Expression of *interstitial collagenase* is restricted to skeletal tissue during mouse embryogenesis

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**SUMMARY**

Collagenases are thought to be involved in physiological and pathological processes that require extracellular matrix remodeling. Using the in situ hybridization technique, we describe the expression of *interstitial collagenase* gene during mouse embryogenesis between E6.5 and E17. We demonstrate that *interstitial collagenase* expression is exclusively detected in one event, namely the onset of bone formation. Transcripts accumulate in hypertrophied chondrocytes, found in the mature cartilaginous matrix of long-bone growth plates or ribs, and in osteoblasts and/or in endothelial cells that have migrated into the shafts of developing long bones. The expression of the *tissue inhibitor of metalloproteinases* (TIMP-2) gene precedes the expression of *interstitial collagenase* in developing bones. These data suggest that *interstitial collagenase* plays a specific role in bone development and that the tight regulation of its activity during development is achieved not only by post-translational mechanisms with TIMPs, as previously suggested, but also at the transcriptional level.

Key words: *interstitial collagenase*, TIMP-2, hypertrophied chondrocyte, bone morphogenesis, embryo

**INTRODUCTION**

Collagen molecules, the most abundant proteins in vertebrate organisms, are structural components of the extracellular matrix. Fourteen different types of collagen have been characterized so far. They form various structures, such as fibrils found in most connective tissues and made of types I, II, III, V and XI collagen, or sheets constituting basement membranes with type IV collagen and Descemet’s membrane with type VIII collagen. The sequence and structural organization of type X collagen are very similar to those of type VIII collagen. This collagen is specifically expressed in hypertrophic chondrocytes (Kirsch and von der Mark, 1990). Other less abundant collagens, types IX, XII, XIV fibril associated collagen and types VII and VI collagen, connect sheets and fibrils with other tissue components (Van Der Rest and Garrone, 1991).

Matrix metalloproteinases are zinc proteinases that degrade at least one of the different components of the extracellular matrix. The matrix metalloproteinase family is divided into three groups: collagenses with interstitial (MMP-1) and neutrophil collagens (MMP-8); gelatinases with gelatinase A or 72 kDa gelatinase (MMP-2) and gelatinase B or 92 kDa gelatinase (MMP-9); and stromelysins with stromelysin-1 (MMP-3), stromelysin-2 (MMP-10) and matrilysin (MMP-7). Gelatinases degrade basement membrane collagens or gelatin; stromelysins can act on several substrates including proteoglycans, type IV collagen, laminin, fibronectin and gelatin. Collagenases are the only known proteinases capable of cleaving the triple helical domain of the native fibrillar collagens, thereby generating fragments which can be degraded by gelatinolytic proteinases (Docherty and Murphy, 1990; Matrisian, 1990; Woessner, 1991). Interstitial collagenase or type I collagenase (EC 3.4.24.7; MMP-1) is secreted into the extracellular medium (Valle and Bauer, 1979) and degrades types I, II and III collagen (Welgus et al., 1981), type VII collagen or anchoring fibril (Seltzer et al., 1989), type VIII collagen (Sage, 1982) and type X collagen (Welgus et al., 1990). Interstitial collagenase, like other metalloproteinases, is made up of four domains: a signal peptide, a propeptide, a catalytic domain and a hemopexin-like domain (Henriet et al., 1992; Matrisian, 1990).

The control of extracellular proteolysis is critical for the organism. Interstitial collagenase and other metalloproteinases can be activated by proteolytic cleavage (for review see Woessner, 1991). They are also regulated by different inhibitors including the tissue inhibitors of metalloproteinases (TIMPs) (for review see Woessner, 1991). TIMP-1 is a secreted glycoprotein that forms a stoichiometric complex with collagenases, stromelysins or the proform of 92 kDa type IV collagenase/gelatinase. TIMP-2 inhibits the enzymatic activity of both 72 kDa type IV collagenase/gelatinase (Goldberg et al.,
The extracellular matrix is a dynamic structure which is remodeled by metalloproteinases during physiological or pathological processes. For example, mouse blastocysts secrete functional matrix degrading proteases (Brenner et al., 1989). During early morphogenesis of mouse salivary glands collagenase inhibits the initiation of the cleft formation whereas collagenase inhibitors stimulate this formation (Nakanishi et al., 1986). Metalloproteinases are also implicated in tumor invasion and metastasis (reviewed by Liotta, 1986). Metalloproteinases are also implicated in tumor invasion and metastasis (reviewed by Liotta, 1986). Metalloproteinases are also implicated in tumor invasion and metastasis (reviewed by Liotta, 1986).

