DEVELOPMENTAL DYNAMICS 208:387–397 (1997)

Collagenase-3 (MMP-13) Is Expressed by Hypertrophic Chondrocytes, Periosteal Cells, and Osteoblasts During Human Fetal Bone Development

NINA JOHANSSON,[1,2,3] ULPU SAARIALHO-KERE,[4] KRISTIINA AIROLA,[4] RIITTA HERVA,[5] LIISA NISSINEN,[3] JUKKA WESTERMARCK,[1,2,3] EERO VUORIO,[2] JYRKI HEINO,[2,3] AND VELI-MATTI KÄHÄRI[1,2,3]*

[1]*Department of Dermatology, Turku University Central Hospital, and University of Turku, Turku, Finland*
[2]*Department of Medical Biochemistry, University of Turku, Turku, Finland*
[3]*MediCity Research Laboratory, University of Turku, Turku, Finland*
[4]*Department of Dermatology, University of Helsinki, Helsinki, Finland*
[5]*Department of Pathology, University of Oulu, Oulu Finland*

ABSTRACT **Collagenase-3 (MMP-13) is a novel matrix metalloproteinase, the expression of which has so far only been documented in human breast carcinomas and osteoarthritic cartilage. In this study we have examined the expression of MMP-13 during human fetal development. Northern blot hybridizations revealed abundant expression of MMP-13 mRNAs in total RNA from fetal cartilage and calvaria at gestational age of 15 weeks. By in situ hybridization MMP-13 transcripts were detected in chondrocytes of hypertrophic cartilage in vertebrae of the spinal column and in the dorsal end of ribs undergoing ossification, as well as in osteoblasts and periosteal cells below the inner periosteal region of ossified ribs. In contrast, no expression of MMP-13 could be detected in osteoclasts. Furthermore, expression of MMP-13 mRNA was detected in osteoblasts and fibroblasts primarily on the inner side of calvarial bone of the skull at 16 weeks of gestation. Expression of MMP-13 mRNA by primary human fetal chondrocytes in culture was enhanced by transforming growth factor-β (TGF-β) and inhibited by bone morphogenetic protein-2 (BMP-2). No expression of MMP-13 mRNA could be noted in other fetal tissues, including the skin, lungs, neural tissue, muscle, and liver. These results suggest that MMP-13 plays an important role in the extracellular matrix remodeling during fetal bone development both via endochondral and intramembranous ossification.** ***Dev. Dyn. 208:387–395, 1997.***

Key words: bone; cartilage; collagenase; matrix metalloproteinase; transforming growth factor-β; bone morphogenetic protein-2

INTRODUCTION

Proteolytic remodeling of extracellular matrix is essential in several physiological situations, including tissue morphogenesis during fetal development, tissue repair, and angiogenesis. On the other hand, excessive breakdown of connective tissue components plays an important pathogenetic role e.g. in autoimmune blistering disorders of skin, dermal photoageing, rheumatoid arthritis, osteoarthritis, and periodontitis, as well as in tumor cell invasion and metastasis (see Birkedal-Hansen et al., 1993). Matrix metalloproteinases (MMPs) are a family of zinc-dependent metalloendopeptidases collectively capable of degrading essentially all extracellular matrix components (see Woessner, 1994). At present, the MMP gene family consists of at least 14 members, which can be divided into subfamilies of collagenases, gelatinases, stromelysins, and membrane type MMPs (MT-MMPs) according to substrate specificity and primary structure (Birkedal-Hansen, 1995).

The original members of the collagenase subfamily, fibroblast interstitial collagenase (MMP-1) and neutrophil collagenase (MMP-8), have long been the only known secreted neutral proteinases capable of initiating the degradation of native fibrillar collagens of type I, II, and III in the extracellular space. Recently, a novel member of the MMP gene family, collagenase-3 (MMP-13), was cloned from human breast carcinoma tissue cDNA (Freije et al., 1994). The substrate specificity of MMP-13 differs from that of other collagenases, MMP-1 and MMP-8. Specifically, MMP-13 degrades type II collagen sixfold more effectively than type I and III collagens and displays almost 50-fold stronger gelatinolytic activity than MMP-1 and MMP-8 (Knäuper et al., 1996; Mitchell et al., 1996). Interestingly, the deduced amino acid sequence of human MMP-13 shows high degree of homology (86%) to rat and murine interstitial collagenases, while its homology to human MMP-1 is markedly lower (50%), indicating that rat and murine interstitial collagenase cDNAs cloned represent counterparts of human MMP-13 instead of MMP-1 (Freije et al., 1994). In comparison to MMP-1, the expression of MMP-13 appears to be limited: so far MMP-13 transcripts have only been detected in human breast carci-

*Correspondence to: Veli-Matti Kähäri, M.D., Ph.D., MediCity Research Laboratory, University of Turku, Tykistökatu 6, FIN-20520 Turku, Finland.
Received 19 September 1996; Accepted 4 December 1996

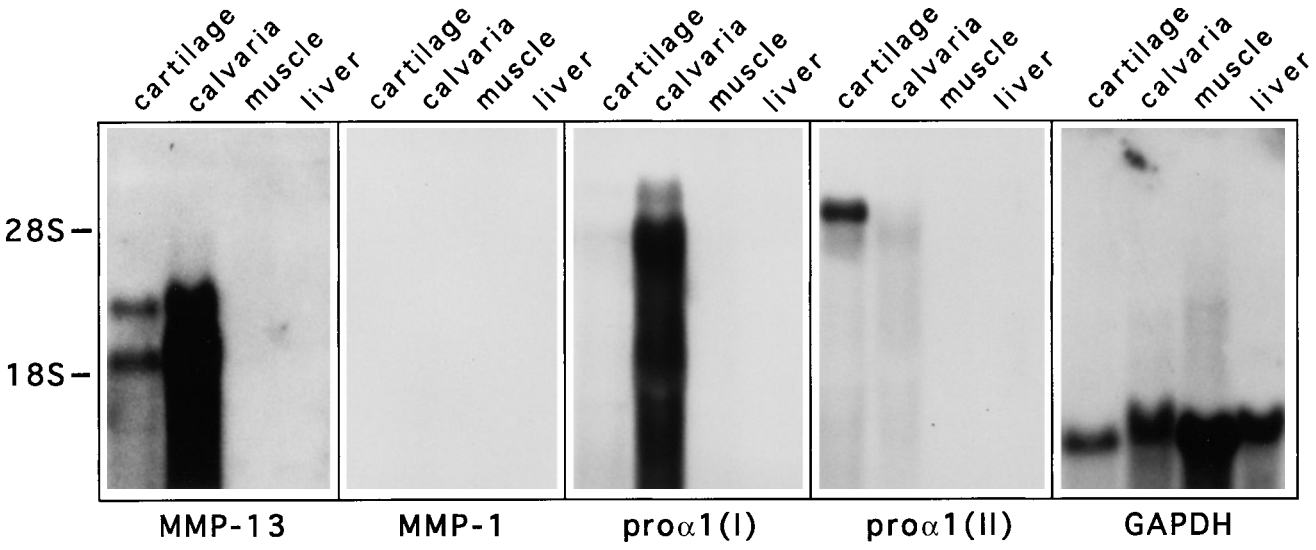

Fig. 1. Collagenase-3 (MMP-13) mRNAs are expressed in fetal cartilage and calvaria. Total cellular RNAs were extracted from human fetal tissues at 15 weeks of gestation. Aliquots of total RNA (25 μg/lane) were analyzed by Northern blot hybridizations for the levels of collagenase-3 (MMP-13), interstitial collagenase (MMP-1), proα1(I) collagen, proα1(II) collagen and GAPDH mRNAs.

noma tissue (Freije et al., 1994) and in osteoarthritic cartilage and chondrocytes (Mitchell et al., 1996; Reboul et al., 1996).

