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THE CHARACTEFIZATION OF THE COLLAGENASE INVOLVED DURING CHONDROCYTE MATURATION IN THE BOVINE FETAL GROWTH PLATE

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ABSTRACT

Studies were designed to investigate the expression and regulation of interstitial collagenase (MMP-1) during endochondral ossification in fetal bovine growth plate chondrocytes *in vitro*. Fetal bovine growth plate chondrocytes were separated into subpopulations representing different maturational stages by using a Percoll discontinuous gradient. Gene expression and synthesis of MMP-1 in these chondrocyte subpopulations were followed in serum-free cultures as they matured from the prehypertrophic to hypertrophic phenotype. However, MMP-1 mRNA expression and protein were undetectable. This expression was also undetectable in interleukin 1α (IL-1α) stimulated growth plate chondrocytes. MMP-1 mRNA and protein were however observed in IL-1α stimulated bovine fetal skin fibroblasts. Although MMP-1 was absent in growth plate chondrocytes, a collagenolytic activity was detectable in culture medium by using ^{14}C labeled type II collagen. It was only seen when the chondrocytes were hypertrophic. By using the polymerase chain reaction (PCR), employing conserved sequences found in mammalian collagenase-3 (MMP-13), a novel bovine collagenase was cloned. The cDNA and predicted amino acid sequence shared a 92% and 90% identity to the human MMP-13 cDNA and amino acid sequence respectively. MMP-13 mRNA and protein were found only when the chondrocytes became hypertrophic. The role of MMP-13 during the maturation of these fetal growth plate chondrocytes was further investigated by using a preferential carboxylate inhibitor of collagenase-3. This inhibitor reduced the generation of denatured type II collagen in chondrocyte cell layers and proteoglycan release into conditioned

medium in a dose dependent manner. Also, types II and X collagen synthesis and matrix calcification were inhibited. These studies indicate that MMP-13 may play a very important role during the maturation of fetal growth plate chondrocyte *in vitro* and during endochondral ossification and that cellular hypertrophy is dependent upon the activity of this enzyme.

RÉSUMÉ

L'objectif de cette étude était d'évaluer l'expression et la régulation de la collagénase interstitielle (MMP-1) lors du processus d'ossification endochondrale à l'aide de chondrocytes isolés à partir de la plaque de croissance de foetus bovins. Les chondrocytes ont été séparés, à l'aide d'un gradient de Percoll discontinu, dans des sous-populations représentant les différentes étapes de maturation cellulaire caractéristiques de la plaque de croissance de foetus bovins. L'expression du gène et la synthèse de MMP-1 ont été étudiés lorsque ces sous-populations de chondrocytes pré-hypertrophiques cultivées en absence de sérum, se differenciaient et exprimaient le phénotype hypertrophique. Cependant, l'expression du mRNA et de la protéine MMP-1 n'ont pas pu être détectés dans ces cultures, même après avoir stimulé les chondrocytes par l'interleukine 1-alpha. Par contre, ces mêmes molécules étaient clairement identifiables dans des cultures de fibroblastes de peau bovine foetale après stimulation par l'interleukine 1-alpha. Bien que la MMP-1 n'ait pas pu être détectée dans les chondrocytes de la plaque de croissance, une activité collagénolytique, mesurée par la dégradation du collagène de type II marqué au ^{14}C, était observée dans le milieu de culture. Cette activité était uniquement présente dans les milieux de culture de chondrocytes hypertrophiques. À l'aide de la réaction en chaîne de la polymérase (PRC), et en utilisant des séquences conservées et présentes dans les collagénases-3 (MMP-13) des mammifères, une nouvelles collagénase bovine a été clonée. Le cADN et la séquence des acides aminés étaient identiques à 92% et 90% réspectivement au cADN et à la sèquence polypeptidique de la MMP-13 humaine. De plus,

l'expression du mARN et de la protéine MMP-13 était propre aux chondrocytes hypertrophiques. Le rôle joué par la MMP-13 lors de la maturation des chondrocytes de la plaque de croissance a été aussi étudié à l'aide d'un inhibiteur de la collagénase-3 de type carboxylique. Cet inhibiteur réduisait, de manière dose-dépendante, la dénaturation de collagène de type II dans la matrice extracellulaire produite par les chondrocytes in vitro, ainsi que le contenu en protéoglycanes libérés dans le milieu de culture. De plus, la synthèse des collagènes de types II et X, et la calcification de la matrice extracellulaire étaient également inhibées. Ces résultats indiquent que la MMP-13 pourrait jouer un rôle très important lors de la maturation des chondrocytes foetaux de la plaque de croissance in vitro, et par conséquence dans le processus d'ossification endochondrale. Ils suggérent également que l'hypertrophie cellulaire est dépendant de l'activité de cette enzyme.

DEDICATION

I wish to dedicate this thesis to my parents, family, and Jennifer, without whose support and encouragement, this work would not have been possible.

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α1(II)	amino acid chain of type II collagen					
Amino acids:	Alanine	A or	Ala	Leucine	L or	Leu
	Arginine	R or	Arg	Lysine	K or	Lys
	Asparagine	N or	Asn	Methionine	M or	Met
	Aspartic acid	D or	Asp	Phenylalanine	F or	Phe
	Cysteine	C or	Cys	Proline	P or	Pro
	Glutamine	Q or	Gln	Serine	S or	Ser
	Glutamic acid	E or	Glu	Threonine	T or	Thr
	Glycine	G or	Gly	Tryptophan	W or	Trp
	Histidine	H or	His	Tyrosine	Y or	Tyr
	Isoleucine	I or	Ile	Valine	V or	Val

APMA	aminophenyl mercuric acetate
bp	base pairs
BSA	bovine serum albumin
COL2-3/4m	intrachain hidden epitope of α1(II) chain
cDNA	copy DNA
CS	chondroitin sulfate
D	Daltons
DEPC	diethyl pyrocarbonate
DMEM	Dulbecco's minimal essential medium
DMMB	dimethylmethylene blue

DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EDTA	ethylene diamine tetraacetate
ELISA	enzyme-linked immunosorbent assay
FCS	fetal calf serum
GAG	glycosaminoglycan
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
IgG	immunoglobulin G
IL-1	interleukin-1
I.T.S.	insulin-transferrin-sodium selenite culture medium supplement
kD	kilodaltons
K_I	dissociation constant of an enzyme-inhibitor complex
KS	keratan sulphate
MMP	matrix metalloproteinase
MMP-1	collagenase-1 or interstitial collagenase
MMP-8	collagenase-2 or neutrophil collagenase
MMP-13	collagenase-3
MT-MMP	membrane-type matrix metalloproteinase
mRNA	messenger RNA
OVA	ovalbumin
PCR	polymerase chain reaction

PTH	parathyroid hormone
PTHrP	parathyroid hormone-related peptide
PBS	phosphate buffered saline
RNA	ribonucleic acid
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
T_3	tri-iodothyronine
T_4	thyroxine
TC^A	collagenase-generated three quarter collagen a chain fragment
TC^B	collagenase-generated one quarter collagen a chain fragment
TGF-β	transforming growth factor beta
TIMP	tissue inhibitor of matrix metalloproteinase

1. INTRODUCTION

During fetal development there is extensive growth and remodeling of tissues. This process involves proteolytic remodeling of the extracellular matrix and is essential in these physiological situations (Alexander and Werb, 1991). One of these events is the process of endochondral ossification which is mediated by the primary and secondary growth plates. Growth plate chondrocytes play a pivotal role in promoting longitudinal bone growth in vertebrates. These chondrocytes undergo a dramatic change in synthesis of their extracellular matrix macromolecules during their maturation from prehypertrophic chondrocytes to hypertrophic chondrocytes (Hunziker *et al.*, 1987; Poole, 1991). During hypertrophy, chondrocytes enlarge and remodel their surrounding matrix (Poole, 1991). This remodeling involves degradation of type II collagen (Poole, 1991; Alini *et al.*, 1992, 1994). Type II collagen can only be cleaved by enzymes called collagenases that belong to the matrix metalloproteinase family (Birkedal-Hansen *et al.*, 1993). The role and regulation of collagenase(s) in the growth plate is poorly understood and there is little knowledge of its regulation and expression during endochondral ossification.