The expression of *interstitial collagenase* during embryogenesis has never been described, since specific probes were not available for such studies. In this study using a mouse collagenase probe (Henriet et al., 1992) we show that *interstitial collagenase* expression is first detected by northern blot analysis of total embryonic RNA on day 15 of gestation, and appears to increase thereafter.

In situ hybridization reveals that this expression is located exclusively in bone formation areas. By comparison between in situ hybridization and histological staining with Alcian Blue, we determined that *interstitial collagenase* is mainly expressed in hypertrophied chondrocytes during mouse development.

**MATERIALS AND METHODS**

**Interstitial collagenase and TIMP-2 probes**

A 2.7 kbp mouse MMP-1 cDNA (Henriet et al., 1992) was inserted between the *Hind*III and *Nofl* sites of a pBluescript plasmid (Stratagene). This cDNA contains a 10 bp 5′ untranslated region, a 1416 bp open reading frame, a 1215 bp 3′ untranslated region containing the polyadenylation signal and a 19 bp poly(A) tail. A 997 bp fragment was prepared by *Xho-NarI* digestion to probe northern blots. The probe used to detect TIMP-2 mRNAs was transcribed from a 791 bp fragment of a human TIMP-2 cDNA. Since TIMP-2 had only 41% amino acid identity until TIMP-1 (Stetler-Stevenson et al., 1989), TIMP-2 probe will not hybridize with TIMP-1 transcripts under the conditions used for our in situ studies. Furthermore, the intensity of the signals detected with our TIMP2 probe decreased drastically from E10.5 to E17 (see Fig. 4) whereas Flenniken and Williams (1990) and Nomura et al. (1989) report that the amount of TIMP1 transcripts during bone formation increases until birth.

**Northern blot**

Embryos from Swiss strain mice were frozen in liquid nitrogen and total RNA was extracted in RXzol (Bioprobe) according to Chomczynski and Sacchi (1987). Samples of 20 μg of total RNAs were electrophoresed in 1.2% agarose formaldehyde gel and transferred to nitro-cellulose membranes (Amersham). These membranes were hybridized at 42°C overnight with the 997 bp 32P-nick translated mouse *interstitial collagenase* cDNA probe in the hybridization solution (formamide 50%, Denhardt 1X, Heps, pH 7.0, 50 mM SSC 3X, calf thymus DNA 250 μg/ml). Membranes were subsequently washed at 60°C in 2X SSC, 0.1% SDS twice during 10 minutes and exposed 6 days with an Amersham MP film.

Poly(A)+ RNAs were purified with oligo(dT)-cellulose columns (mRNA purification kit; Pharmacia). Samples of 2 μg of poly(A)+ RNAs were used for northern blot analysis in the same conditions as used with total RNA.

Equal loading of total RNA in various lanes was assessed with a GAPDH probe.

**In situ hybridization**

Embryos were fixed in 4% paraformaldehyde and embedded in paraffin. 5 μm sections were collected on glass slides previously treated with 3-aminopropyltriethoxysilane (TESPA, Aldrich).

Sense and antisense [35S]CTP labelled RNA probes were transcribed with T7 or T3 RNA polymerases from the pBluescript plasmids previously linearised. In situ hybridization was performed as described by Quéva et al. (1992). Since a human TIMP-2 probe was used in these studies with mouse embryos, hybridization and washes were carried out at 55°C instead of 60°C. Control sense probe hybridization never displayed signals.

**Histological staining**

The sections were hydrated and stained with Alcian Blue as described by Lev and Spicer (1964). They were counterstained with Nuclear Red, rinsed, dehydrated with ethanol and treated with xylene before mounting under coverslips with Xam (BDH Gurr).