It is conceivable that collagenases play an important role in the remodeling of collagenous extracellular matrix during mammalian fetal development. Immunostaining for MMP-1 has been detected in human fetal skin at the gestational age of 8 and 12 weeks, in basal epidermal keratinocytes and dermal fibroblasts, as well as in cells in and around developing hair follicles, blood vessels, and nerves (McGowan et al., 1994). Expression of MMP-1 mRNA has also been noted during human fetal intrahepatic bile duct development (Terada et al., 1995). In murine embryonal development, interstitial collagenase and stromelysin-1 transcripts have been detected at the zygote and cleavage stages and their expression was increased at the blastocyst stage and during endoderm differentiation (Brenner et al., 1989). In addition, expression of interstitial collagenase mRNA has been noted in developing bone during murine fetal development (Gack et al., 1995). However, since the murine interstitial collagenase cloned is the homologue of MMP-13 and it is likely that mice lack the counterpart of MMP-1, it is difficult to interpret the results on the developmental expression of murine interstitial collagenase in the context of human development.

In this study we have examined the expression of a novel collagenase, collagenase-3 (MMP-13), during human fetal development. We show that MMP-13 gene transcripts are exclusively expressed by chondrocytes in hypertrophic cartilage and by periosteal cells and osteoblasts during ossification of ribs and vertebrae. In addition, MMP-13 mRNA is expressed by osteoblasts and fibroblasts in calvarial bone of the developing skull. We also show that the expression of MMP-13 gene by cultured primary fetal chondrocytes is enhanced by transforming growth factor-β (TGF-β) and suppressed by bone morphogenetic protein-2 (BMP-2). These results suggest that MMP-13 plays an important role in human fetal bone development both via endochondral and intramembranous ossification.

RESULTS

Expression of MMP-13 mRNA in Fetal Cartilage and Calvaria

To elucidate the tissue specificity of MMP-13 gene expression during human fetal development, we initially assayed MMP-13 mRNA levels in total RNAs extracted from different tissues of 15-week-old fetuses. Using Northern blot hybridizations two distinct MMP-13 mRNAs of 2.0 and 2.5 kb were detected in RNA from epiphyseal cartilage (Fig. 1). Interestingly, the highest levels of MMP-13 transcripts were noted in RNA from calvarial bone of the skull (Fig. 1). In contrast, no expression of interstitial collagenase (MMP-1) mRNA could be noted in either cartilage or calvaria RNA samples, indicating that the predominant collagenase expressed in these developing tissues is MMP-13 (Fig. 1). No expression of MMP-13 or MMP-1 mRNAs was noted in RNAs prepared from fetal skeletal muscle or liver (Fig. 1).

To confirm the identity of the cartilage and calvaria RNA preparations, the Northern blot was rehybridized with cDNA probes for cartilage-specific type II collagen mRNA and calvaria and bone-specific type I collagen mRNA. As expected (Sandberg and Vuorio, 1987), marked expression of proα1(II) collagen mRNA was detected in cartilage RNA sample, but not in calvarial

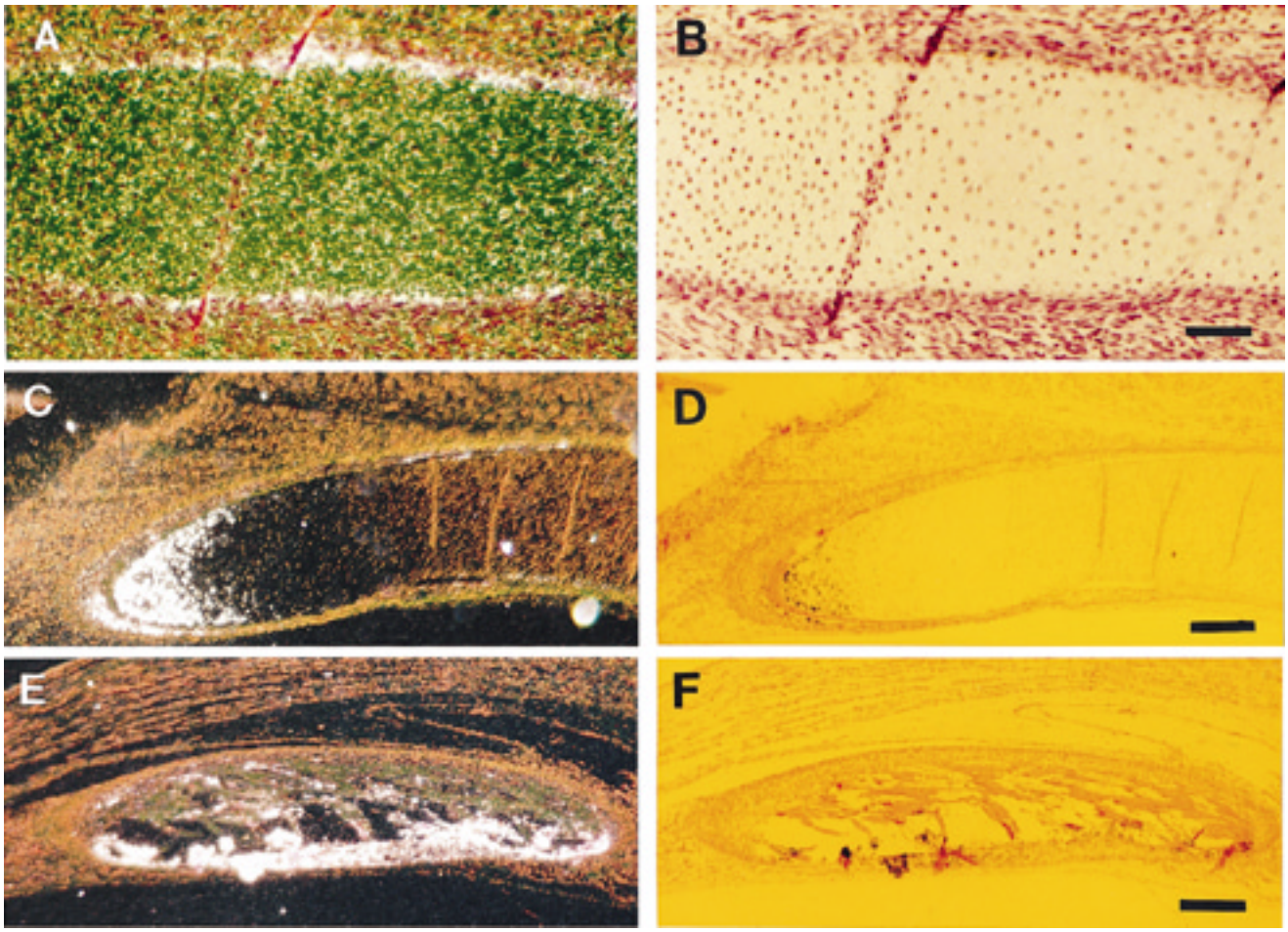

Fig. 2. Expression of collagenase-3 (MMP-13) mRNA in developing human bones. **A:** Dark-field exposure showing MMP-13 transcripts in perichondrium of iliac bone of a 10-week-old fetus. **B:** Corresponding bright-field micrograph of A. **C:** Dark-field exposure showing MMP-13 mRNA expression in the dorsal end of a developing rib of a 12-week-old fetus. **D:** The corresponding bright-field photomicrograph to C. **E:** Dark-field exposure showing expression of MMP-13 mRNA along the inner curvature of an ossified part of the rib of a 12-week-old fetus. **F:** Corresponding bright-field photomicrograph to E. Bars: A, B, 24 µm; C, D, E, F, 60 µm.