The specific aim of this thesis is to address the gene expression and biochemical events of collagenase(s) during growth plate chondrocyte maturation from the prehypertrophic to hypertrophic phenotype. This process will be studied in isolated bovine fetal growth plate chondrocytes. These chondrocytes are purified by Percoll discontinuous gradient centrifugation that allows for separation

of prehypertrophic chondrocytes from hypertrophic chondrocytes (Lee *et al.*, 1990; Carey *et al.*,1993). Furthermore, these chondrocytes in culture undergo the same biochemical and maturation changes as observed *in vivo* (Alini *et al*, 1994) and can be cultured in serum free conditions (Alini *et al.*, 1996). When these studies were initiated, there was only one collagenase that was known to be present in chondrocytes: interstitial collagenase (MMP-1). With the cloning of human collagenase-3 (MMP-13) (Freije *et al.*, 1994) and demonstrating that it was the homologue of rodent collagenase, it has further made it difficult to interpret the results on developmental expression of collagenases during endochondral ossification. Therefore it was essential to establish which collagenase was involved in bovine fetal growth plate maturation and to develop specific reagents to discriminate between them.

As background, this introduction deals briefly with the biology and biochemistry of the growth plate along with the MMP and inhibitors that are involved in matrix turnover. The relationship between MMPs and the growth plate will also be discussed.

1.1 Overview of Growth Plate

1.1.1 Morphology and physiology of the growth plate

The cartilaginous growth plate lies in the distal and proximal ends of developing mammalian long bones (Poole, 1991) (Fig. 1.1) and mediate endochondral ossification. The primary growth plate is organized into zones (Fig. 1.1) that are perpendicular to the main axis of growth. The thickness of each zone varies according to the level of growth activity. The primary growth plate regulates the longitudinal growth of the physis while the secondary growth plate which is circular in shape regulates the epiphyseal growth.

The growth plate chondrocytes are organized into long columns within each zone. The growth plate can be subdivided into different zones: reserve, proliferating, maturational, and upper and lower hypertrophic (Fig 1.1). The reserve zone cells are the stem cells and show little or no cell division (Kember, 1960). They in turn give rise to daughter cells that make up the proliferative zone which undergoes rapid cell division (Hunziker *et al.*, 1987). These chondrocytes divide perpendicular to the longitudinal axis of growth giving rise to a column and thus mediate the elongation of the growth plate. They then start to enlarge and begin to form the maturation zone in which they will mature into hypertrophic chondrocytes. The hypertrophic zone is easily distinguishable from the other zones due to the enlarged round cells. The chondrocytes enlarge five- to tenfold which results in a reduction of matrix volume per unit volume of tissue (Hunziker *et al.*, 1987). Due to this increase in cell size, it is believed that matrix degradation

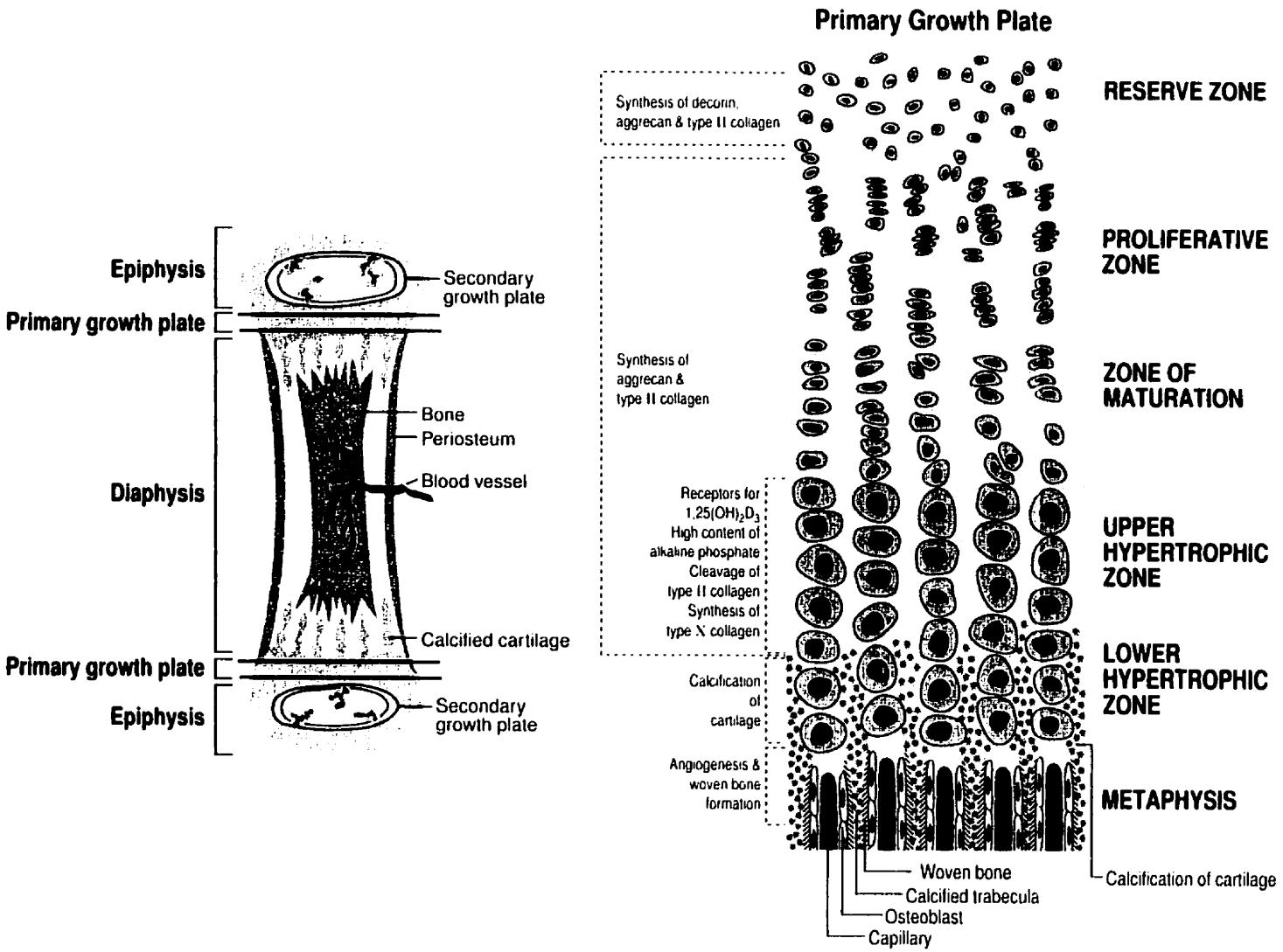


Fig. 1.1 Diagrammatic representation of the organization of a mammalian long bone and primary growth plate.

begins in this zone. It is also in this zone where chondrocytes prepare for matrix mineralization (Howell and Dean, 1992). The hypertrophic zone is also where we first see the synthesis of type X collagen (Gibson and Flint, 1985; Schmid and Linsenmayer, 1987; Poole and Pidoux, 1989), and the up-regulation of alkaline phosphatase (Väänänen, 1980; de Bernard *et al.*, 1986; Alini *et al.*, 1992) which leads to calcification of the extracellular matrix.