**RESULTS**

Previous studies have shown that the size of mouse collagenase mRNA was about 3.0 kb (Henriet et al., 1992). On a northern blot of total RNA from mouse embryos the *interstitial collagenase* probe hybridized with a 3 kb mRNA species (Fig. 1). These transcripts were first detected at E15 and their amount increased thereafter.

Since the absence of hybridization signal at E13 could be due to the poor sensitivity of northern analysis, we used the in
situ hybridization technique to assess the expression of *interstitial collagenase* during the early stages of mouse development. RNA probes were transcribed from 2.7 kb templates using [35S]CTP at about 1300 Ci mmol⁻¹. Assuming that 10% hybridization efficiency is achieved and that the probability of getting one silver grain from a β particle emitted by the disintegration of 35S is 10% (Rogers, 1979), the sensitivity of the detection of collagenase transcripts will be about 0.6 grain per mRNA molecule per week of exposure to the emulsion. Despite a low estimate for the hybridization efficiency this evaluation indicates that the limit of the sensitivity after a two week exposure will be about one mRNA molecule/cell. At E6.5, embryos with the surrounding maternal tissue, and at E10.5 and E13, the entire embryos, were sectioned and

![Image](image.png)

**Fig. 2.** In situ hybridization analysis of *interstitial collagenase* expression in mouse embryos. Alcian Blue staining (A,C,E) visualized by bright field and distribution of silver grains (B,D,F) visualized by dark field. (A-B) Sagittal sections at E13. The little arrows show cartilaginous primordia of cervical vertebrae. *interstitial collagenase* transcripts were not detected. The liver (li), at this stage as well as later during development, and the heart ventricle (ve) displayed a non specific light scattering due to the presence of erythrocytes. mx, maxillary; md, mandible. (C-D) Sagittal sections at E15. (C) According to their state of differentiation, young chondroblasts (e.g. in ventral ribs or in future cranial bones, arrowheads) displayed large amounts of matrix and consequently were strongly stained. In contrast, hypertrophied chondrocytes (e.g. in dorsal ribs or in femur; arrows) were faintly stained. (D) Only the cells of the mature cartilaginous matrix expressed *interstitial collagenase* (arrows). lu, lung. (E-F) Sagittal sections at E17. The cartilaginous matrix was stained in ribs, in caudal vertebrae (arrowheads), in scapula (arrow), in mandibles and in nasal and cervical bones. *interstitial collagenase* expression was detected in the mature cartilaginous matrix cells of the scapula (arrow) and of the dorsal ribs whereas cells found in immature cartilaginous matrix were negative (e.g. caudal vertebrae, arrowhead). Bar, 1 mm.
hybridized with the \textit{interstitial collagenase} probe. No hybridization signal was detected (Fig. 2B), suggesting that the \textit{interstitial collagenase} gene was not expressed at these stages.

We subsequently attempted to identify the cells where this gene was expressed at later stages of development. In view of the detection of collagenase activity during bone morphogenesis (Eeckhout et al., 1986), sections were stained with Alcian Blue to visualize cartilaginous matrix accumulation within the developing skeletal tissue. Interestingly, at E13 the primordium of cervical vertebrae was stained, indicating the cartilaginous matrix deposition in these skeletal tissues where no \textit{interstitial collagenase} transcripts were detected (compare Fig. 2A and B).

At E15, an intense hybridization signal with the \textit{interstitial collagenase} probe was conspicuous in the dorsal ribs, in the femur and in the clavicle area (Fig. 2D). No signal was detected in the ventral ribs or elsewhere in the embryo, in the lung, in the liver or in the brain, for example. As shown with the Alcian Blue staining, at E15 the maturation of cartilaginous matrix was obvious: dorsal ribs displayed hypertrophied chondrocytes within a faint Alcian Blue staining whereas younger chondrocytes were found in the humerus head (Fig. 2C). The \textit{interstitial collagenase} signal was localized in hypertrophied chondrocytes while immature cartilaginous matrix in cranial cartilaginous primordia, humerus head, tibia, fibula and metacarpals (Fig. 2C) were negative.

