Overlay for In Situ Proteinase Activity

A method of fluorescent substrate overlay developed by our laboratory permits detecting in situ proteinase activity in intact frozen tissue sections [Sarment et al., 1999]. This assay is based on the fact that the fluorescence is quenched when substrate is in its native conformation; however, cleavage by proteinases present in the tissue section causes the quenched substrate to become fluorescent [Haugland, 1996] Embryonic day 18 chick sternae were frozen, embedded, and sectioned as described above. Six-micron transverse sections were immediately overlaid with 20-μl rhodamine-labeled β-casein (4 mg/ml in 10 mM Tris, pH 7.8) or 20-μl fluorescein isothiocyanate (FITC)-labeled gelatin (2 mg/ml in 50 mM Tris, pH 7.6). Fluorescent-labeled substrates were obtained from Molecular Probes (Eugene, OR). Coverslips were applied and the samples were incubated in a humid chamber at 37°C for 3 h. Areas of proteolytic activity were visualized by fluorescence microscopy and were compared with parallel samples inhibited by EDTA added along with the substrate.

RESULTS

MMP-13 mRNA Is Produced During Chondrocyte Hypertrophy

MMP-13 gene expression during chondrocyte hypertrophy was analyzed in prehypertrophic chondrocytes from the upper (cephalic) region of day 15 chick embryo sternae, cultured under conditions that induce maturation. Although these chondrocytes do not undergo hypertrophy-related changes when cultured with 10% fetal bovine serum or NuSerum, addition of BMP-2 or ascorbate phosphate induces expression of both type X collagen and alkaline phosphatase, and transfer to serum-free medium accelerates this process [Leboy et al., 1997]. Because long-term growth of chondrocytes under serum-free conditions induces apoptosis [Venezian et al., 1998], chondrocytes were cultured with BMP-2 and ascorbate phosphate in the presence of 10% NuSerum and were transferred to serum-free medium 48 h before harvesting. Northern blots of RNA from upper sternal chondrocytes, hybridized to a cDNA probe for avian MMP-13 (Fig. 1A), showed a single band at 2.3 kb corresponding to the previously reported size for avian MMP-13 mRNA [Lei et al., 1999]. Comparison of the relative levels of MMP-13 mRNA and type X collagen mRNA suggested that both mRNAs were induced at the same time (Fig. 1B). Neither MMP-13 mRNA nor type X mRNA

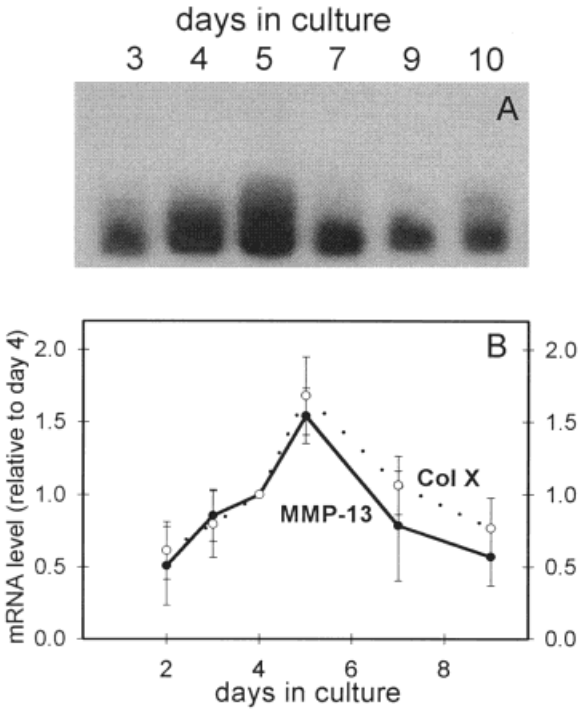

Fig. 1. MMP-13 mRNA is expressed coordinately with type X collagen mRNA in upper sternal chondrocytes. Chondrocytes isolated from day 15 avian embryo upper (prehypertrophic) sterna were cultured in serum-free conditions as described in Materials and Methods. One hundred fifty micromolar ascorbate phosphate and 30 ng/ml BMP-2 were added to cultures to induce hypertrophy. Total cellular RNA was extracted, and 5–10 μg RNA samples were glyoxalated and run on 1% agarose gels. **A:** Representative Northern blot, containing 10 μg RNA/lane, hybridized with a cDNA prepared from the avian MMP-13 sequence [Lei et al., 1999]. **B:** Comparison of MMP-13 mRNA and type X collagen mRNA levels, relative to the level of each mRNA at day 4. Results are the average of Northern blot scans from at least six independent preparations of upper sternal chondrocytes. For abbreviations, see list.

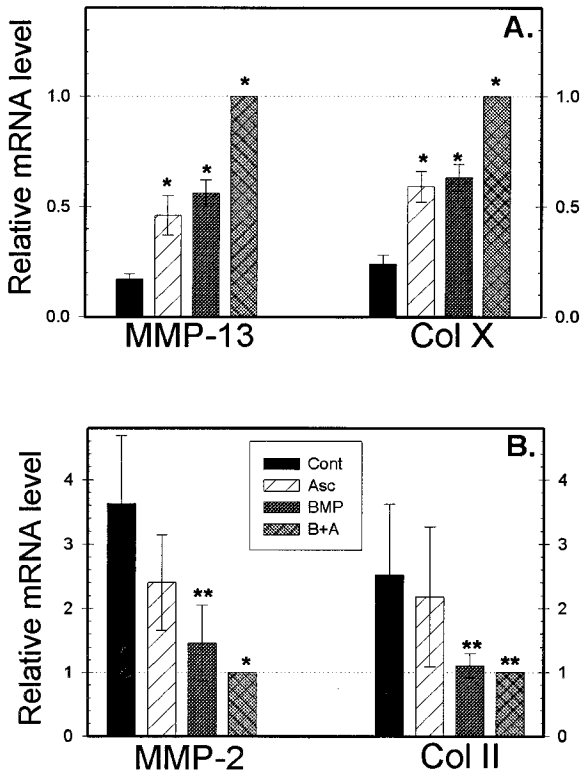

Fig. 2. Effects of BMP-2 and ascorbate phosphate on expression of MMP-13, MMP-2, type II collagen and type X collagen. RNA from upper sternal chondrocyte cultures, transferred to serum-free medium at day 2 and harvested at day 4, was analyzed by Northern blot hybridization as described in Figure 1. Each value represents the average of Northern blots derived from at least four independent chondrocyte cultures, and mRNA level is expressed relative to that seen with chondrocytes cultured in the presence of BMP plus ascorbate phosphate. **A:** The pattern of induction of MMP-13 mRNA is not significantly different from that seen for type X collagen; both BMP and ascorbate phosphate increase mRNA levels, with maximal stimulation seen with both inducers. **B:** The pattern of induction of MMP-2 mRNA at day 4 of culture is similar to that seen for type II collagen; both BMP and BMP plus ascorbate phosphate significantly diminish mRNA levels. The effect of ascorbate phosphate alone on levels of MMP-2 and type II collagen mRNA was highly variable at day 4. *Significantly different from value with no inducer at $P < 0.001$. **Significantly lower than value with no inducer at $P < 0.01$. For abbreviations, see list.