RNA (Fig. 1). Furthermore, in accordance with previous observations (Sandberg et al., 1989), marked expression of proα1(I) collagen mRNAs was detected in calvarial RNA, but not in cartilage RNA (Fig. 1), corroborating the tissue source of these RNA preparations.

Expression of MMP-13 mRNA in Hypertrophic Cartilage and Developing Bone During Fetal Development

In order to identify and localize the cells expressing the MMP-13 gene during human fetal development we performed *in situ* hybridizations using tissue sections from fetuses with gestational ages ranging from 8 to 17 weeks. No signal for MMP-13 mRNA could be detected in the tissue sections obtained from 8-week-old fetuses (not shown). In a sagittal section of a 10-week-old fetus, a weak but specific signal for MMP-13 mRNA was detected symmetrically in the perichondrium of a developing iliac bone, while the remaining cartilaginous portion of this developing bone was entirely devoid of MMP-13 mRNA (Fig. 2A, B).

Additional *in situ* hybridizations of a cross section of a 12-week-old fetus with MMP-13 specific antisense RNA probe revealed a strong signal for MMP-13 mRNA in the dorsal end of developing ribs in the region representing hypertrophic cartilage, while no signal for MMP-13 mRNA could be detected in the remaining cartilaginous part of the rib (Fig. 2C, D). At higher magnification, MMP-13 mRNA was detected in hypertrophic and degenerating chondrocytes of hypertrophic cartilage (Fig. 3A–C). Interestingly, a strong expression of MMP-13 mRNA was also noted in cells located in the inner cambium layer of periosteum, particularly near the dorsal end of the ossifying rib (Figs. 2C, D; 3A–C). In addition, expression of MMP-13 mRNA was detected in hypertrophic cartilage of developing vertebrae of the spinal column (not shown).

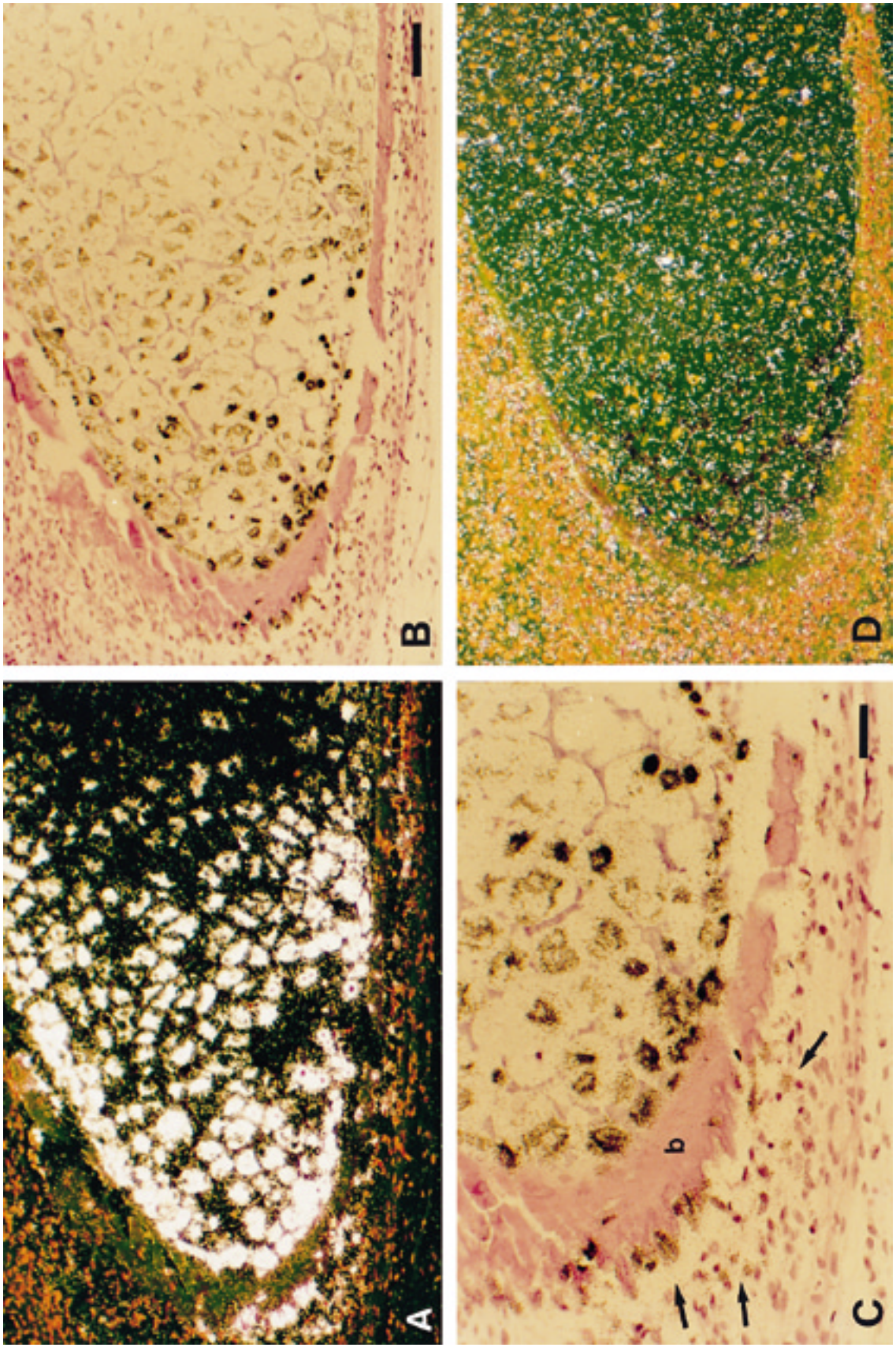

Fig. 3. Expression of collagenase-3 (MMP-13) transcripts in hypertrophic cartilage. **A:** Dark-field exposure showing MMP-13 mRNA positive hypertrophic chondrocytes in a developing rib of a 12-week-old fetus. In addition, expression of MMP-13 mRNA is noted in cells lining the periosteum. **B:** The corresponding bright-field photomicrograph to A. **C:** Higher magnification of B, showing hypertrophic chondrocytes and periosteal cells (arrows) expressing MMP-13 mRNA; b, cortical bone. **D:** No signal is detected in a parallel section hybridized with the sense MMP-13 probe. Bars: A, B, D, 28 µm; C, 14 µm.