In the primary bovine growth plate (and probably in most other mammals), the process of calcification occurs in the longitudinal septa, whereas the transverse septa remains uncalcified. The exact mechanism of this process is still not known. Many believe that it may be mediated by matrix vesicles which are rich in alkaline phosphatase (Anderson, 1969, 1976; Arsenault and Hunziker, 1988; Hunziker *et al.*, 1989; Kirsch and Wuthier, 1994; Kirsch *et al.*, 1994), while others have postulated a role for matrix glycoproteins (Landis and Glimcher, 1982), C-propeptide of type II collagen (Poole *et al.*, 1984; Poole, 1991; van der Rest *et al.*, 1986) or proteoglycans such as aggrecan (Hunter, 1987; Poole, 1991). In the cartilage matrix, proteoglycans have been reported to be focally concentrated in mineralizing sites (Shepard and Mitchell, 1985). Proteoglycans can bind calcium via their chondroitin sulfate (CS) chains (Bowness and Lee, 1967; Dziewiakowski, 1987; Hunter, 1987, Hunter *et al.*, 1988; Poole, 1991). Calcium has been shown to be displaced by increasing phosphate concentration that is provided by alkaline phosphatase and therefore allows for the formation of hydroxyapatite (Hunter, 1987).

It is thought that the fate of the lowermost hypertrophic chondrocytes is an apoptotic death due to mineralization of their extracellular matrix (Farnum *et al.*, 1987; Zenmyo *et al.*, 1996; Amling *et al.*, 1997). There is also evidence that some hypertrophic chondrocytes may give rise to precursors of bone forming cells (Cancedda *et al.*, 1992; Roach *et al.*, 1995).

It is at this final stage, that the process of angiogenesis occurs at the metaphysial junction with the invasion of capillary sprouts into the last transverse septum (Schenk *et al.*, 1967, 1968; Arsenault, 1987, Hunter and Arsenault, 1990; Lewinson and Silbermann, 1992). This physiological invasion is tightly controlled and carefully balanced with the continual production of new cells and cartilage matrix in the growth plate. Therefore, growth plate height remains fairly constant (Thorngren and Hansson, 1981).

1.1.2 Hormones

1.1.2.1 Parathyroid Hormone (PTH), Parathyroid hormone-related peptide (PTHrP) and PTH/PTHrP receptor

Parathyroid hormone (PTH) is synthesized by the parathyroid glands and plays an important part in calcium homeostasis. It acts primarily on the renal cortex and bone for the regulation of calcium concentrations in the circulation (Goltzman *et al.*, 1975; Potts *et al.*, 1982) In contrast, parathyroid hormone-related peptide (PTHrP) is synthesized in multiple tissues with very specific temporal and spatial patterns of expression (Broadus and Stewart, 1994). PTHrP was initially discovered as a product of tumors that gave rise to humoral

hypercalcemia during malignancy (Broadus and Stewart, 1994). Although PTHrP does not normally circulate and primarily acts as a paracrine factor (Broadus and Stewart, 1994), patients with these tumors produced significant quantities of PTHrP which entered the circulation and cross-reacted with PTH receptors in the bone and kidneys.

The PTH and PTHrP genes arose from a gene duplication event. They share a similar genomic organization and a homologous amino-terminal peptide sequence that interacts with their specific G protein-coupled receptor: PTH/PTHrP receptor (Jüppner, 1995). The human PTHrP gene is comprised of 9 exons (Martin *et al.*, 1991) and utilizes three promoters for its transcription. The mRNA is alternatively spliced which leads to three major isoforms of the mature peptide, one of 139, 141 and 173 amino acids. There is only one transcript found in rat (Thiede and Rodan, 1988; Yasuda *et al.*, 1989) and mouse (Mangin *et al.*, 1990) which encodes a 141 and 139 amino acid PTHrP, respectively. The highest amount of homology in PTHrP lies in the N-terminal and midregion of the protein. The PTH/PTHrP receptor from the different mammalian species ranges from 585-593 amino acids in size. There does not seem to be alternative splicing in the coding region of the genes and only the human kidney PTH/PTHrP receptor differs in it's 5' noncoding region (Jüppner, 1995). The PTH/PTHrP receptor is composed of a N-terminal extracellular domain of approximately 180 amino acids, seven transmembrane-spanning helixes, and an intracellular C-terminal tail of about 120 amino acids. Although it is similar in structure to other G-protein

coupled receptors, it is now classed into its own protein family (Jüppner, 1994).

The first indication that PTHrP was important as a paracrine factor in skeletal development arose when a null mouse for PTHrP was generated (Karaplis *et al.*, 1994). These mice died postnatally due to asphyxia and exhibited abnormalities in endochondral ossification. The proliferative zones of these mice were greatly reduced and their hypertrophic zones were enlarged. Their growth plates undergo an accelerated maturation and bone formation. The reduced size of the epiphyseal growth plate was due to diminished numbers of chondrocytes in the reserve and proliferative zones (Amizuka *et al.*, 1994). In the normal litter mates, PTHrP was shown to be localized in the chondrocytes of the resting, proliferative maturation zones. These studies indicated that PTHrP must be important in the resting zone and proliferative zones. PTHrP probably controlled chondrocyte proliferation and maturation. The PTH/PTHrP receptor expression was also analyzed in the PTHrP null mouse (Amizuka *et al.*, 1996; Lee *et al.*, 1996). Both groups showed the PTHrP receptor mRNA and protein to be present in the resting and proliferative zone but maximal at the maturation zone in both normal and mutant mice. PTHrP's role in the growth plate is to regulate chondrocyte maturation by delaying terminal differentiation into the hypertrophic phenotype. These observations were confirmed when PTHrP was overexpressed in murine growth plates utilizing the type II collagen promoter (Weir *et al.*, 1996). These mice developed a novel form of chondrodysplasia due to a delay in endochondral ossification. These mice are born with a cartilaginous endochondral skeleton.

Their growth plates are comprised of mainly proliferating and prehypertrophic chondrocytes. This clearly demonstrated that PTHrP's function is to delay the differentiation of prehypertrophic chondrocytes to hypertrophic chondrocytes. These phenotypes can also be seen in the PTH/PTHrP receptor mutations. Patients with Jansen-type metaphyseal chondrodysplasia have a constitutively active PTH/PTHrP receptor (Schipani *et al.*, 1995). This constitutive receptor mimics the action of overexpression of PTHrP and results in delayed hypertrophy and mineralization. PTHrP receptor null mice exhibited accelerated differentiation of chondrocytes (Lanske *et al.*, 1996). Their growth plates undergo premature hypertrophy and mineralization. These mice exhibited a similar phenotype to the PTHrP null mice.