were detectable in lower sternal chondrocytes cultured with BMP plus ascorbate phosphate. To confirm that MMP-13 is induced as a hypertrophy-related gene, the pattern of induction by BMP-2 and ascorbate phosphate at day 4 was compared with that for type X collagen (Fig. 2A). Like type X collagen, MMP-13 mRNA levels were increased by either BMP or ascorbate phosphate, with highest levels requiring both BMP and ascorbate phosphate. In contrast, MMP-2 mRNA levels, like those for type II collagen, were suppressed in upper sternal chondrocytes at day 4 by inducers of hypertrophy (Fig. 2B). However, while type II collagen expression continues to decline in the presence of inducers [Leboy et al., 1989], MMP-2 mRNA levels are lowest at early times of hypertrophy and subsequently increase in the presence of BMP plus ascorbate phosphate (see Fig.7B).

Zymography of Conditioned Medium From Cultured Chondrocytes

Production of active metalloproteinases in chondrocyte cultures was assayed by gelatin zymography of 48-h conditioned medium (Fig.

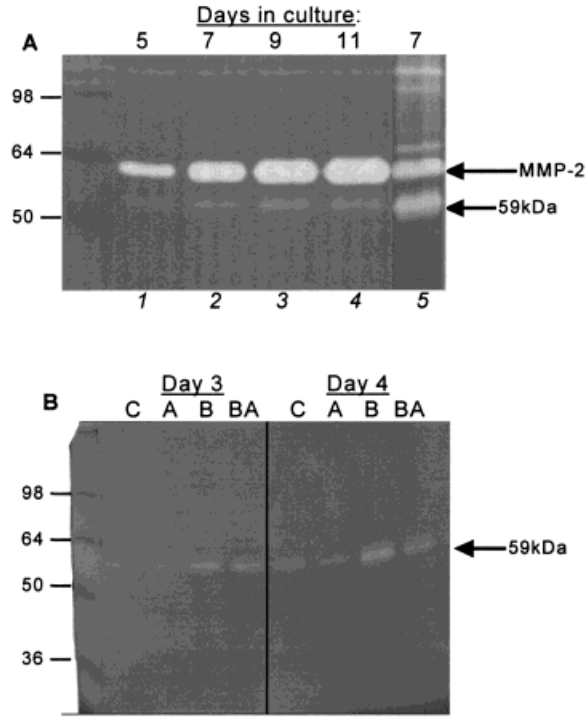

Fig. 3. Zymographic analysis of metalloproteinase activity during chondrocyte hypertrophy. Upper sternal chondrocytes were transferred to serum-free medium 48 h before harvesting conditioned media samples. Two hundred nanograms protein was loaded in each lane, and zymography was performed as described in Materials and Methods. **A:** Gelatin zymography. Conditioned medium was loaded on 8% polyacrylamide gels containing 0.1% gelatin and electrophoresis was performed under nonreducing conditions for 1½ h. **Lanes 1–4:** Day 15 (prehypertrophic) chondrocytes were cultured in monolayer with BMP plus ascorbate and the media were harvested at days 5, 7, 9, and 11. Both the 64-kDa MMP-2 and the 59-kDa activity increases with time in culture. **Lane 5:** Day 17 (hypertrophic) chondrocytes were cultured in alginate beads (see Materials and Methods) for 7 days, with no added inducers. **B:** β-Casein zymography. Medium samples from day 3 and 4 cultures in the absence of inducers (C), with 150 μM ascorbate-phosphate (A), with 30 ng/ml BMP-2 (B), or with both BMP and ascorbate (BA) were loaded on 12% polyacrylamide gels containing 0.1% β-casein and electrophoresis was performed under nonreducing conditions for 2 h. Both day 3 and day 4 samples expressed low levels of the 59-kDa proteinase, and samples from cultures incubated with BMP or BMP plus ascorbate showed elevated levels. β-Casein is a substrate for MMP-13 but not MMP-2 [Lemaitre et al., 1997]. For abbreviations, see list.

3A). Prehypertrophic chondrocytes from day 15 sternae, induced to hypertrophy by addition of BMP plus ascorbate phosphate, produced a major band of proteinase activity with M_r of approximately 64 kDa, along with another band at 59 kDa , and the level of both enzymes increased up to day 9 of culture (lanes 1–4). Hypertrophic (day 17) chondrocytes cultured in alginate beads to maintain the hypertrophic state showed a marked increase in the 59-kDa protein (lane 5), and several additional bands of proteinase activity were seen. When zymography was carried out in the presence of 20 mM EDTA, both the 64-kDa band and the 59-kDa band were absent, indicating that these enzymes were metalloproteinases (data not shown). Furthermore, activity was unaffected by trypsin or APMA activation, implying that both proteinases were present in conditioned medium in a form that was active on SDS-PAGE zymography. Because chondrocytes are known to constitutively produce MMP-2, which has an active form at 64 kDa, we performed Western blotting using antibody raised against avian MMP-2 [Aimes et al., 1994]. As demonstrated in Figure 4C, immunoreactive avian MMP-2 migrated with the same mobility as the major 64-kDa band seen on zymography.