In situ hybridizations of adjacent horizontal sections of the same 12-week-old fetus containing ossified parts of ribs demonstrated a high expression of MMP-13 mRNA in cells located primarily in the inner aspect of the curvature of the rib (Fig. 2E, F). Examination of these sections with high magnification revealed that both bone lining cells and periosteal cells expressed MMP-13 mRNA (Fig. 4A–C), whereas no expression of MMP-13 mRNA was detected in multinuclear osteoclastic cells (Fig. 4C). While a majority of the bone lining cells appeared to be osteoblasts, the presence of other mononuclear cell types containing MMP-13 mRNA could not be ruled out. In the tissue sections examined, no MMP-13 mRNA expression could be noted in other tissues examined, including skin, skeletal muscle (Fig. 2E, F), lungs, neural tissue, and liver (not shown). Furthermore, no expression of MMP-1 mRNA could be detected in any of the skeletal tissues analyzed (not shown). No signal was detected in tissues hybridized with a labeled sense probe for MMP-13 cDNA (Fig. 3D).

Expression of MMP-13 mRNA in Calvarial Bone of the Skull

Since abundant expression of MMP-13 mRNA was detected in fetal calvarial RNA, we also wanted to localize the cells expressing MMP-13 mRNA in developing fetal calvarial bone. A strong signal for MMP-13 transcripts was noted in the calvarial bone of the skull of a 16-week-old fetus (Fig. 5A, B). Interestingly, MMP-13 mRNA localized mainly to the cells in the inner side of the calvarial bone in the intratrabecular mesenchyme and periosteum, and in some areas also to the osteoblastic cells lining the bone spicules (Fig. 5A–C). In contrast, the upper convex side of the growing calvarial bone was remarkably devoid of hybridization signal (Fig. 5A, B). No signal for MMP-1 mRNA could be detected in calvaria (not shown).

Expression of MMP-13 mRNAs by Cultured Fetal Chondrocytes Is Enhanced by TGF-β and Suppressed by BMP-2

Previous studies have shown that TGF-β1 mRNA is expressed in the growth plate of developing bones, suggesting a role for it in stimulation of bone extracellular matrix deposition (Sandberg et al., 1988a). In this context, we wanted to elucidate the effect of TGF-β on the expression of MMP-13 mRNA in cultured primary human fetal chondrocytes. Treatment of chondrocytes with TGF-β1 and - 2 (5 ng/ml) for a period of 24 hr resulted in enhancement (2.5- and 10.0-fold, respectively) of MMP-13 mRNA levels in fetal chondrocytes (Fig. 6, Table 1). In parallel, fetal chondrocytes were also treated with BMP-2, a potent inducer of cartilage and bone formation (see Reddi, 1994; Wozney, 1995). Interestingly, treatment of fetal chondrocytes with BMP-2 (50 ng/ml) resulted in potent suppression of MMP-13 mRNA levels (by 80%), as compared to the untreated chondrocytes (Fig. 6, Table 1). Very low levels of MMP-1 mRNA were detected only in cells treated with TGF-β2 (Fig. 6).

In order to confirm the chondrocytic phenotype of these cells, we hybridized the RNA blot with type I and II collagen-specific cDNA probes. As shown in Figure 6, these cells readily expressed proα1(II) collagen mRNA, indicating that they had retained chondrocytic phenotype in culture. However, these cells also expressed detectable levels of proα1(I) collagen mRNAs, indicating that they had started to dedifferentiate in culture (Fig. 6). In these chondrocytes expression of type I collagen mRNAs was enhanced 5.0- and 3.0-fold, and type II collagen mRNA levels 3.6- and 2.0-fold by TGF-β1 and - 2, respectively (Fig. 6, Table 1). Interestingly, BMP-2 potently enhanced type II collagen mRNA levels (8.7-fold), while the levels of type I collagen mRNAs were minimally affected by BMP-2 (Fig. 6, Table 1). These results show that MMP-13 is expressed by fetal chondrocytes in culture and that its expression is differently modulated by two potent stimulators of bone and cartilage formation, namely TGF-β and BMP-2.

DISCUSSION

In the present study, we have examined the expression of a novel MMP, collagenase-3 (MMP-13), in developing human fetal tissues at the gestational ages ranging from 8 to 17 weeks during which period the expression of MMP-13 transcripts was noted exclusively in developing skeleton. MMP-13 expression was first detected in cells in the perichondreal region of developing iliac bone at the age of 10 weeks. Next, at 12 weeks of gestation, a strong signal for MMP-13 mRNA was observed in hypertrophic chondrocytes of developing ribs and vertebral column, but not in cartilage. These results show that the expression of MMP-13 is specifically and potently induced in hypertrophic cartilage during endochondral ossification, suggesting that MMP-13 plays a role in degradation of type II collagen, the major component of cartilage. A recent report shows that MMP-13 degrades type II collagen about sixfold more effectively than fibrillar collagens of type I and III (Knäuper et al., 1996), but at this point it is not known whether MMP-13 also degrades type X collagen, a major and specific component of hypertrophic cartilage.

In addition to hypertrophic cartilage, marked expression of MMP-13 mRNA was noted in osteoblasts or mononuclear bone lining cells and periosteal cells in the inner aspect of ossified parts of ribs. It is possible that MMP-13 participates in degradation of type I collagen of the bone extracellular matrix on the inner side of the growing rib, while extracellular matrix is being deposited on the outer side of the rib. Similarly, in calvarial bone of developing skull, expression of MMP-13 was noted mainly in osteoblastic and fibroblastic cells residing on the inner side of the calvarial bone. Previously, it has been shown that the cells expressing type I collagen mRNAs are located on the outer side of the developing calvarial bone of the skull (Sandberg et al., 1988b). It is