At E17, collagenase mRNAs were detected in the dorsal ribs, as observed at E15, but also in the lower jaw in the central part of Meckel’s cartilage, in a narrow band of the dorsal part of the scapula (Fig. 2F) and in the cervical vertebrae (data not shown). Alcian Blue staining demonstrated again that this expression was restricted to hypertrophied chondrocytes while immature chondrocytes (e.g. in caudal vertebrae) were negative (Fig. 2E). \textit{interstitial collagenase} transcripts were never localized in vascular, nervous or urogenital systems, or in the gut and its derivatives.

In order to determine if \textit{interstitial collagenase} might be expressed in other cell types during bone morphogenesis, we analysed its expression in E18 forelimb long bones. In sagittal sections of long bones, all steps of the cartilaginous maturation during endochondral ossification can be identified (Fig. 3A and C). In the humerus, \textit{interstitial collagenase} transcripts were detected not only in hypertrophied chondrocytes but also in bone areas in the shaft where osteoblasts, endothelial cells and probably also osteoclasts had migrated (Fig. 3B and D).

Therefore it appeared that \textit{interstitial collagenase} was exclu-

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**Fig. 3.** In situ hybridization analysis of \textit{interstitial collagenase} expression in the humerus at E18. Alcian Blue staining (A,C) and distribution of silver grains (B,D). (A-B) The epiphysis staining corresponds to the cartilaginous matrix; between the arrows are the growth plate and the forming bone. \textit{Interstitial collagenase} expression was detected in the growth plate and in the bone up to the middle of the shaft. (C-D) In the growth plate the resting zone (rz), proliferating zone (pz), hypertrophic zone (hz) and forming bone (b) can be easily identified after Alcian Blue staining. \textit{Interstitial collagenase} gene expression began to be detected in hypertrophied chondrocytes of the hypertrophic zone (hz) and remained high in the forming bone (b) where endothelial cells, future osteoblasts and probably osteoclasts were migrating. Bar, 300 µm.
sively expressed at the onset of bone formation during mouse development. It was of interest to see how this expression correlated with that of metalloproteinase inhibitors. In contrast to interstitial collagenase, TIMP-2 expression had already occurred at E13. TIMP-2 transcripts were detected at the periphery of the dorsal ribs of mouse embryos (Fig. 4) whereas interstitial collagenase transcripts were found only at E15 and inside the ribs (Fig. 2). TIMP-2 transcripts were expressed in large areas around the dorsal ribs at E13 and the amount of transcripts decreased thereafter (Fig. 4). This expression was closely restricted to the rib periphery at E17 (Fig. 4E).

**DISCUSSION**

Since interstitial collagenase is able to degrade different collagen molecules (types I, II, III, VII, VIII and X), its expression could have been expected in a variety of tissues in the mouse embryo. Interestingly, however, our in situ hybridization analysis revealed a restricted expression of interstitial collagenase mRNAs within developing bones suggesting that uncontrolled collagenase activity may represent a danger for the integrity of the embryo. At E6.5, during mouse embryo implantation in the uterine wall, interstitial collagenase transcripts were never detected. This result indicates that interstitial collagenase is not involved during mouse embryo implantation; at that time urokinase-type plasminogen activator (uPA) transcripts are expressed in invading embryonic tissues (Grevin et al., 1993; Sappino et al., 1989) whereas they are not detected in the embryo proper until E10.5.

The absence of interstitial collagenase expression outside the developing bones in embryos is in sharp contrast to its conspicuous expression in adult tissues under pathological conditions, in stromal fibroblasts of colonic neoplasia (Gray et al., 1993), oral squamous cell carcinoma (Gray et al., 1992), breast carcinoma (Polette et al., 1993) and other invasive carcinomas (Wernert et al., in the press), and also in keratinocytes and epithelial cells in cutaneous wound repair after burn injury (Stricklin et al., 1993). This indicates that the remodeling of the extracellular matrix during invasive processes requires different proteases in the embryo and in tumors.

The comparison between our in situ hybridization and histological staining results demonstrates that interstitial collagenase is mainly expressed in hypertrophied chondrocytes.