Because β-casein is a substrate for MMP-13 [Lemaitre et al., 1997], we performed zymography with 0.1% β-casein as substrate; this resulted in only one band of activity at 59 kDa that was present at low levels in day 3 and day 4 cultures of upper sternal chondrocytes (Fig. 3B). Conditioned medium from lower sternal chondrocytes, which are not induced to hypertrophy, showed no proteinase activity on β-casein zymography (data not shown). Furthermore, when upper sternal chondrocyte cultures were incubated with BMP-2 to promote hypertrophy, conditioned medium consistently showed more than 10-fold higher levels of the 59-kDa proteinase than in the absence of BMP (Fig. 3B). Both of these results suggested that the 59-kDa band is associated with chondrocyte hypertrophy, and might be MMP-13.

Western Blot Analysis of MMP-13 Protein

To confirm the identity of the 59-kDa proteinase, a 13 amino acid peptide was constructed based on sequence information from the avian MMP-13 cDNA. This peptide contained a region, located between a hemopexin domain and a cysteine switch domain, corresponding to amino acids 272–284 of rat, mouse, and human MMP-13. It was designed to cross react with both mammalian and avian MMP-13, but not with other known MMPs. Polyclonal antibody was then raised against this peptide, and was used for Western blot analysis of conditioned medium from

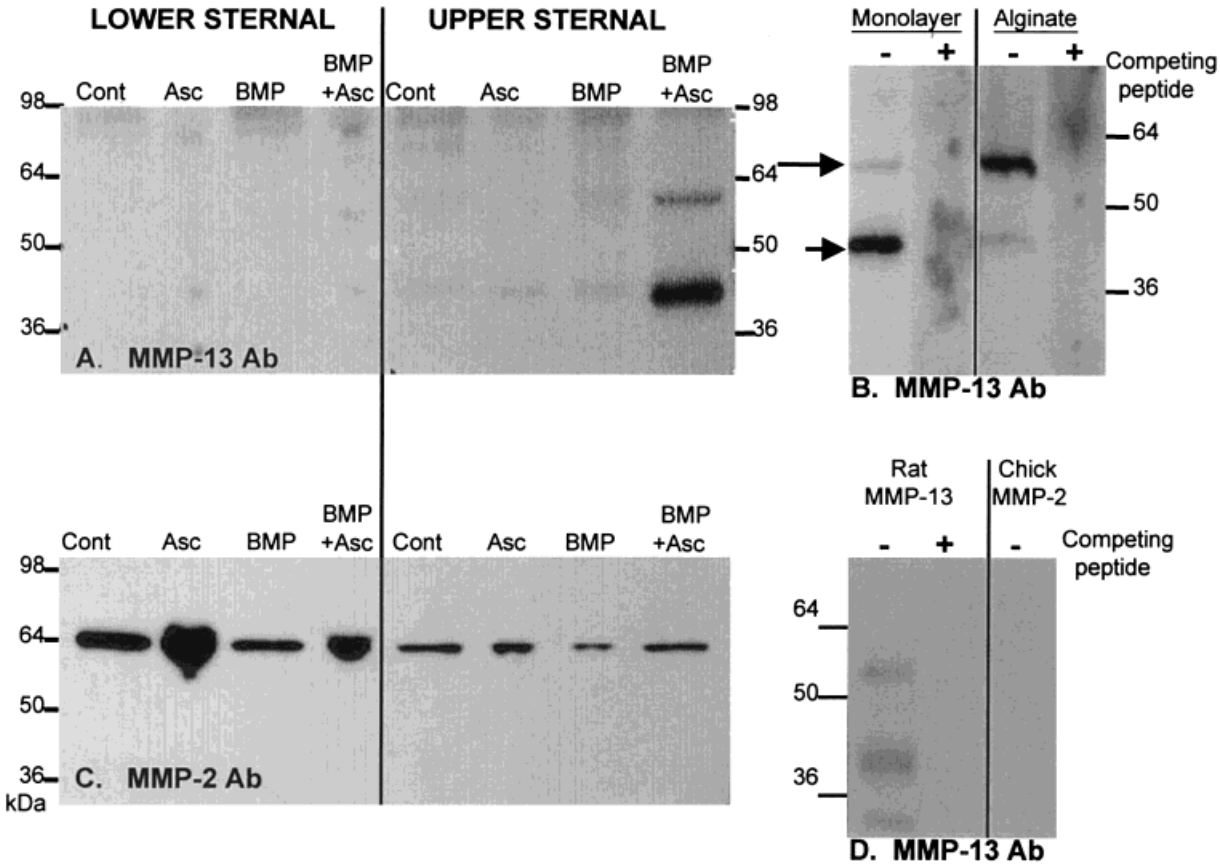

Fig. 4. Western blots of conditioned media demonstrate that the 64-kDa proteinase is MMP-2 and the 59-kDa proteinase is MMP-13. Electrophoresis of conditioned media samples was performed on Tris-glycine gradient gels as described in the legend to Figure 3. The samples were then blotted onto nitrocellulose and subjected to Western blot analysis as described in Materials and Methods. **A:** Western blotting of 4–20% gradient gels with antibody against avian MMP-13. Media from lower sternal chondrocyte cultures at day 10 showed no bands of immunoreactivity except for several faint bands with BMP plus ascorbate samples. Media from upper sternal chondrocytes at day 10 showed one reactive band at 59kDa and a stronger band at 43kDa; these two bands were markedly increased in cultures treated with BMP plus ascorbate. **B:** Western blotting of 8–16% gradient gels with antibody against avian MMP-13 and peptide competitor. The first two lanes contain media samples from day 15 upper sternal chondrocytes cultured in monolayer until day 10 in the presence of BMP plus ascorbate; however, the right lane was incubated with the anti-MMP-13 antibody in the presence of 25 μg of the peptide used to raise the antibody. The last two lanes contain media samples from day 17 upper sternal chondrocytes cultured in alginate beads until day 7. Both monolayer and alginate cultures produced a 59-kDa band and a 43-kDa band (arrows); however, although monolayer cultures yielded primarily the 43-kDa protein, alginate cultures produced primarily the 59-kDa band. Immunoreactivity of both 43- and 59-kDa bands was abolished by competing peptide. **C:** Western blotting of 4–20% gradient gels with antibody against avian MMP-2. This polyclonal antibody identified MMP-2 as a single band of 64kDa that reacts with day 9 conditioned media from both lower sternal and upper sternal chondrocyte cultures. Levels of MMP-2 protein are elevated in lower sternal cultures treated with ascorbate, and decreased in chondrocyte cultures treated with BMP alone. **D:** Western blotting of purified rat MMP-13 and recombinant chick MMP-2 demonstrates that the antibody raised against chick MMP-13 peptide cross reacts with rat MMP-13, but this antibody does not react with chick MMP-2. Cont, control; Asc, ascorbate. For other abbreviations, see list.