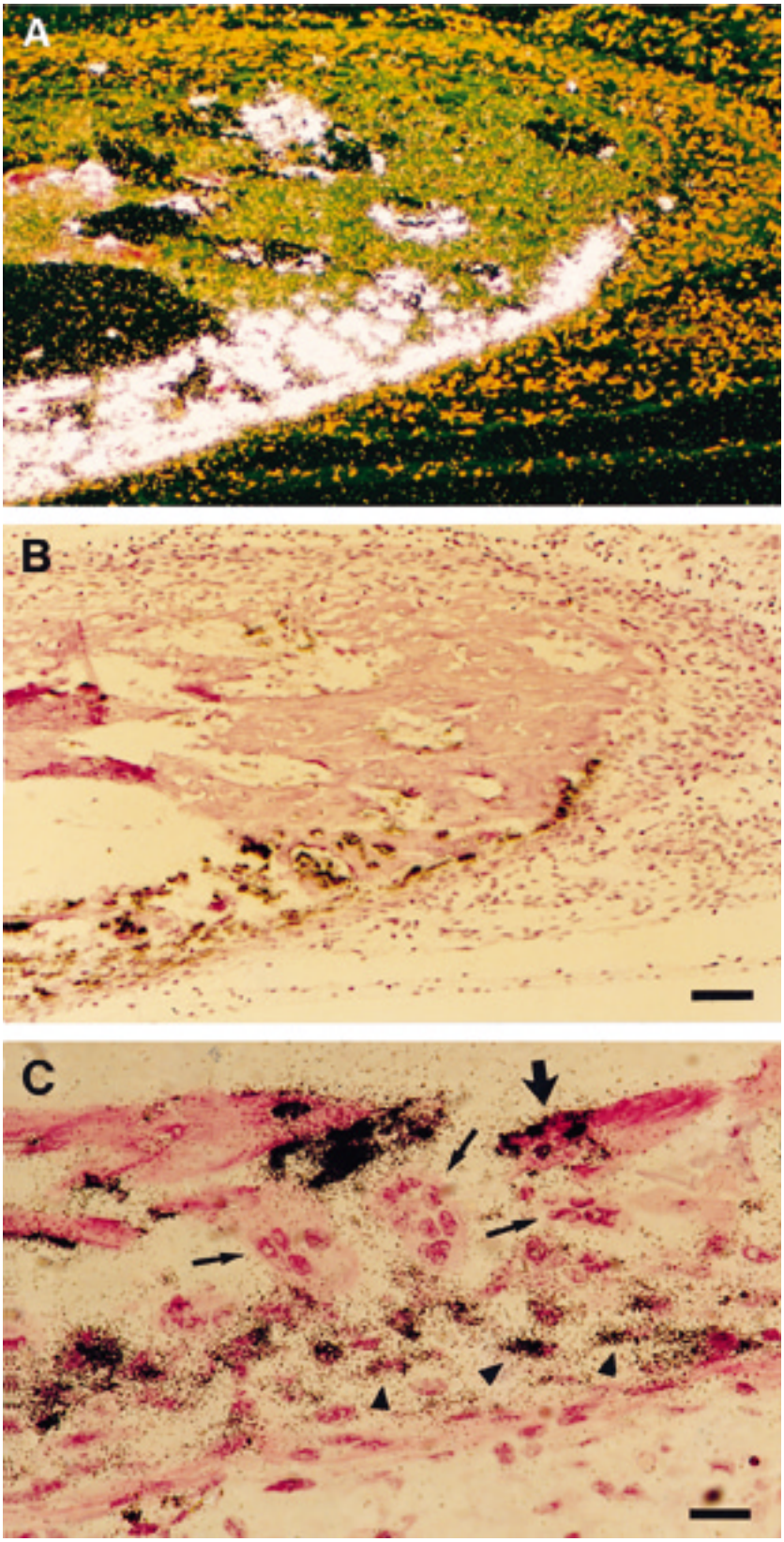

Fig. 4. Expression of collagenase-3 (MMP-13) mRNA in periosteal cells and mononuclear bone lining cells in the ossified part of a growing rib. **A:** Dark-field exposure showing MMP-13 mRNA expression predominantly in the inner surface of the rib from a 12-week-old fetus. **B:** The corresponding bright-field photomicrograph to A. **C:** Higher magnification of B. No signal for MMP-13 mRNA is detected in multinuclear osteoclasts (small arrows), while osteoblasts (large arrow) and periosteal cells (arrowheads) show strong hybridization. Bars: A, B, 28 µm; C, 6 µm.

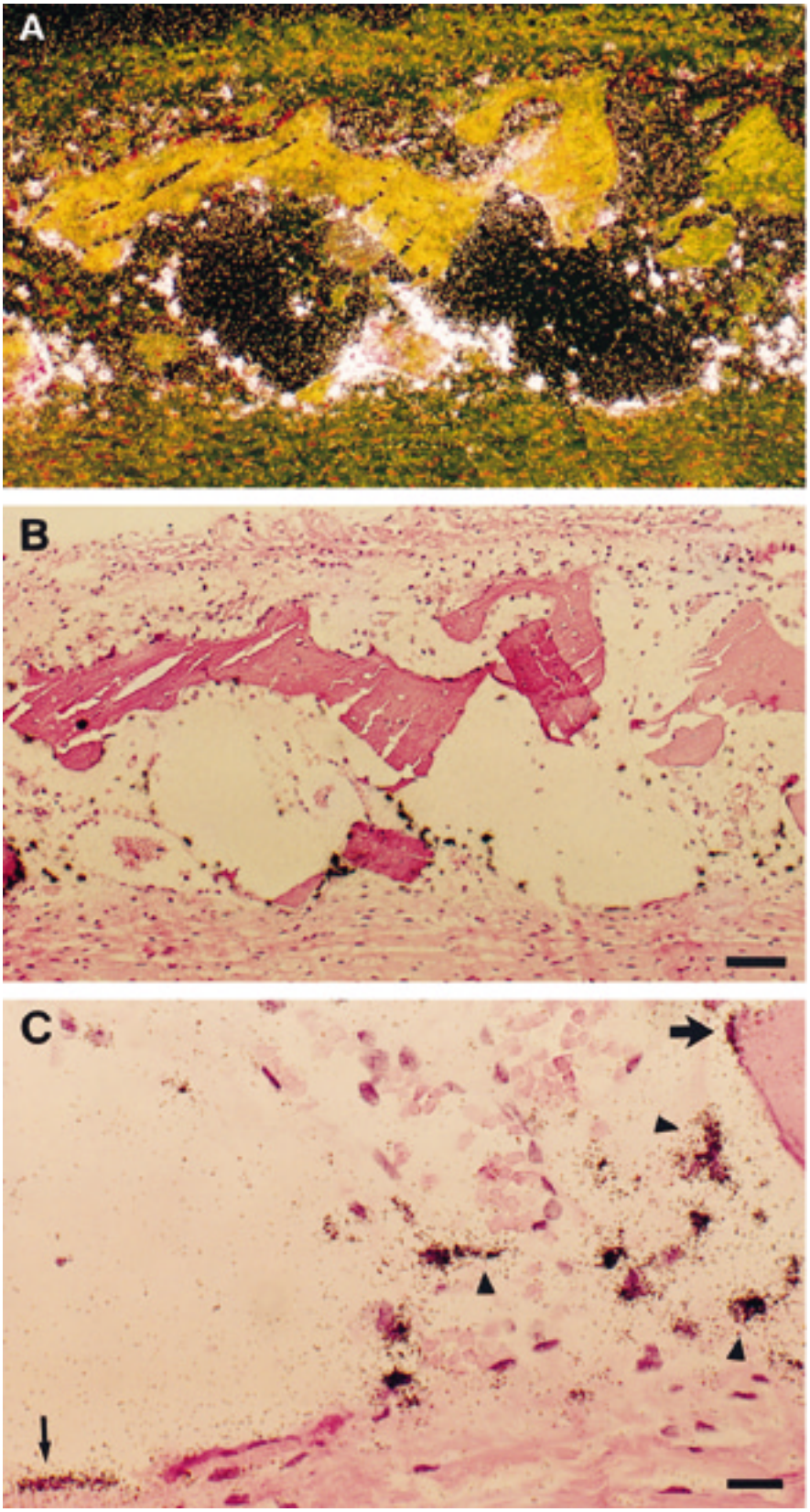

Fig. 5. Expression of collagenase-3 (MMP-13) in fetal calvaria. **A:** Dark-field exposure, showing expression of MMP-13 mRNA in the calvaria of the skull of a 16-week-old fetus, predominantly on the inner (concave) aspect of the bone. **B:** Corresponding bright-field photomicrograph to A. **C:** Higher magnification of B, showing MMP-13 mRNA positive osteoblast (large arrow), periosteal cell (small arrow) and fibroblastic cells (arrowheads) of the intratrabecular mesenchyme. Bars: A, B, 24 μm; C, 6 μm.