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**Fig. 4.** In situ hybridization analysis of TIMP-2 expression around the dorsal ribs. Hybridization with the antisense (A,C,E) or the sense (B,D,F) TIMP-2 RNA probe. TIMP-2 transcripts were detected at the periphery of the dorsal ribs (arrows) in the mouse embryo at E13 (A-B), at E15 (C-D) as well as at E17 (E-F). The intensity of the signal decreases upon development. lu, lung. Bar, 200 µm.
The cartilaginous matrix of the future bones appears at about E13. Endochondral ossification occurs from this cartilaginous matrix (Kaufman, 1992). During this process proliferating chondrocytes in the cartilaginous growth plate differentiate into hypertrophied chondrocytes (reviewed by Farnum and Wilsman, 1987) and the surrounding cartilaginous matrix is extensively remodelled. Therefore the localization of interstitial collagenase RNA in hypertrophied chondrocytes is not surprising. Moreover, other studies have shown the involvement of collagenase in bone morphogenesis. Collagenase activity was detected in sections of the growth plate containing hypertrophied chondrocytes from rats developing a rachitic phenotype (Brown et al., 1989). Metalloproteinases including collagenase were detected by immunolocalization, not only in hypertrophied chondrocytes but also in the chondrocytes of the resting and proliferating zones in femur growth plates dissected from newborn rabbits (Brown et al., 1989). In the mouse embryo forelimb at E13, endochondral ossification is evident with the complete degradation of the cartilage in cultured chick embryo tibia. Moreover, osteoblasts (Heath et al., 1984), osteoclasts (Delaissé et al., 1993) and endothelial cells (Herron et al., 1986; M. B. Raes, unpublished results) are able to express and secrete collagenase.

Interstitial collagenase activity is regulated by both TIMP-1 and TIMP-2. Furthermore TIMP-2 may be able to block the autoproteolytic activation of interstitial collagenase (DeClerck et al., 1991). At E13.5, TIMP-1 transcripts have been detected in the mandible and in the clavicle, around the ribs and the femurs of mouse embryos (Flenniken and Williams, 1990; Nomura et al., 1989) and TIMP-1 has been localized in rabbit growth plate chondrocytes (Brown et al., 1989). We also detected TIMP-2 transcripts at E13 in bone formation areas including at the periphery of dorsal ribs. Therefore both TIMP-1 and TIMP-2 transcripts are expressed before the onset of interstitial collagenase gene expression and their presence thereafter makes it likely that TIMP-1 and TIMP-2 take part in a security mechanism preventing the dissemination of active collagenase molecules.

The tight regulation of interstitial collagenase activity during development seems to be achieved not only by post-translational mechanisms, as previously suggested, but also at the transcriptional level. The activity of the collagenase gene promoter has been shown to be regulated by c-Fos/c-Jun (AP1) complexes and by Ets (PEA3) transcription factors (Gutman and Wasylyk, 1990; Wasylyk and Wasylyk, 1992; reviewed by Mauviel, 1993; Woessner, 1991). c-fos and c-jun expression during mouse development has been previously described. c-fos transcripts were detected in the perichondrium enclosing the extremities of E17 fetal long bones (Dony and Gruss, 1987) and c-jun transcripts were found in cartilaginous and perichondrial cells in ribs and limbs at E14.5 (Wilkinson et al., 1989). In addition, c-fos overexpression induces bone tumors in transgenic mice (Rüther et al., 1989) and targeted disruption of this gene by homologous recombination generates bone defects in homozygous mutants (Wang et al., 1992). In contrast c-ets1 transcripts were not expressed in hypertrophied chondrocytes at E17 where interstitial collagenase transcripts accumulated (data not shown). In view of the expression pattern of interstitial collagenase described in this paper, the features of c-fos and c-jun expression suggest that c-fos/c-jun AP1 complexes might be involved in interstitial collagenase gene regulation during bone morphogenesis. However, the expression of c-fos and c-jun is not restricted to this process since c-fos is also detected in the outer spongiosotrophoblast of the 13-day murine placenta and c-jun in developing muscle or in the nervous system (Dony and Gruss, 1987; Wilkinson et al., 1989), for example. Therefore, it is likely that transcription factors distinct from AP1 are involved in the specific activation of interstitial collagenase gene transcription during mouse skeletal development, and/or in the inhibition of this transcription in other tissues.

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