lower and upper sternal chondrocytes. As seen in Figure 4A, the antibody showed no reactivity with medium from day 9 cultures of lower sternal chondrocytes, whereas upper sternal chondrocytes showed low levels of two immunoreactive proteins, one migrating at 59 kDa and a stronger band at 43 kDa. Like the mRNA for MMP-13, both proteins were increased when cultures were treated with BMP plus ascorbate phosphate. Figure 4B presents Western blots of conditioned medium from upper sternal chondrocytes in which antibody binding was carried out in the absence or presence of peptide used to

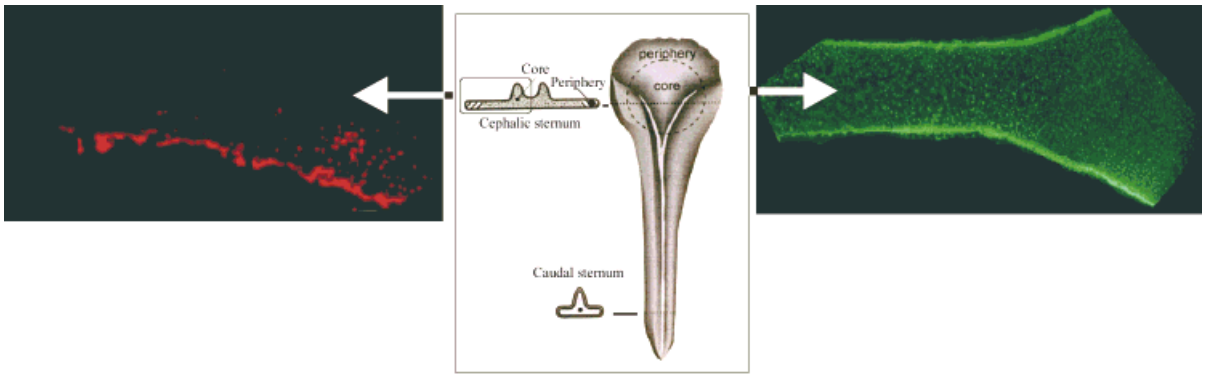

Fig. 5. Substrate overlay demonstrates that active collagenase-3 is found primarily in the hypertrophic region of avian day 18 embryonic upper sternum. Six-micron transverse sections of frozen day 18 embryonic upper sternum were overlaid with rhodamine-labeled β-casein (**left**) or fluorescein isothiocyanate–labeled gelatin (**right**) and substrate allowed to digest for 3 h at 37°C. Samples were then examined by fluorescence microscopy to detect the cleaved, fluorescent substrate.

raise anti-MMP-13 antibody. Although both monolayer and alginate bead cultures yielded the same two immunoreactive bands, the 43-kDa band predominated in monolayer cultures of prehypertrophic chondrocytes induced with BMP plus ascorbate, but the 59kDa was the major product from alginate cultures of day 17 hypertrophic chondrocytes. The chick MMP-13 peptide competed with both the 59-kDa and 43-kDa bands. These data, combined with zymographic results showing hypertrophy-related MMP activity at 59 kDa, imply that the 59-kDa band is active MMP-13. The second 43-kDa form is presumably an inactive degradation product of MMP-13 that is released into conditioned medium in monolayer culture of induced prehypertrophic chondrocytes and is less abundant in alginate-cultured mature hypertrophic chondrocytes. To confirm the specificity of the MMP-13 antibody, we performed Western blotting with purified rat MMP-13 and chick MMP-2 (Fig. 4D). As expected, the antibody cross reacted with the rat MMP-13, but not with the avian MMP-2. The bands seen with rat MMP-13 are consistent with a 52-kDa latent form and a 42-kDa active form reported for this enzyme [Woessner, 1998], along with a smaller band at approximately 30 kDa.

Analysis of Active Metalloproteinase in Developing Sternal Cartilage

To determine whether MMP-2 and MMP- 13 are active in the avian sternum in vivo, we incubated frozen tissue sections from day 18 sternae with an overlay of commercially available fluorescein- or rhodamine-labeled proteinase substrates. At this stage in development the core portion of the cranial sternum contains hypertrophic chondrocytes, whereas the peripheral region surrounding the core is primarily proliferating chondrocytes [D'Angelo and Pacifici, 1997]. As shown in Figure 5, proteinase activity was present throughout both the hypertrophic core region and the proliferating peripheral region of upper (cephalic) sternae when fluorescein-labeled gelatin was used as substrate (right). However, when rhodamine-labeled β-casein was the substrate, active enzyme was primarily confined to the hypertrophic core region, and the lower edge region of the section containing perichondrium. Most of the activity with either substrate was eliminated when sections were incubated in the presence of EDTA [Sarment et al., 1999], and no activity was observed with sections from the lower sternum (data not shown). The presence of EDTA-sensitive, β-casein degrading activity, which is localized in the hypertrophic core of the upper sternum, suggests that MMP-13 is active in this region. The degradation of gelatin substrate throughout both the hypertrophic and proliferating regions of the upper sternum implies that the gelatinases MMP-2 and/or MMP-9 may also be active during sternal development.