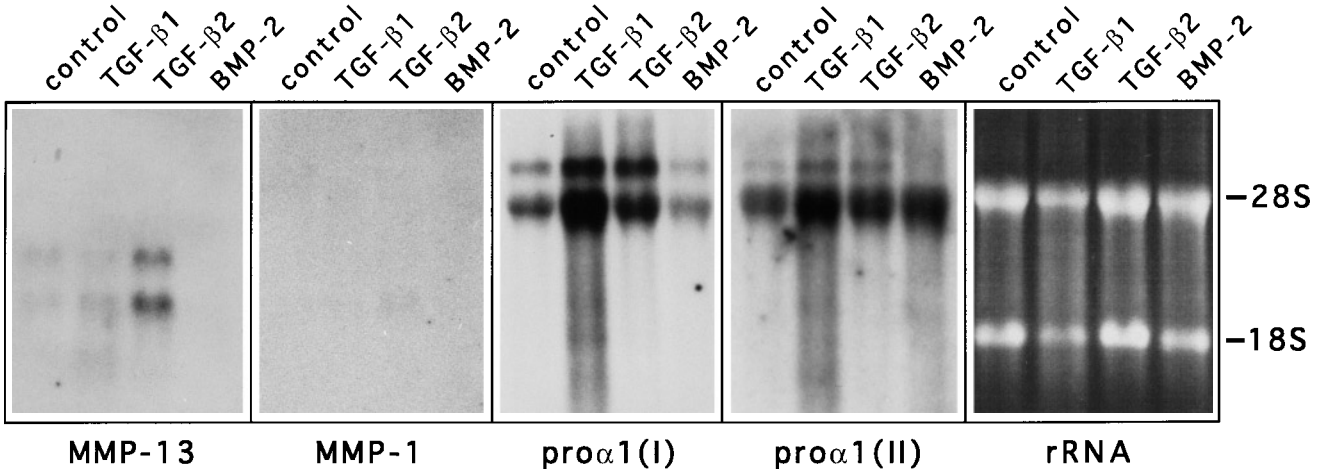

Fig. 6. TGF-β stimulates and BMP-2 suppresses collagenase-3 mRNA levels in cultured fetal chondrocytes. Human fetal chondrocytes in culture were treated for 24 hr with TGF-β1, TGF-β2 (5 ng/ml each) or with BMP-2 (50 ng/ml). Total cellular RNA was extracted and 5 μg aliquots were used for assay of collagenase-3 (MMP-13), interstitial collagenase (MMP-1), proα1(I), and proα1(II) collagen mRNA levels with Northern blot hybridizations. 28S and 18S rRNAs were visualized by ethidium bromide staining.

TABLE 1. Quantitative Estimation of Collagenase-3 (MMP-13), Type I and Type II Collagen mRNA Levels in Primary Human Fetal Chondrocytes in Culture

	Treatment		
	TGF-β1	TGF-β2	BMP-2
MMP-13	2.5	10.0	0.2
proα1(I)	5.0	3.0	1.7
proα1(II)	3.6	2.0	8.7

Primary human fetal chondrocytes were treated for 24 hr with TGF-β1, TGF-β2 (5 ng/ml each), or BMP-2 (50 ng/ml), as described in the legend for Figure 6. MMP-13, proα1(I) and proα1(II) collagen mRNA levels were quantitated by scanning densitometry of the autoradiographs shown in Figure 6, and corrected for the levels of rRNA in the same samples. The values indicate fold induction of the levels for each mRNA by a given treatment, as compared to the untreated control cultures (1.00).

likely that in ribs and calvaria of the skull, which both grow primarily by increasing the diameter of their curvature and less by increasing their thickness, degradation of the collagenous extracellular matrix takes place on the inner side. Interestingly, no expression of MMP-1 could be noted either in cartilage or calvaria, providing evidence that the expression of MMP-1 is not crucial for extracellular matrix remodeling during human fetal bone development. It is therefore likely that the predominant collagenase expressed during both endochondral and intramembranous ossification is MMP-13. Although the expression of MMP-13 appears to very strictly regulated spatially and temporally, we cannot rule out the possibility that the expression of MMP-13 may take place in other tissues besides bone after the developmental period examined in this study.

TGF-β is a potent stimulator of extracellular matrix formation, which enhances expression of a number of matrix genes, including type I, III, and IV collagens, elastin, fibronectin, biglycan, and versican (Kähäri et al., 1990, 1991a,b, 1992). In this study, MMP-13 expression by fetal chondrocytes in culture was enhanced by TGF-β1 and -2. During human fetal bone development TGF-β1 mRNA is expressed specifically in hypertrophic cartilage and bone, but not in epiphyseal cartilage (Sandberg et al., 1988a). It is possible that MMP-13 expression in these tissues is enhanced by TGF-β, which apparently plays a role in the development of bone via endochondral ossification, by autocrine or paracrine stimulation. In contrast to cartilage, expression of TGF-β1 mRNA in calvaria is diffuse (Sandberg et al., 1988b) and does not co-localize with the expression of MMP-13 mRNA, suggesting that induction of MMP-13 expression in calvaria is mediated by modulators other than TGF-β. Interestingly, BMP-2, which enhances formation of cartilage *in vivo*, simultaneously suppressed MMP-13 expression and enhanced type II collagen mRNA levels in primary fetal chondrocytes in culture. This is interesting in the context of our observation that MMP-13 mRNAs were not expressed by chondrocytes in cartilage. Our results suggest that the ability of BMP-2 to promote formation of cartilage is not only due to its ability to enhance expression of type II collagen, but it is also a result of inhibition of type II collagen degradation due to reduced expression of MMP-13 by chondrocytes. Finally, our results showing up-regulation of MMP-13 by TGF-β and down-regulation by BMP-2 suggest distinct roles for these two polypeptide growth factors in the extracellular matrix remodeling during bone development.

In addition to fetal chondrocytes, expression of MMP-13 has been noted in adult human chondrocytes from osteoarthritic cartilage, in which its expression was enhanced by IL-1α (Mitchell et al., 1996). We have also observed expression of MMP-13 mRNAs by transformed human epidermal keratinocytes, including

HaCaT cells, in which MMP-13 expression is enhanced by treatment with TGF-β1 and -2, and with TNF-α (Johansson et al., 1997). Furthermore, we have noted expression of MMP-13 in human osteosarcoma cell lines (Johansson et al., unpublished results). Together the observations on MMP-13 gene expression *in vivo* and by cultured cells indicate restricted tissue-specific expression, as compared to MMP-1. It is possible that due to its ability to degrade both fibrillar collagens and gelatin, MMP-13 is too destructive for controlled remodeling of extracellular matrices of several developing and adult tissues. Nevertheless, this unique combination of collagenolytic and gelatinolytic capacity of MMP-13 may be beneficial in situations such as fetal development of bone, in which rapid and effective removal of type II and type I collagen fibrils is required. In this context, it should be noted that expression of MMP-9 (92-kDa gelatinase, gelatinase B) during murine fetal development appears to be restricted to cells of osteoclastic lineage (Reponen et al., 1994). Since osteoclasts in human fetal bone do not appear to express MMP-13, it is possible that a major substrate for osteoclast-derived MMP-9 is denatured type I collagen initially degraded by osteoblast-derived interstitial collagenase in mice and by MMP-13 in humans.