1.1.2.2 Thyroid hormone

Thyroid hormones are important in the regulation of growth and development in vertebrates. Changes in the secretion of thyroid hormones can lead to retardation of skeletal growth and deformities (Dott, 1923; Simpson *et al.*, 1950; Ray *et al.*, 1954; Wilkins, 1965; Sibermann, 1983). Thyroid hormone (T_3) and thyroxine (T_4) are synthesized in the thyroid gland. The major thyroid hormone that is secreted by the cells in the thyroid follicle is T_4. T_3 is derived from the enzyme-catalyzed 5' -deiodination of T_4 (Leonard and Visser, 1986). Type I T_4 5'-deiodinase is the enzyme which catalyzes this reaction with T_4 from the circulation and is found mainly in the liver and kidney (Chopra, 1977). Type II T_4 5'- deiodinase which is found in the brain and pituitary, produces T_3 that

contributes directly to thyroid receptor occupancy (Larsen *et al.*,1981). Several studies *in vivo* (Simpson *et al.*, 1950; Ray *et al.*, 1954; Lewison *et al.*, 1989, 1994) in hypothyroid rats have implicated thyroid hormone or thyroxine in skeletal growth and function. These rats exhibited a dramatic decrease in their epiphyseal and metaphyseal trabecular bone volume. The cell volumes in the growth plates were reduced and there was a significant decrease in the number and size of hypertrophic chondrocytes (Lewison *et al.*, 1989). There was also a decrease in matrix turnover and the hypertrophic zone was separated from the subchondral bone marrow by a mineralized barrier that was not resorbed. The chondrocytes do not undergo full stages of hypertrophy and the process of endochondral ossification is impaired. However, administration of thyroid hormone or thyroxine reversed these effects (Lewison *et al.*, 1989). There have also been several in vitro studies on T_3 or T_4 effects on pig scapulae tissue (Burch and Lebovitz, 1982), rat epiphyseal chondrocytes (Ohlsson *et al.*,1992; Ballock and Reddi, 1994), chick embryo sternal chondrocytes (Bohme *et al.*, 1989; Quarto *et al.*, 1992) and fetal bovine growth plate chondrocytes (Alini *et al.*, 1996). In all of these studies, T_3 or T_4 promoted type X synthesis and chondrocyte hypertrophy. Also in the bovine system, T_3 or T_4 promoted matrix calcification with T_3 being more potent (Alini *et al.*, 1996). These studies indicate that T_3 or T_4 play an important role in chondrocyte maturation and hypertrophy.

1.1.2.3 Vitamin D

Vitamin D has been known to be very important in calcium metabolism

and bone homeostasis. It is essential in the early development of long bones during endochondral ossification. In vitamin D deficiency, both the bone matrix and cartilage growth are affected. The normal growth in the long bones that is mediated by the growth plate is affected: there is impaired calcification with the lengthening of the hypertrophic zone, resulting in rickets (Raisz and Kream, 1983a, b) Treatment of patients with rickets with the active metabolite of vitamin D, 1,25-dihydroxyvitamin D_3, restores mineralization and bone formation. Receptors for 1,25-dihydroxyvitamin D_3 are expressed by the growth plate chondrocytes and are maximal in the hypertrophic zone (Iwamoto *et al.*, 1989). There has also been evidence that another metabolite of vitamin D, 24,25-dihydroxyvitamin D_3, is important in bone formation during endochondral ossification and binding has been shown by auto-radiography to be localized in the proliferative zone of the growth plate (Corvol *et al.*, 1980; Fine *et al.*, 1985). In healthy rats, both metabolites seem to be required to maximally stimulate calcification *in vitro* (Hinek and Poole, 1988) while in rachitic rats, the resting zone require 24,25-dihydroxyvitamin D_3 to mature (Atkin *et al.*, 1985). Others have shown that cellular responses to vitamin D_3 metabolites are specific to different zones of rat costochondral cartilage (Boyan *et al.*, 1988) and in normal male rats (Seo *et al.*, 1996). Resting chondrocytes respond primarily to 24,25-dihydroxyvitamin D_3 while growth zone chondrocytes responded primarily to 1,25-dihydroxyvitamin D_3. 1,25-dihydroxyvitamin D_3 has also been shown to promote the expression of the hypertrophic phenotype in chick sternal

prehypertrophic cells (Gerstenfeld *et al.*, 1990a, b). These observations would point to different responses to each metabolite at different stages of maturation of the chondrocyte.

The growth plate chondrocytes at each zone are undergoing specific changes and are under the direction of different hormones and cytokines. PTHrP is very important in regulating the maturation of these chondrocytes, especially at the junction between the transition zone between proliferative and hypertrophic cells (as reviewed above). Recently, it has been shown that rat PTHrP gene is suppressed by 1,25-dihydroxyvitamin D_3 (Kremer *et al.*, 1996). The effect of other vitamin D metabolites on PTHrP have yet to be investigated. Since deficiencies and overexpression of these hormones can influence the rates of growth and maturation at different zones of the growth plate, these differential effects by vitamin D metabolites may be due to its interaction with other hormones at this critical transitional region where cells are maturing from prehypertrophic to hypertrophic chondrocytes.

1.2 Biochemistry of the Growth Plate

1.2.1 Collagens

1.2.1.1 Type II collagen

Type II collagen was first discovered in chick cartilage and was shown to be a homotrimer (Miller and Matukas, 1969). In fact, type II collagen is the major collagen found in all hyaline cartilages. Type II collagen belongs to the family of fibrillar collagens and its function is to provide tensile strength to cartilage. It

provides articular cartilages the ability to resist shear forces. Type II collagen is synthesized as a procollagen that is comprised of three identical pro α1(II) chains that form a triple helix. The procollagen is then converted to its processed form by cleavage leading to the removal of its non-helical N- and C-propeptides. This is accomplished by two different proteinases: N- and C-proteinases. Recently, the C-proteinase has been identified as the molecule formally known as bone morphogenic protein-1 (BMP-1) (Kessler *et al.*, 1996; Li *et al.*, 1996). It belongs to the astacin family of metalloproteinases (Sarras Jr., 1996). The processed collagen consists of short non-helical telopeptide regions at both the C- and N-terminal ends and the triple helix.

Type II collagen is found throughout the cartilage matrix of the growth plate. (Mayne, R. 1989; Lee *et al.*, 1990). It's expression pattern has been studied *in situ* in rat (Balmain *et al.*, 1995), chick (Iyama *et al.*, 1994), bovine (Sandell *et al.*, 1994) and human (Sandberg and Vurio, 1987; Mundlos, *et al.*, 1990; Vornehm *et al.*, 1996) growth plates. Its expression was found to be throughout the different zones of the growth plate, with the strongest signals in the proliferative and maturational zones. Interestingly, in the chick and rat growth plates, type II collagen mRNA was found to be less abundant in the hypertrophic and calcifying zones (Iyama *et al.*, 1994; Balmain *et al.*, 1995). This finding was not observed in the bovine growth plates (Sandell *et al.*,1994). In the human growth plates, type II collagen expression was observed to be down-regulated (Mundlos, *et al.*, 1990). Alini *et al.* (1992) found that total type II collagen content, as measured by

hydroxyproline, rapidly decreased in the hypertrophic zone in fetal bovine growth plates. There was also increased denaturation of type II collagen in the hypertrophic zone. These results could arise from increased degradation in the hypertrophic zone or decreased synthesis or a combination of both mechanisms.

1.2.1.2 Type IX collagen

Type IX collagen is a nonfibrillar collagen of cartilage that is composed of three distinct gene products, α1(IX), α2(IX) and α3(IX) (van der Rest and Garrone, 1991). It belongs to the subgroup of collagen molecules called fibril-associated collagens with interrupted triple helices (FACIT) (Gordon and Olsen, 1990) The molecule is comprised of three collagenous domains (COL1-3) and four non-collagenous domains (NC1-4). It can also exist in a proteoglycan form by having a chondroitin/dermatan sulfate glycosaminoglycan attached to its NC3 domain on its α2(IX) chain (Vasios *et al.*, 1988). There is also another form of type IX collagen that is found in the vitreous humor that lacks the NC4 domain at its N-terminus, and this arises by transcription of α1(IX) using an alternate promoter (Nishimura *et al.*, 1989; Muragaki *et al.*, 1990). Type IX collagen has been immunolocalized in cartilage and is associated with type II collagen fibrils. It is bound to type II collagen by covalent cross-links in an anti-parallel orientation (Eyre *et al.*, 1987; van der Rest and Mayne, 1988). It has been proposed that its association with type II collagen and its localization to the perifibrillar space in cartilage, that it may be important in the interactions of type II collagen fibrils with other matrix molecules (Shaw and Olsen, 1991). To test this hypothesis,

Fassler *et al.*, 1994, generated homozygous mutant mice that harbored a null mutation for type IX collagen. They were viable and showed no detectable abnormalities at birth. As the mice aged 4 months or older, they developed severe degeneration of their articular cartilage that resembled human osteoarthritis.