Immunohistochemical Localization of MMP-13

The polyclonal antibody raised against MMP-13 was also used to examine MMP-13

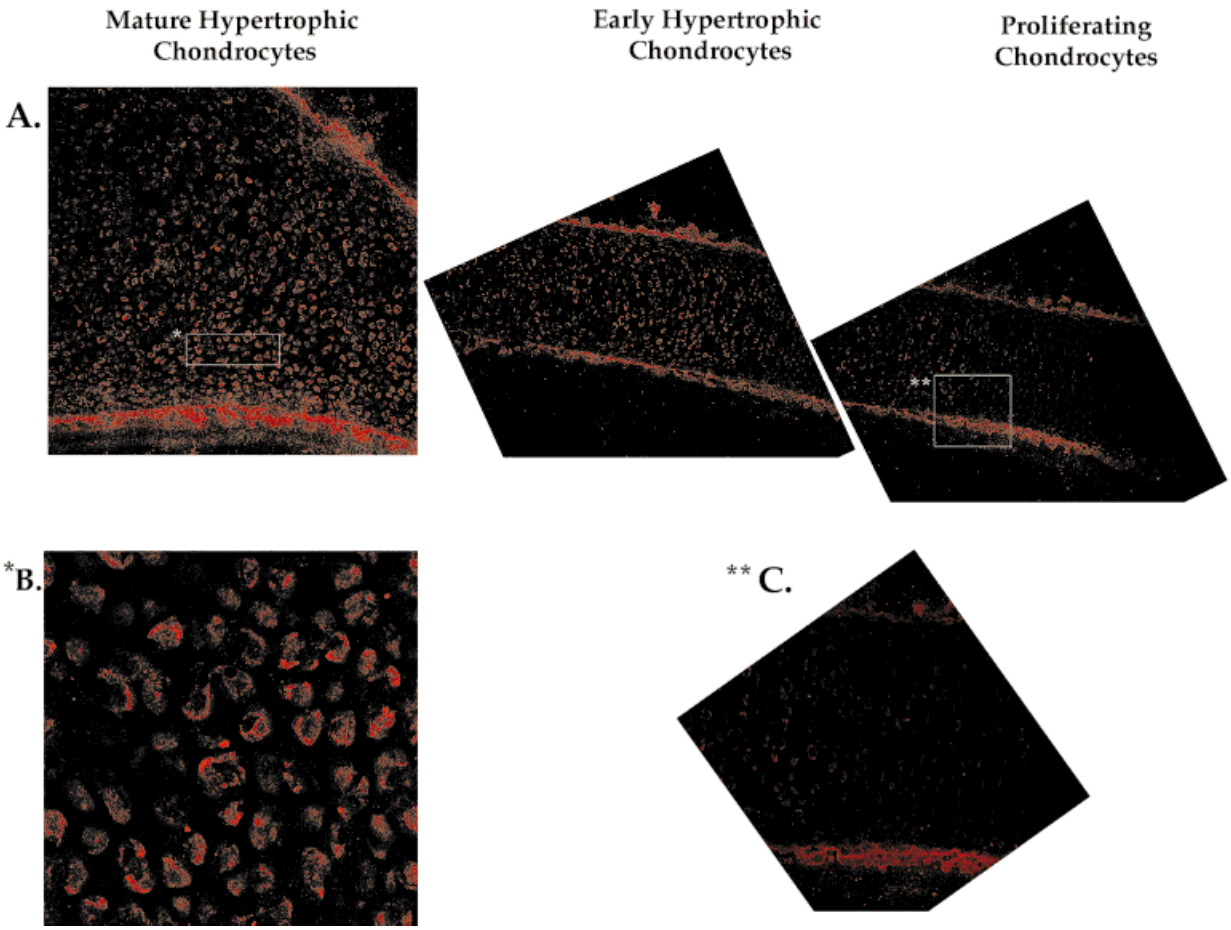

Fig. 6. Immunohistochemistry indicates that collagenase-3 is present primarily in the hypertrophic region of day 18 chick embryo sternum. Five-micron transverse sections of frozen day 18 embryonic upper sternum were reacted with a 1:5 dilution of affinity-purified chick collagenase-3 antibody and rhodamine-conjugated second antibody. They were maintained in a humidified atmosphere and viewed using confocal laser scanning microscopy (excitation filter = 488 nm; emission filter = 585 IF). **A:** Three adjacent sections of the upper sternal region with images captured using identical instrument settings. The mature hypertrophic region (left image) corresponds to the core region (see Fig. 5) and the two images at the right extend into the peripheral, less mature, region of the upper sternum. Magnification, ×200. Chondrocyte staining increases as the cells become more mature, and all stain was abolished in the presence of competing peptide. Edges of the tissue are apparent as regions of high rhodamine staining. **B:** A ×800 view of the boxed region in A, indicated by a single asterisk, shows cell-associated staining of mature hypertrophic chondrocytes. **C:** A ×400 view of the boxed region in A indicated by double asterisks displays lighter staining around early hypertrophic chondrocytes on the left side and little immunoreactivity of the proliferating chondrocytes on the right side.

expression in sternae from 18-day chick embryos. Confocal laser scanning views of a transverse section through the upper sternum of day 18 chick embryos are presented in Figure 6. Strong immunoreactivity was observed in the core region containing mature hypertrophic chondrocytes (Fig. 6A, left panel), and this reactivity decreased in regions containing fewer hypertrophic chondrocytes (right panels). At higher magnification (Fig. 6B,C), the staining was seen to be pericellular, suggesting that MMP-13 may be membrane associated.

Relationship Between MMP-13 Activity and MMP-2 Expression

Using conditioned medium from prehypertrophic upper sternal chondrocytes cultured with BMP plus ascorbate phosphate, the amount of MMP-13 activity found in 48-h conditioned medium increased significantly between days 4–5 and day 9 (Fig. 3A). Thus, although mRNA for MMP-13 reached maximal levels at days 4–5 of culture (Fig.1B and Fig.7A), the level of active enzyme was rela-