In summary, the results of the present study demonstrate for the first time that the expression of MMP-13 during human fetal development is confined to endochondrally and intramembranously developing bones, suggesting an important role for MMP-13 in the extracellular matrix remodeling during bone development. Our results are in accordance with those of a recent study on murine fetal development, in which expression of interstitial collagenase was noted in hypertrophic chondrocytes and osteoblasts during endochondral and intramembranous bone development (Gack et al., 1995). However, as mentioned above, murine interstitial collagenase appears to represent a homologue of human MMP-13, not MMP-1, and no counterpart for MMP-1 gene has been found in murine genome (Gack et al., 1995). It is likely that murine interstitial collagenase also serves in the role of MMP-1 in connective tissue remodeling and its expression may be regulated differently from human MMP-13. Therefore, observations on the expression of interstitial collagenase in murine development are most likely not directly applicable to human development. Thus, examination of MMP-13 expression during human development is required to elucidate the role of this MMP in the extracellular matrix remodeling. Based on the strictly regulated expression of MMP-13 both spatially and temporally during human development, it can be speculated that deficient expression or activity of MMP-13 during fetal development may result in severe skeletal abnormality. Furthermore, it is conceivable that unveiling of the mechanisms responsible for tissue specific regulation of human MMP-13 gene may prove feasible in development of novel therapies for disorders in which excessive collagenolytic activity plays a role, including autoimmune blistering disorders of skin, osteoarthritis, tumor cell invasion and metastasizing.

EXPERIMENTAL PROCEDURES

RNA Analysis

Tissue samples for RNA extractions were obtained from 15-week-old human fetuses from medical abortions with permission from the Joint Ethical Committee of the Turku University Central Hospital and the University of Turku, Turku, Finland. Total cellular RNA was isolated from fetal tissues and fetal chondrocyte cultures using the guanidine thiocyanate/cesium chloride method (Chirgwin et al., 1979). Northern blot hybridizations were performed as described previously (Westermarck et al., 1994, 1995) with cDNAs labeled with [α-^{32}P]dCTP using random priming. Three MMP-13 cDNA fragments generated by RT-PCR and subcloned to plasmid Bluescript (Johansson et al., 1997) were used as probes. The first, MMP13HT1, corresponds to nucleotides 57 to 547 and the second, MMP13HT2, to nucleotides 786 to 1420 in the coding region; the third, MMP13HT3, corresponds to nucleotides 1532 to 2042 in the 3′-untranslated region (Freije et al., 1994). All MMP-13 cDNA fragments were isolated and used as probes in the same Northern blot hybridizations. In addition, the following cDNAs were used for hybridizations: a 2.0 kb human collagenase (MMP-1) cDNA (Goldberg et al., 1986); a 0.7 kb human proα1(I) collagen cDNA (Mäkelä et al., 1988); a 550 bp human proα1(II) collagen cDNA (Elima et al., 1987); and a 1.3 kb rat cDNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Fort et al., 1985). [^{32}P]cDNA-mRNA hybrids were visualized by autoradiography and quantitated by densitometry.

In Situ Hybridizations

All fetal material used for *in situ* hybridizations originated from medical abortions at 8, 10, 12, 15, 16, and 17 weeks of gestation. Formalin-fixed, paraffin-embedded specimens (n = 9) were obtained from the Department of Pathology, University of Oulu, Oulu, Finland. Fetal age was estimated by menstrual age and histologic examination. Sections from breast carcinoma tissue, previously shown to express MMP-13 mRNA (Freije et al., 1994), were used as positive controls in each *in situ* hybridization.

Tissue sections were hybridized with [^{35}S]-labeled RNA probes (3×10^4 cpm/μl of hybridization buffer) at 50°C and were washed under stringent conditions, including treatment with RNAse A, as described (Prosser et al., 1989; Saarialho-Kere et al., 1993a). After autoradiography for 14 to 30 days, the photographic emulsion was developed and the slides were stained with hematoxylin and eosin. *In vitro* transcribed antisense and sense RNA probes were labeled with [α-^{35}S]-UTP as described (Saarialho-Kere et al., 1993b). For this, MMP-13 cDNA plasmid MMP13HT1 was linearized within the multiple cloning site with *Xho*I and *Kpn*I, and plasmid MMP13HT3 was linear-

ized with *Hind*III and *Eco*RI, to allow transcription of antisense and sense RNAs, respectively. By FASTA alignment, the maximal homology between the probes used and other members of the metalloproteinase gene family (MMP-1, MMP-3) was 61–62% making unspecific hybridization at high stringency unlikely. The results obtained with the two MMP-13 antisense RNA probes were identical.

Chondrocyte Cultures

Human primary fetal chondrocyte cultures were initiated as described previously (Elima and Vuorio, 1989) and cultured in Dulbecco's modified Eagles Minimum Essential Medium (DMEM; Flow Laboratories, Irvine, UK) supplemented with 50 µg/ml streptomycin sulfate, 100 IU/ml penicillin, and 10% (v/v) fetal calf serum (FCS) (Gibco Biocult, Paisley, UK). The cells used in experiments had been subcultured five to eight times. For the experiments, the chondrocytes were first incubated for 18 hr in culture medium containing 1% FCS and subsequently treated for 24 hr with 5 ng/ml of bovine TGF-β1 or TGF-β2 (kindly provided by Dr. David R. Olsen, Celtrix Co., Santa Clara, CA), or with 50 ng/ml of human recombinant BMP-2 (provided by Genetics Institute, Cambridge, MA) prior to extraction of total RNA.

ACKNOWLEDGMENTS

The expert technical assistance of Marita Potila, Eeva Virtanen, Alli Tallqvist, and Liisa Sund is gratefully acknowledged. This work was supported by the Medical Research Council of the Academy of Finland, Sigrid Jusélius Foundation, Turku University Foundation, Paulo Foundation, the Cancer Research Foundation of Finland, and by a grant from the Turku University Central Hospital.

REFERENCES

Birkedal-Hansen, H. (1995) Proteolytic remodeling of extracellular matrix. Curr. Opin. Cell Biol. 7:728–735.

Birkedal-Hansen, H., Moore, W.G.I., Bodden, M.K., Windsor, L.J., Birkedal-Hansen, B., DeCarlo, A., and Engler, J.A. (1993) Matrix metalloproteinases: A review. Crit. Rev. Oral Biol. Med. 4:197–250.

Brenner, C.A., Adler, R.R., Rappolee, D.A., Pedersen, R.A., and Werb, Z. (1989) Genes for extracellular matrix-degrading metalloproteinase and their inhibitor, TIMP, are expressed during early mammalian development. Genes Dev. 3:848–859.

Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J., and Rutter, W.J. (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18:5924–5929.

Elima, K. and Vuorio, E. (1989) Expression of mRNAs for collagens and other matrix components in dedifferentiating and redifferentiating human chondrocytes in culture. FEBS Lett. 258:195–198.

Elima, K., Vuorio, T., and Vuorio, E. (1987) Determination of the polyadenylation site of the human type II collagen gene. Nucleic Acids Res. 15:9499–9504.

Fort, P., Marty, L., Piechaczyk, M., El Sabrouty, S., Dani, C., Jeanteur, P., and Blanchard, J.M. (1985) Various rat adult tissues express only one major mRNA species from the glyceraldehyde-3-phosphate-dehydrogenase multigenic family. Nucleic Acids Res. 13:1431–1442.