Type IX collagen's mRNA expression *in situ* has been studied in the rat (Balmain *et al.*, 1995) and human fetal (Verneham *et* al., 1996) growth plates. In both studies, type IX collagen mRNA was most abundant in the proliferative, maturational and upper hypertrophic zones but was down-regulated in the lower hypertrophic and calcifying zones.

1.2.1.3 Type X collagen

Type X collagen is a unique collagen that is found predominantly in endochondral cartilages committed to calcification (Gibson and Flint, 1985; Schmid and Linsenmayer, 1985). However, its mRNA expression (Hoyland *et al.*, 1991) and protein (von der Mark, 1992; Walker *et al.*, 1995) has also been shown in human osteoarthritic cartilage. Its mRNA has also been found in human osteophytes (Aigner *et al.*, 1995). This collagen, along with type II collagen are the major extracellular components that are synthesized by hypertrophic chondrocytes (Mayne and Irwin, 1986; Schmid and Linsenmayer, 1987). Type X collagen is a homotrimer comprised of three identical α1(X) chains. It has a short triple helix, a small N-terminal domain (NC2) and a large C-terminal globular domain (NC1). The mammalian type X collagen contains two additional cysteine residues in the triple helix which are disulfide linked. These residues are absent in

the chicken molecule (Kirsch and von der Mark, 1991; Marriott *et al.*, 1991).

In situ hybridization studies of type X collagen mRNA expression have consistently shown it to be restricted to the hypertrophic zone of growth plates (LuValle *et al.*, 1989; Linsenmayer *et al.*, 1991; Reichenberger *et al.*, 1991; Iyama *et al.*, 1994). This observation would lead to the conclusion that hypertrophic chondrocytes employ a unique transcriptional mechanism during their maturation in order to express type X collagen (LuValle *et al.*, 1992). In fact, it was shown that the transcription of the type X collagen gene is always turned on, but repressed in all cell types except in hypertrophic chondrocytes (LuValle *et al.*, 1993).

The function of type X collagen is still unknown. Since it is present in the hypertrophic and calcifying zones, it is believed to play a role in mineralization. Although it is associated with type II collagen fibrils, it is not concentrated in the focal sites of calcification nor is it associated with matrix vesicles (Poole and Pidoux, 1989; Schmid and Linsenmayer, 1990). However, others have shown that type X collagen can interact with matrix vesicles and activate calcium influx into matrix vesicles and mediates mineralization (Kirsch and Wuthier, 1994; Kirsch *et al.*, 1994). Type X collagen becomes insoluble after biosynthesis and can only be extracted following the addition of cross-link inhibitors such as β-aminopropionitrile (Reginato and Jimenez, 1991). Also, further solubilization requires pepsin digestion and reduction. This indicates that its disulfide bonds may be involved in its association to the extracellular matrix (Reginato and Jimenz,

1990). This tight association would indicate a molecule that had some structural function. Electron microscopic studies have shown type X collagen to form a hexagonal lattice and this is thought to be mediated by the association of their NC1 domains (Kwan *et al.*, 1991). Type X collagen contains two collagenase cleavage sites in its triple helix and can be cleaved by vertebrate collagenases resulting in a 32 kD triple helical fragment (Schmid *et al.*, 1986; Cole *et al.*, 1993; Knäuper *et al.*, 1997). The melting temperature of this fragment is 43^0C and therefore would remain triple helical at physiological temperature (Schmid *et al.*, 1986). Therefore, the importance of its degradation in the growth plate is still uncertain. Recently, it was shown that the 32 kD triple helical fragment could be digested only by cathepsin-B at acidic pH and this may be the mechanism in which invading osteoclasts remove this molecule from the calcified cartilage during endochondral ossification (Sires *et al.*, 1995). In humans, a frameshift mutation that alters the amino acids of the NC1 domain of type X, causes Schmid metaphyseal chondrodysplasias (SMCD) (Warman *et al.*, 1993). The affected individuals are short in stature but not severely dwarfed. They have a normal spine and hands, an almost normal pelvis, reduced femoral shaft-neck angle (coxa vara), distal femoral metaphyseal involvement and rib changes (Lachman, *et al.*, 1988). The mutated collagen α1(X) chains were shown to be unable to form trimers in *in vitro* assembly and cell transfection experiments (Chan *et al.*, 1995). Therefore it has been postulated that SMCD patients have no type X collagen in their matrix. Recently two groups have generated null mutants of type X collagen in mice

(Rosati *et al.*, 1994; Kwan *et al.*,1997). Rosati *et al.*, showed that their mice harbored no abnormalities and no signs of SMCD. However, Kwan *et al.* (1997) found that their mice had abnormalities that resembled SMCD. These mice had abnormal trabecular bone architecture, had developed coxa vara, a reduction in the thickness of the resting zone in the growth plate and atypical distribution of matrix components within the growth plate. These findings would indicate that type X collagen has some role in endochondral ossification, but the exact function is still unclear.

1.2.1.4 Type XI collagen

Type XI collagen is a fibrillar and heterotrimeric collagen found in cartilage and in the vitreous of the eye. It is composed of three distinct chains, α1(XI), α2(XI), and α3(XI) (Morris *et al.* 1987). Further analysis of the molecule revealed that α1(XI) and α2(XI) are distinct gene products (Bernard *et al.*, 1988; Kimura *et al.*, 1989), but α3(XI) is closely related to the α1(II) chain of type II collagen. The α3(XI) has overmodified hydroxylysine residues as compared to type II collagen (Furuto, and Miller, 1983). Since there is only one gene for α1(II), these two collagen chains must arise from the same gene, but can be assembled differently into their respective collagen molecules (Kimura *et al.*, 1989; Mendler, *et al.*, 1989).

It has been hypothesized that type XI collagen may play a role in regulating the diameter of type II collagen fibrils similar to type V collagen's proposed role in regulating type I collagen fibril diameter (Linsenmayer *et al.*, 1993). Type XI

collagen molecules are cross-linked together in a head to tail arrangement. It forms a microfibril around type II collagen molecules Since there is incomplete processing of the amino propeptide of type XI collagen (Linsenmayer *et al.*, 1993) this may lead to the presence of N-terminal extensions on type XI collagen that block the addition of type II collagen molecules onto the existing collagen fibril. Keene *et al.* (1995) showed with immunolocalization, that type XI collagen was present on the fibril surface and associated with predominantly small fibrils which were less than 25 nm in diameter in human newborn epiphyseal cartilage, human juvenile rib growth plate and fetal calf cartilage. Type XI collagen was not present in thick fibrils but was associated with fibrils of all sizes. Recently, it was shown that mice that were autosomal recessive for chondrodysplasia (cho) and died at birth with abnormal cartilage in their limbs, ribs, mandible and trachea, were mutants that harbored a deletion in their α1(XI) gene (Li *et al.*,1995). Further analysis showed that they had a complete absence of α1(XI) collagen in their extracellular matrix. Their growth plates were also completely disorganized. Also, these mice exhibited abnormally thick collagen fibrils. These observations demonstrated that type XI collagen's role in regulating fibril diameter is involved during growth plate organization and skeletal morphogenesis.