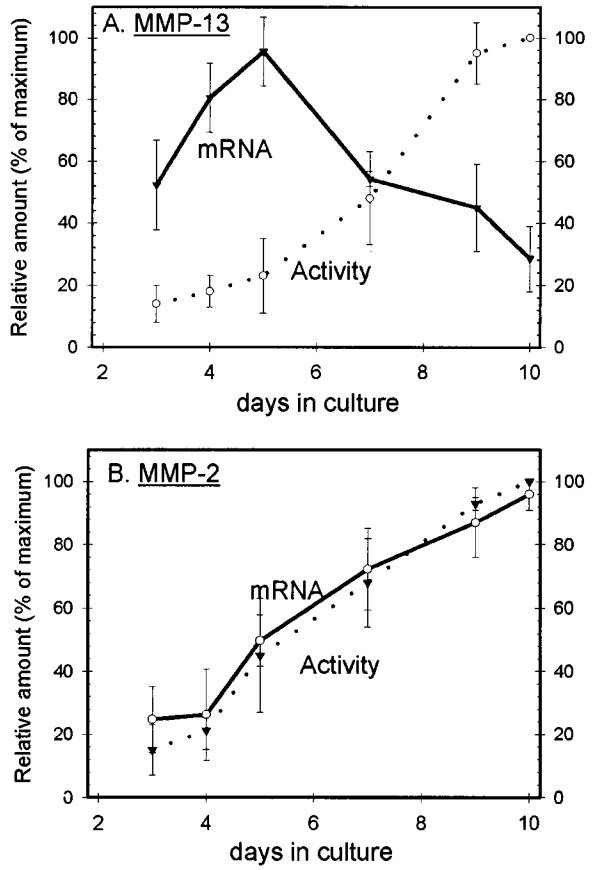

Fig. 7. Relationship between expression of MMP-13 and MMP-2 in upper sternal chondrocytes induced to hypertrophy with BMP plus ascorbate. Total RNA was prepared from chondrocytes cultured for the last 48 h in serum-free medium and Northern blots prepared using 5–10 μg RNA/lane for MMP-13 and 20 μg RNA/lane for MMP-2. Activity of each proteinase was determined by digital scanning of zymograms on a Molecular Dynamics densitometer. **A:** Although mRNA levels for MMP-13 peak at days 4–5 of culture with inducers of hypertrophy, activity levels are relatively low at this time, and gradually rise to maximal levels at days 9–10. **B:** Both MMP-2 RNA and MMP-2 activity are low until cultures approach confluence (days 5–6), and then rise in parallel. The time course for MMP-13 activity is more similar to the time course for MMP-2 mRNA and MMP-2 activity. For abbreviations, see list.

tively low at this time but increased markedly at later times (Fig.7A). Furthermore, although highest levels of MMP-13 mRNA at days 4–5 were seen with a combination of BMP plus ascorbate, MMP-13 activity at day 4 with BMP alone was the same as with BMP plus ascorbate, and the stimulatory effects of BMP plus ascorbate were only apparent at later times (Table I). These observations suggest that after mRNA for MMP-13 has been induced, MMP-13 activity is regulated by one or more other factors. Because MMP-2 has been reported to activate MMP-13 [Knäuper et al., 1996b], we examined the possibility that levels of active MMP-13 correlate with increasing MMP-2 activity. Relatively high levels of active MMP-2 are produced by cultured sternal chondrocytes in the absence of inducers of hypertrophy. This is consistent with reports that MMP-2 is a constitutively expressed enzyme [Birkedal-Hansen et al., 1993]. In the presence of BMP plus ascorbate phosphate, which induces highest levels of MMP-13 mRNA, the amount of MMP-2 mRNA was low at early culture times (Fig. 2B) and increased approximately fourfold by day 9 (Fig.7B). Because this increase starts after day 4 of culture, it may be correlated with the onset of confluence. Similarly, levels of activated MMP-2 enzyme also increased four- to fivefold between day 4 and day 9 (Fig.7B). The onset of elevated MMP-13 activity coincided with increased MMP-2 activity (Fig.7A), suggesting that time-dependent increases in MMP-2 seen with inducers of hypertrophy may be responsible for increasing amounts of activated MMP-13 found at later stages of hypertrophic chondrocyte cultures.

MMP Expression in Nonhypertrophic Chondrocytes

Lower sternal chondrocytes were characterized by high levels of MMP-2, and culture with ascorbate consistently increased levels of both MMP-2 activity and MMP-2 protein (Fig. 4C). These permanent chondrocytes also produced high levels of type II collagen mRNA but no detectable MMP-13 or type X collagen mRNA (Table II), even when cultured with BMP plus ascorbate phosphate. Comparing upper sternal and lower sternal chondrocytes after 4 days in serum-free culture, upper sternal chondrocytes without added inducers produced less MMP-2 and type II collagen mRNA, but more MMP-13 and type X collagen mRNA than lower sternal chondrocytes cultured with inducers. This is consistent with previous reports that prehypertrophic chondrocytes will initiate some hypertrophy-related changes when placed under serum-free conditions with insulin and thyroxine, without requiring additional inducers [Böhme et al., 1992; Quarto et al., 1992; Leboy et al., 1997].

TABLE I. Effects of BMP-2 and Ascorbate on Levels of Active MMP-13 in Conditioned Medium of Upper Sternal Chondrocytes[a]

	Additions to serum-free culture			
Day of culture	None	+Asc	+BMP-2	BMP + Asc
Day 4	3.05 ± 0.50	3.30 ± 0.36 $P = 0.03$*	33.63 ± 17.95	25.53 ± 13.33 $P > 0.05$
Day 9	0.23 ± 0.12	11.83 ± 3.51 $P = 0.003$	54.70 ± 8.42	120.30 ± 34.71 $P = 0.03$

[a]Levels of MMP-13 in conditioned medium (48-h samples) were analyzed by casein zymography. Samples (200 ng protein) were loaded on gels without prior activation. Gels were stained with Coomassie Blue and cleared regions were quantitated by densitometry. Results are expressed in arbitrary densitometry units, with the negative values (representing regions of proteinase activity) converted to positive numbers. For abbreviations, see list.
*P values indicate probability that the value is significantly different from that with BMP alone.

TABLE II. BMP Plus Ascorbate (Asc) Induces mRNA for MMP-13 and Type X Collagen in Prehypertrophic Upper Sternal Chondrocytes But Not in Permanent Lower Sternal Chondrocytes[a]

		mRNA levels (relative to USC with BMP + Asc)			
Chondrocytes	Additions	MMP-13	MMP-2	Type X	Type II
Upper sternal	None	0.17 ± 0.03	3.63 ± 0.06	0.24 ± 0.04	2.52 ± 0.72
Upper sternal	BMP + Asc	1.00	1.00	1.00	1.00
Lower sternal	BMP + Asc	<0.1	5.2 ± 1.15*	<0.1	3.36 ± 0.21*

[a]Chondrocytes from upper or lower sternal regions of day 15 chick embryos were cultured in monolayers in the presence or absence of BMP-2 and ascorbate phosphate, and total RNA was analyzed by Northern blot hybridization. Chondrocytes were transferred to serum-free medium at day 2, and RNA was prepared from cultures at day 4.
*Significantly higher than upper sternal chondrocytes (USC) cultured without BMP + Asc ($P < 0.02$).