Freije, J.M.P., Díez-Itza, I., Balbín, M., Sánchez, L.M., Blasco, R., Tolivia, J., and López-Otín, C. (1994) Molecular cloning and expression of collagenase-3, a novel human matrix metalloproteinase produced by breast carcinomas. J. Biol. Chem. 269:16766–16773.

Gack, S., Vallon, R., Schmidt, J., Grigoriadis, A., Tuckermann, J., Schenkel, J., Weiher, H., Wagner, E.F., and Angel, P. (1995) Expression of interstitial collagenase during skeletal development of the mouse is restricted to osteoblast-like cells and hypertrophic cartilage. Cell Growth Differ. 6:759–767.

Goldberg, G.I., Wilhelm, S.M., Kronberger, A., Bauer, E.A., Grant, G.A., and Eisen, A.Z. (1986) Human fibroblast collagenase. Complete primary structure and homology to an oncogene transformation induced rat protein. J. Biol. Chem. 261:6600–6605.

Johansson, N., Westermarck, J., Leppä, S., Häkkinen, L., Koivisto, L., López-Otín, C., Peltonen, J., Heino, J., and Kähäri, V.-M. (1997) Collagenase-3 (MMP-13) gene expression by HaCaT keratinocytes is enhanced by tumor necrosis factor-α and transforming growth factor-β. Cell Growth Differ 8:243–250.

Kähäri, V.-M., Chen, Y.Q., Su, M.W., Ramirez, F., and Uitto, J. (1990) Tumor necrosis factor-α and interferon-γ suppress the activation of human type I collagen gene expression by transforming growth factor-β1: Evidence for two distinct mechanisms of inhibition at transcriptional and post-transcriptional level. J. Clin. Invest. 86: 1489–1495.

Kähäri, V.-M., Larjava, H., and Uitto, J. (1991a) Differential regulation of extracellular matrix proteoglycan (PG) gene expression: Transforming growth factor-β1 upregulates biglycan (PG I), and versican (large fibroblast PG) but down-regulates decorin (PG II) mRNA levels in human fibroblasts in culture. J. Biol. Chem. 266:10608–10615.

Kähäri, V.-M., Peltonen, J., Chen, Y.Q., and Uitto. J. (1991b) Differential modulation of basement membrane gene expression in human fibrosarcoma HT-1080 cells by transforming growth factor-β1. Enhanced type IV collagen and fibronectin gene expression correlates with altered culture phenotype of the cells. Lab. Invest. 64:807–818.

Kähäri, V.-M., Olsen, D.R., Rhudy, R.W., Carrillo, P., Chen, Y.Q., and Uitto, J. (1992) Transforming growth factor-β up-regulates elastin gene expression in human skin fibroblasts. Evidence for post-transcriptional modulation. Lab. Invest. 66:580–588.

Knäuper, V., López-Otín, C., Smith, B., Knight, G., and Murphy, G. (1996) Biochemical characterization of human collagenase-3. J. Biol. Chem. 271:1544–1550.

Mäkelä, J.K., Raassina, A., Virta, A., and Vuorio, E. (1988) Human proα1(I) collagen cDNA sequence for the C-propeptide domain. Nucleic Acids Res. 16:349.

McGowan, K.A., Bauer, E.A., and Smith, L.T. (1994) Localization of type I human skin collagenase in developing embryonic and fetal skin. J. Invest. Dermatol. 102:951–957.

Mitchell, P.G., Magna, H.A., Reeves, L.M., Lopresti-Morrow, L.L., Yocum, S.A., Rosner, P.J., Geoghegan, K.F., and Hambor, J.E. (1996) Cloning, expression, and type II collagenolytic activity of matrix metalloproteinase-13 from human osteoarthritic cartilage. J. Clin. Invest. 97:761–768.

Prosser, I.W., Stenmark, K.R., Suthar, M., Crouch, E.C., Mecham, R.P., and Parks, W.C. (1989) Regional heterogeneity of elastin and collagen gene expression in intralobar arteries in response to hypoxic pulmonary hypertension as demonstrated by *in situ* hybridization. Am. J. Pathol. 135:1073–1088.

Reboul, P., Pelletier, J.-P., Tardif, G., Cloutier, J.-M., and Martel-Pelletier, J. (1996) The new collagenase, collagenase-3, is expressed and synthesized by human chondrocytes but not synoviocytes. A role in osteoarthritis. J. Clin. Invest. 97:2011–2019.

Reddi, A.H. (1994) Bone and cartilage differentiation. Curr. Opin. Genet. Dev. 4:737–744.

Reponen, P., Sahlberg, C., Munaut, C., Thesleff, I., and Tryggvason, K. (1994) High expression of 92-kD type IV collagenase (gelatinase B) in the osteoclast lineage during mouse development. J. Cell Biol. 124:1091–1102.

Saarialho-Kere, U.K., Chang, E.S., Welgus, H.G., and Parks, W.C. (1993a) Expression of interstitial collagenase, 92 kDa gelatinase, and TIMP-1 in granuloma annulare and necrobiosis lipoidica diabeticorum. J. Invest. Dermatol. 100:335–342.

Saarialho-Kere, U.K., Kovacs, S.O., Pentland, A.P., Olerud, J.E.,

Welgus, H.G., and Parks, W.C. (1993b) Cell-matrix interactions modulate interstitial collagenase expression by human keratinocytes actively involved in wound healing. J. Clin. Invest. 92:2858–2866.

Sandberg, M. and Vuorio, E. (1987) Localization of types I, II, and III collagen mRNAs in developing human skeletal tissue by in situ hybridization. J. Cell Biol. 104:1077–1084.

Sandberg, M., Vuorio, T., Hirvonen, H., Alitalo, K., and Vuorio, E. (1988a) Enhanced expression of TGF-β and c-fos mRNAs in the growth plates of developing human long bones. Development 102: 461–470.

Sandberg, M., Autio-Harmainen, H., and Vuorio, E. (1988b) Localization of the expression of types I, III, and IV collagen, TGF-β1 and c-*fos* genes in developing human calvarial bones. Dev. Biol. 130:324–334.

Sandberg, M., Mäkelä, J.K., Multimäki, P., Vuorio, T., and Vuorio, E. (1989) Construction of a human proα1(III) collagen cDNA clone and localization of type III collagen gene expression in human fetal tissues. Matrix 9:82–91.

Terada, T., Okada, Y., and Nakanuma, Y. (1995) Expression of matrix proteinases during human intrahepatic bile duct development. Am. J. Pathol. 147:1207–1213.

Westermarck, J., Lohi, J., Keski-Oja, J., and Kähäri, V.-M. (1994) Okadaic acid-elicited transcriptional activation of collagenase gene expression in HT-1080 fibrosarcoma cells is mediated by JunB. Cell Growth Differ. 5:1205–1213.

Westermarck, J., Häkkinen, L., Fiers, W., and Kähäri, V.-M. (1995) TNF-R55-specific form of human tumor necrosis factor-α induces collagenase gene expression by human skin fibroblasts. J. Invest. Dermatol. 105:197–202.

Woessner, J.F. (1994) The family of matrix metalloproteinases. Ann. N.Y. Acad. Sci. 732:11–30.

Wozney, J.M. (1995) The potential role of bone morphogenetic proteins in periodontal reconstruction. J. Periodontol. 66:506–510.