Type XI collagen mRNA has been studied *in situ* in both rat and human growth plates (Balmain *et al.*,1995; Vornehm *et al.*, 1996). In both studies, type XI mRNA was lowest in the resting zone and increased slightly in the proliferative zone but stayed constant in the maturing and hypertrophic zones. In the rat,

however, there was a decrease in the calcifying zone which may be related to the decreased expression of type II collagen mRNA in the same zone.

1.2.2 Aggrecan

Aggrecan is a large chondroitin sulphate proteoglycan that accounts for about 10% of the total dry weight of cartilage. It interacts with link protein to form a stable complex with hyaluronan (HA) (Hascall and Heinegård, 1974). Aggrecan is found as a part of a large aggregate that can contain as many as 100 proteoglycan molecules per HA molecule (Neame, 1993). The primary function of aggrecan is to provide cartilage with its compressive stiffness and resistance to indentation and deformation. It allows cartilage to swell and hydrate its matrix .

Aggrecan is composed of about 90% carbohydrate and a 10% core protein. It has a core protein molecular weight of 237-248 kD., depending on which exons are spliced into the mature mRNA (Doege *et al.*, 1991) and a total molecular weight of 1-5 x 10^6 D. The core protein is comprised of 7 domains. The first domain at the N-terminus consists of a globular domain called G1. G1 consists of 335 amino acids. It is followed by the interglobular domain (IGD) of 130 amino acids and the G2 globular domain with 200 amino acids. The G1 domain binds to HA and closely resembles a glycoprotein, link protein, that helps stabilize the aggregate. Link protein was shown to be in cartilage and an essential component of the aggregate (Hascall and Sajdera., 1969; Buckwalter *et al.*, 1984). G1 contains 2 different structures: a region that belongs to the immunoglobulin superfamily (Bonnet, *et al.*, 1986) and a region that is related to a lymphocyte

homing receptor (Goldstein *et al.*, 1989; Stamenkovic *et al.*, 1989). The G2 domain closely resembles the G1 domain but does not bind to HA, collagen, link protein or other aggregating proteoglycan monomers (Fosang and Hardingham, 1989). There is a keratan sulfate (KS) attached to G2, although the exact location is not known (Fosang and Hardingham, 1991). This may interfere with its ability to bind HA. The next region of the molecule contains the majority of the GAGs; a KS containing domain followed by a CS containing domain. The exact function of the KS region is unknown. It contains a hexamer repeating sequence which in turn contains a serine residue in both bovine and human aggrecans (Antonsson *et al.*, 1989; Doege *et al.*, 1991). This serine has been suggested to be the attachment site for the KS chain (Antonsson *et al.*, 1989). The CS domain is the major part of the protein core of aggrecan. Repeats of serine-glycine amino acid pairs dominate this region and the serines are the sites of attachment for the CS chains (Bourdon *et al.*, 1985, 1987). The C-terminal region is a globular domain called G3. Various forms can arise from alternative splicing of its mRNA. It may contain regions with similarities to lectins, EGF, and complement regulatory proteins (Balwin *et al.*, 1989; Stoolman, 1989). The G3 domain can thereby vary in size from 156 to 255 amino acids. The exact function of the G3 domain is still unknown.

The turnover of aggrecan has been studied extensively. Studies have shown two major sites of proteolytic cleavage within the IGD between amino acid residues Asp_{341}-Phe_{342} and Glu_{373}-Ala_{374} (Sandy *et al.*, 1991, 1992; Flannery *et al.*, 1992; Lark *et al.*, 1995). Cleavages in this site result in the separation of the core

protein from the G1 domain that remains attached to HA. The first site between Asn_{341}-Phe_{342} has been termed the "MMP site" due to the ability of MMPs 1, 2, 3, 7, 8, 9, and 13 to cleave this site *in vitro* (Fosang *et al.*, 1992, 1993, 1996). The protease(s) that generate the second site between Glu_{373}-Ala_{374} has yet to be discovered and has been given the name "aggrecanase" based on its ability to cleave the aggrecan core protein. However, *in vitro* studies with recombinant MMP-8 at very high concentration (160 μg/ml) and in the presence of polyethylene glycol 4000, showed that it was able to generate the Glu_{373}-Ala_{374} cleavage, although it still preferred the MMP cleavage site (Fosang *et al.*, 1994). Recently, Arner *et al.*, (1997), using a specific MMP-8 inhibitor could not inhibit the generation of the aggrecanase cleavage product in adult bovine nasal cartilage and postulated that MMP-8 is not aggrecanase.

Aggrecan is present throughout the matrix in the growth plate (Hardingham, and Fosang, 1992). Its mRNA is expressed in all zones of the growth plate (Sandell *et al.*, 1994). Studies on the turnover of aggrecan in the growth plate have produced conflicting results. There have been reports of a loss of matrix proteoglycan just before or during the initiation of calcification in the growth plate (Hirschmann and Dziekwaitkowski, 1966; Lohmander and Hjerpe, 1974; Boyde and Shapiro, 1980; Franzen *et al.*, 1982). However, others have found no evidence for this loss (Howell and Carlson, 1968; Poole *et al.*, 1982; Scherft and Moskalweski, 1984; Hargest *et al.*, 1985). There may be a loss of proteoglycan after initiation of calcification (Mitchell *et al.*, 1982; Hargerst *et al.*,

1985). In more recent studies, where data was expressed per unit volume of extracellular matrix, there was a progressive increase in proteoglycan content in the growth plate (Matsui *et al.*, 1991; Alini *et al.*, 1992). There was, however, a net reduction in proteoglycan per unit weight of tissue when the chondrocytes enlarged in size and matrix volumes are considerably reduced (Matsui *et al.*, 1991; Alini *et al.*, 1992).

1.3 Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are metal-dependent endopeptidases with activity against most extracellular matrix macromolecules (Woessner, 1994). At least eighteen MMPs have been identified, and twelve are summarized in Table 1.1. These enzymes all share a number of conserved structural and functional domains but differ in their substrate specificities. Their structures and sequences are highly conserved among species, indicating important roles and functions of these enzymes. They play a pivotal role in normal tissue remodeling during ovulation, embryonic growth and differentiation (Birkedal-Hansen, *et al.*, 1993). They have been implicated in pathological conditions such as the arthritides and in tumor cell invasion. Although these enzymes have been studied extensively, many aspects of their regulation and function *in vivo* remain unknown.

1.3.1 Functional domains of MMPs

The MMPs, which are shown in Table 1.1 are comprised of domains which are conserved among the members of the family (Birkedal-Hansen, *et al.*, 1993; Woessner, 1994; Murphy and Knäuper, 1997) (Fig. 1.2). The MMPs consist of