MMP-13 Expression in Chondrocyte Cultures Is Inhibited by Serum

Comparisons of serum-free and serum-containing chondrocyte cultures have suggested that the major effect of serum is to decrease the rate of hypertrophy; although serum-free culture is more effective in short-term culture, serum-free and serum-containing conditions yield comparable increases in markers of hypertrophy with extended culture times [Ecarot et al., 1992; Quarto et al., 1992]. The present studies confirmed that upper sternal chondrocytes, cultured in either serum-free or serum-containing media until day 9, show similar increases in alkaline phosphatase activity and type X collagen mRNA; they also express similar levels of type II collagen and MMP-2 mRNA (data not shown). However, little MMP-13 mRNA or activity was detected in cultures maintained with either NuSerum or fetal bovine serum. Exposure to serum-free conditions for only 4 h before harvesting yielded MMP-13 mRNA levels that were 50–60% of those found after 48 h in serum-free culture. In contrast, no MMP-13 mRNA was seen if 24-h exposure to serum-free medium at days 4–5 was followed by 2 days of culture in serum-containing medium (data not shown). These results suggest that serum contains an inhibitor of MMP-13 transcription.

DISCUSSION

Our results demonstrate that chondrocyte hypertrophy is associated with major changes in the expression of both MMP-2 and MMP-13. MMP-13 gene expression in cultures of prehypertrophic chondrocytes is induced by modulators that promote expression of both type X collagen and alkaline phosphatase during initiation of hypertrophy, and the active proteinase is found both at low levels during early stages of hypertrophy and at higher levels after development of mature chondrocytes. This pattern of MMP-13 expression is confirmed by histochemical analyses of developing chick embryo sternae, and is consistent with reports that MMP-13 is found in hypertrophic and calcifying cartilage of mammalian growth plate

[Mattot et al., 1995; Johansson et al., 1997; Wu et al., 1997]. Neither mRNA nor protein for MMP-13 is found in lower sternal chondrocytes, which do not undergo hypertrophy during development in vivo and do not develop hypertrophic markers in vitro. Thus, expression of MMP-13 mRNA is associated with chondrocytes undergoing hypertrophy. In contrast, MMP-2 mRNA is present at high levels in both lower sternal chondrocytes and in nonhypertrophic upper sternal chondrocytes. MMP-2 gene expression is transiently downregulated during early stages of hypertrophy, but rises gradually as chondrocytes mature, in parallel with increases in levels of active enzyme. While MMP-13 mRNA is expressed along with other hypertrophy-related mRNAs, production of high-level MMP-13 activity is modulated posttranscriptionally, and is associated with more mature chondrocytes. Furthermore, the parallel between late-stage increases in MMP-2 and MMP-13 activity are consistent with a role for MMP-2 in activating MMP-13 before matrix mineralization.

The hypothesis that cartilage-derived proteinases were required for initiation of mineralization was proposed 25 years ago [Kuettner et al., 1975], and was followed by the demonstration that hypertrophic chondrocytes of bovine growth plate produced neutral proteoglycanases that were inhibited by EDTA [Mercier et al., 1987]. In the late 1980s, Dean and Howell provided evidence that matrix metalloproteinases were associated with cell enlargement [Dean et al., 1989], and subsequently reported that MMP-3 (stromelysin-1) and MMP-2 (72-kDa gelatinase) were present in preparations of matrix vesicles implicated in matrix mineralization [Dean et al., 1992; Schmitz et al., 1996; Dean et al., 1996]. However, direct evidence of a causal relationship between proteinase expression and either cell enlargement or degradation of mineralization inhibitors is limited. Yasuda et al. [1995] reported that the calcium-dependent neutral cysteine proteinase, m-calpain, was increased during rat chondrocyte hypertrophy, and that addition of a cysteine proteinase inhibitor decreased matrix mineralization. However, there is no evidence that calpain functions as an extracellular proteinase. In contrast, changes in MMP expression seen during chondrocyte hypertrophy imply that these proteinases are more likely to be responsible for matrix degradation during endochondral bone formation.

Degradation of cartilage matrix to permit cell enlargement should require MMPs capable of attacking the major matrix components: type II collagen and the proteoglycan, aggrecan. Similar substrate specificity would be expected for MMPs involved in degrading mineralization inhibitors, because both type II collagen cleavage and proteoglycan degradation have been implicated as key factors associated with calcifying cartilage [Hunter, 1991; Alini et al., 1992; Alini et al., 1994; Boskey et al., 1997]. Although type X collagen degradation might also be involved in mineralization of cartilage matrix [Cole et al., 1993], Alini et al. [1994] have reported that this collagen shows reduced synthesis, rather than degradation, during growth plate calcification. MMP-13 is a collagenase capable of cleaving not only type II and type X collagen, but also aggrecan [Fosang et al., 1996b; Mitchell et al., 1996; Knäuper et al., 1997]. This proteinase may therefore contribute to both collagen and proteoglycan degradation during either cell enlargement or matrix calcification. In addition, MMP-13 expression during development is confined to skeletal tissues, where it is restricted to hypertrophic chondrocytes and osteoblasts [Johansson et al., 1997; Stähle-Bäckdahl et al., 1997] implying that it has a specific function in bone formation. In contrast, MMP-2 is a gelatinase with widespread expression that is thought to serve a housekeeping function in removing abnormal or degraded collagen [Matrisian, 1994]. However, because MMP-2 can also activate pro-MMP-13 [Knäuper et al., 1996b, 1997], it may play a more specific role in hypertrophic cartilage, as a regulator of MMP-13 activity.

Because MMP-13 expression is associated with osteoarthritic lesions [Mitchell et al., 1996; Reboul et al., 1996; Borden et al., 1996; Shlopov et al., 1997; Fernandes et al., 1998], its regulation in articular cartilage has been actively investigated. These studies have suggested that control of MMP-13 primarily involves regulation of mRNA levels. Both interleukin-1 (IL-1) and transforming growth factor beta (TGF-β1) elevate MMP-13 mRNA in human articular chondrocytes [Mitchell et al., 1996; Borden et al., 1996; Moldovan et al., 1997]. However, in rat epiphyseal plate chondrocytes, MMP-13 transcription was stimulated by IL-1 and 1,25-dihydroxyvitamin D_3

[Grumbles et al., 1996] but inhibited by TGF-β1, under conditions where TGF-β also inhibited hypertrophy [Ballock et al., 1993]. Our results, demonstrating a correlation between induction of MMP-13 mRNA and other hypertrophic markers (Figs.1 and 2), are consistent with the hypothesis that modulators that promote hypertrophy will increase MMP-13 mRNA, whereas those that block hypertrophy will suppress MMP-13. In accord with this hypothesis, BMP-2 stimulated both mRNA levels and activity of MMP-13 at all time points tested (Fig.2A and 3B). However, ascorbate, another inducer of hypertrophy, elevated MMP-13 mRNA but not active enzyme at days 3–4 (Fig. 3B), and ascorbate-mediated increases in MMP-13 activity were only seen at day 9 (Table I). These results implied that MMP-13 activity was not regulated solely by levels of mRNA, a conclusion supported by the observation that elevated MMP-13 activity occurred several days after maximal levels of mRNA were reached (Fig. 7A). The parallel time course for MMP-13 activity and MMP-2 activity (Fig. 7A,B) suggested that changes in MMP-2 mRNA and activity might be implicated in the control of MMP-13 activity.