Table 1.1 The major groups of matrix metalloproteinases

Enzyme	MMP No.	A.A.	Latent	Active	Chromosome	Substrates
Interstitial collagenase	1	469	55 000	45 000	11q22.3-23	Collagens I, II, III, VII, X
Neutrophil collagenase	8	467	75 000 53 000	58 000 43 000	11q21-q22	Collagens I, II, III Aggrecan
Collagenase 3	13	471	65 000	55 000	11q22.3	Collagens I, II, III, X; N-telopeptides of collagens I, II
Gelatinase A	2	662	72 000	66 000	16q21	Gelatin Collagens IV, V, VII, X Elastin
Gelatinase B	9	707	92 000	86 000	16q20	Same as gelatinase A
Stromelysin-1	3	477	55 000	45 000	11q22.3-23	PG core protein; Fibronectin; Laminin; N-telopeptides of type I collagen; Collagen IV, V, IX, X; Elastin; Procollagenase
Stromelysin-2	10	476	60 000	55 000	-	Same as Stromelysin-1
Stromelysin-3	11	488	65 000	45 000	-	α1 Proteinase inhibitor; α2 macroglobulin
Matrilysin	7	267	28 000	20 000	11q21-22	Fibronectin; Laminin; Collagen IV gelatin, procollagenase; PG core protein
MT1-MMP	14	582	73 000	63 000	14q11	Collagen I, MMP-2
MT2-MMP	15	669	70 000	60 000	16q13-q21	MMP-2
MT3-MMP	16	604	70 000	60 000	8q21.3-q22.1	-

the following domains: a 17-29 residue hydrophobic signal sequence which is followed by a 77-87 residue propeptide, a catalytic domain that contains its active site including the Zn^{2+}binding site, and a 5-50 residue proline-rich hinge region just before the 200 residue hemopexin domain (Sanchez-Lopez *et al.*, 1993). The two gelatinases, MMP-2 and MMP-9, contain an extra domain in the catalytic domain that consists of three tandem repeats of fibronectin type II-like sequences which allow them to bind to gelatin (Collier *et al.*, 1988; Goldberg *et al.*, 1989). Matrilysin lacks the hemopexin domain and is smaller than other members in this family. Recently a new group of MMPs have been discovered and have been termed membrane-type matrix metalloproteinases (MT-MMP). In total, there have been four types found: MT1-MMP (Sato *et al.*,1994), MT2-MMP (Will and Hinzmann, 1995), MT3-MMP (Takino *et al.*, 1995) and MT4-MMP (Puente *et al.*, 1996). All have a recognizable transmembrane domain that anchors them to the cell membrane. MT1-MMP has been implicated in the role of the activation of progelatinase A (Sato *et al.*, 1994; Cao *et al.*, 1995).

The active site of each of the MMPs contain a very conserved sequence, HE(L/I/F)GH. This sequence is believed to coordinate the zinc ion together with the catalytically essential glutamic acid residue. Conserved asparagine residues located on either side of this motif act as binding sites for calcium which is required for enzyme stability (Clark and Cawston, 1989; Nagase *et al.*, 1990; Murphy *et al.*, 1992). The C-terminal hemopexin domain, which is present in all members of the MMPs except matrilysin, confers substrate binding specificity.

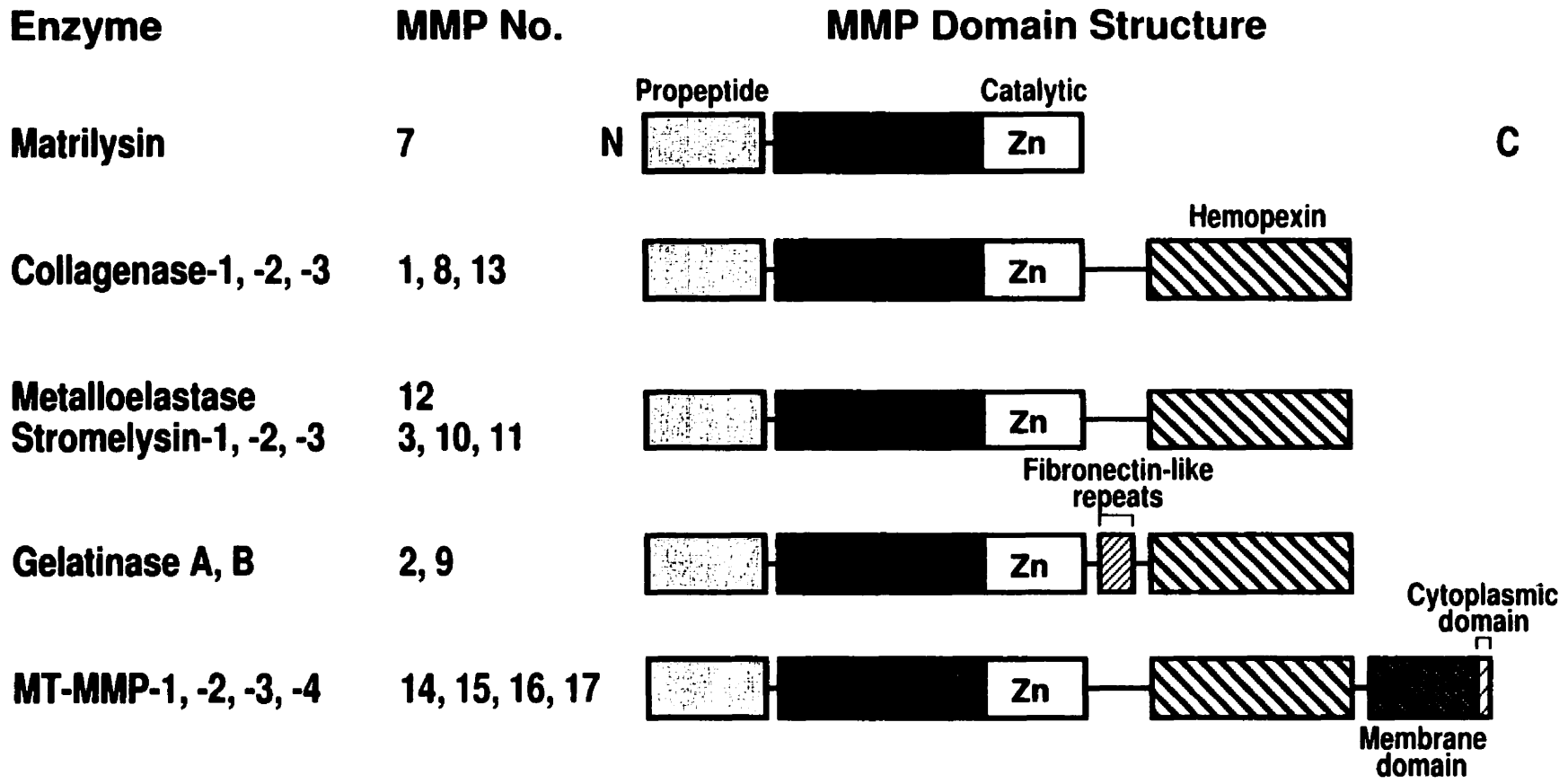


Fig. 1.2. Domain structure and organization of MMPs.

Deletion analysis of this domain produced enzymes which lose their ability to bind its preferred substrate (Murphy and Knäuper, 1997). Truncated forms of MMP-1, MMP-8 and MMP-13 lose their ability to cleave native triple helical collagen (Clark and Cawston, 1989; Murphy *et al.*, 1992; Knäuper *et al.*, 1993, 1997).

1.3.2 MMP Gene Structure and Genomic Localization

The MMP genes also show a high degree of homology. Most of the genomic locations of human MMPs have been mapped (Table 1.1). MMPs 1, 3, and 10 genes each contain ten exons and nine introns in 8-12 kb of DNA. MMPs 2 and 9 are considerably larger (26-27 kb) and contain three additional exons which code for the fibronectin type II-like domains. MMP 1, 3, 8, 10, 13 (Formstone *et al.*, 1992; Pendas *et al.*, 1995,1996) genes have been mapped to the long arm of chromosome 11 while the two gelatinases (MMP 2 and 9) are located on chromosome 16 (Birkedal-Hansen *et al.*, 1993). MMP 14, 15, and 16 of the MT-MMP family have been mapped to chromosomes 14, 16, and 8 respectively (Mattei *et al.*, 1997). It is thought that these MMPs must have arisen from gene duplication because of their high degree of homology.