Regulation of MMP-2 expression in chondrocytes appears complex. MMP-2 activity is high in normal growth plates [Wardale and Duance, 1996; Kawashima-Ohya et al., 1998], but decreases in tibial dyschondroplasia, which is associated with impaired hypertrophy [Wardale and Duance, 1996]. In addition, treatment of confluent avian growth plate chondrocyte cultures with retinoic acid, which increases hypertrophy, causes elevated MMP-2 mRNA and activity [Nie et al., 1998]. These observations imply that mature hypertrophic chondrocytes are characterized by high MMP-2 mRNA and protein. However, treatment of rabbit growth plate chondrocytes with PTH or PTH-related peptide, which maintain proliferating chondrocytes in a nonhypertrophic state, also causes elevated MMP-2 mRNA and protein [Kawashima-Ohya et al., 1998]. It is therefore plausible that high MMP-2 is found both in actively proliferating chondrocytes and in late-stage hypertrophic chondrocytes. Our results are consistent with this hypothesis. Highest MMP-2 mRNA and protein was observed with cultured proliferating lower sternal chondrocytes (Table II and Fig. 4C). Because these chondrocytes are derived from cartilage that does not hypertrophy during development, and do not express significant amounts of type X collagen or alkaline phosphatase even in the presence of inducers [Leboy et al., 1997], it is clear that hypertrophy is not a prerequisite for high MMP-2 expression. When hypertrophy was induced with BMP and ascorbate phosphate, MMP-2 mRNA levels were low initially, but increased as cultures approached confluence (Fig. 7B). By day 9 of culture with BMP plus ascorbate, MMP-2 had recovered to levels seen with non-induced cultures as indicated by both Western blots (Fig. 4C) and mRNA analyses.

Cowell et al. [1998] have proposed that a proteinase cascade is involved in cartilage degradation. Their data are compatible with a sequence of events in which membrane-bound MT1-MMP (MMP-14) can either directly activate MMP-13 or cleave pro-MMP-13 via activation of pro-MMP-2 [Knäuper et al., 1996b]. Avian MT1-MMP has not been identified; however, the human enzyme migrates with an estimated size of 59–63 kDa [Sato et al., 1994; Cowell et al., 1998]. Although our studies have not examined MT1-MMP levels, gelatin zymography using large volumes of conditioned medium consistently indicated the presence of a metalloproteinase with an apparent M_r of 60–62 kDa, which was elevated along with MMP-2 (data not shown). It is therefore possible that our cultured chondrocytes modulate MT1-MMP and MMP-2 coordinately, and that either or both are responsible for activating MMP-13 in postconfluent hypertrophic cultures. Although changes in the level of active TIMPs are frequently implicated in regulating the activity of MMPs, Dean et al. [1990] have provided evidence that changes in collagenase activity during chondrocyte hypertrophy are not associated with significant changes in TIMP levels. This suggests that the elevated MMP-13 and MMP-2 activity seen with increasing hypertrophy is primarily modulated by gene transcription and, perhaps, the MT1-MMP/MMP-2/MMP-13 activation cascade described above, with relatively little regulation by changing TIMP levels.

One surprising observation is that upper sternal chondrocytes do not produce MMP-13 until cultures are transferred to serum-free media. This is not true for MMP-2 expression; MMP-2 mRNA levels in NuSerum-containing day 9 cultures with BMP and ascorbate phosphate are at least as high as those switched to

serum-free media. Like most cartilage, the growth plate is relatively avascular except at the calcified zone, where blood vessels invade [Cancedda et al., 1995]. It is therefore possible that MMP-13 induction in vivo is facilitated by low nutrient levels, and that transfer of chondrocytes to serum-free media mimics these conditions. Cole et al. [1992] reported that when intact embryonic chick tibia were placed in organ culture, growth plate chondrocytes underwent hypertrophy and synthesized type X collagen; however, no cartilage degradation was observed unless the tibia were placed in serum-free culture. They subsequently reported that degradation under serum-free conditions proceeded in two stages: early degradation of proteoglycan, which coincided with expression of a 62–66-kDa gelatinase (MMP-2), and later type X collagen degradation associated with production of a 50–57-kDa collagenase (presumably MMP-13) [Cole et al., 1993]. After MMP activation by APMA, samples from the later time point showed bands at 40–42 kDa that may correspond to the 43-kDa degradation product of MMP-13 seen in our Western blots (Fig. 4A, B). Although these results would suggest that cartilage-derived MMPs produced under serum-free conditions can degrade much of the matrix in calcifying cartilage, the authors noted that removal of the entire matrix was not accomplished without the presence of some bone or marrow cells. This supports the assumption that although chondrocyte-derived proteinases may be required for cell enlargement and matrix calcification, they are not sufficient to completely degrade cartilage remnants during ossification. Recent studies with mice lacking MMP-9 show normal chondrocyte hypertrophy and cartilage calcification, but delayed endochondral ossification [Vu et al., 1998], suggesting that this final step requires osteoclast-derived MMP-9.

The role of proteinases in cartilage hypertrophy and matrix mineralization has been a subject of long-standing interest. One school of thought is that cartilage matrix is normally nonmineralizable, and must undergo degradation of inhibitors such as proteoglycan [Hunter, 1991; Boskey et al., 1992; Tenenbaum and Hunter, 1993; Boskey et al., 1997]. It has also been suggested that matrix degradation by proteinases may be essential for chondrocyte enlargement, which occurs at a stage before the onset of mineralization [Hunter, 1991]. Our results, indicating that production of MMP-13 and MMP-2 is regulated at both early and late stages of chondrocyte maturation, are compatible with a role for these proteinases either in hypertrophy or in mineralization of cartilage matrix.

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