1.3.3 Transcriptional Regulation of MMPs

MMPs are tightly regulated at the transcriptional level. This is essential due to their ability to degrade extracellular macromolecules. MMPs can be regulated by growth factors which may stimulate or repress their mRNA levels. IL-1 and TNF-α have been shown to be potent stimulators of MMP expression while TGF-β is a repressor of MMP expression in chondrocytes and synovial

fibroblasts (Birkedal-Hansen *et al.*, 1993; Benbow and Brinckerhoff, 1997). This, however, does not hold for all cell types. Phorbol esters such as 12-*0*-tetradecanoyl-phorbol-13 acetate (TPA) can also induce high levels of MMP expression through the phorbol ester-response element (TRE) in the MMP genes (Angel *et al.*, 1987). Cytokines and growth factors are believed to mediate MMP expression through the AP-1 binding site in the MMP genes. These factors turn on expression of a *c-fos* and *jun* dependent pathway which binds to the AP-1 site (Schonthal *et al.*, 1988). This site has been identified in most MMP 5' sequences but is absent in MMP-2. Although *fos* and *jun* activation can lead to MMP activation, it is not always so. More than one molecular event determines the up-regulation of these MMPs. TGF-β also works through a *c-fos* dependent pathway in repression of MMP expression (Kerr *et al.*, 1990). Recently, TGF-β was shown to either activate or repress collagenase gene expression by induction of distinct oncogenes of the Jun family (Mauviel *et al.*, 1996). TGF-β activated *c-jun* in basal keratinocytes to activate collagenase expression, while in fibroblasts it activated *jun-B* which repressed collagenase expression. These observations indicate that different cytokines may elicit different MMP expression by recruiting different family members of the AP-1 binding site.

1.3.4 Activation of Metalloproteinase Zymogens

MMPs are secreted as proenzymes which are latent. In order to have enzymatic activity, their N-terminal propeptide must be removed. The propeptide is bound through a Cys-Zn^{2+} bond to the active site displacing the H_20 molecule

needed for catalysis (Springman *et al.*, 1990). The activation has been termed " the cysteine switch" (van Wart and Birkedal-Hansen, 1990). This bond can be disrupted by a number of methods such as cleavage of the propeptide by another protease or by organomercurials, metal ions, thiol reagents and oxidants. This disruption of the Cys-Zn^{2+} bond opens up the enzyme and active site allowing for removal of the propeptide from the active site resulting in activation of the enzyme. Once the enzyme is stabilized in the open form it can catalyze several autolytic cleavages to generate the fully processed form (Suzuki *et al.*, 1990). The exact N-terminal sequence generated will determine the amount of activity the MMP will have, probably due to the new formation of a salt link between the NH^{3+} group of the new N-terminus with the asparigine residue within the active site (Reinemer *et al.*, 1994; Becker *et al.*, 1995). Recently, a furin sequence was found in the propeptide of the MT-MMPs and stromelysin-3 and is absent in the other MMPs (Pei and Weiss, 1995, 1996). Furin belongs to the family of prohormone convertases that are present in the Golgi and pericellular space. These observations lead to experiments that demonstrated intracellular processing of the propeptides of MT-MMPs and stromelysin-3 and secretion of the active form of the enzymes (Pei and Weiss, 1995, 1996).

1.3.5 Collagenases

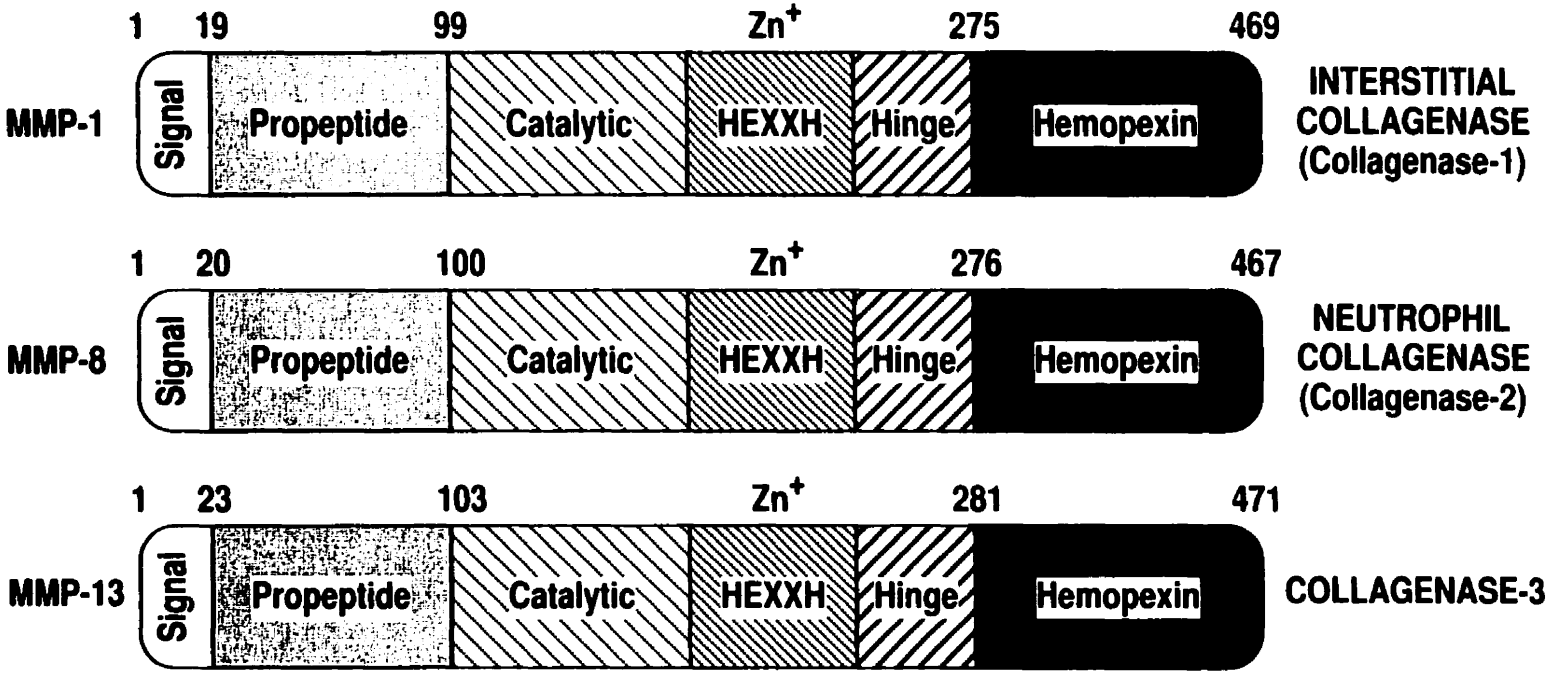
The enzyme collagenase was first described by Gross and Lapiere, in 1962, who proposed a role in collagen degradation during the morphogenesis of the tadpole tail. Although they never purified this enzyme, they showed it had activity

against triple helical collagen. Interstitial collagenase (MMP-1) has been cloned in human (Whitham *et al.*, 1986), rabbit (Fini *et al.*, 1987), porcine (Clarke *et al.*, 1990), rat (Quinn *et al.*, 1990), mouse (Henriet *et al.*, 1992) and bovine (Tamura *et al.*, 1994) species. A second distinct collagenase was cloned from human neutrophils and was called neutrophil collagenase (MMP-8) (Hasty *et al.*, 1990). All these collagenases have the ability to cleave the alpha chains of types I, II, III, collagens to produce fragments of approximately 3/4 and 1/4 the size of the original molecule. The enzymes however, differ in their specificity for their different collagens. MMP-1 from human, rabbit, porcine and bovine species, cleaved types I and III collagens much more rapidly than type II. While MMP-1 from mouse and rat cleaved types I, II and III collagens efficiently, it cleaved type II collagen much more rapidly then MMP-1 found in other species (Welgus *et al.*, 1983). MMP-8 was shown to have a preference for type I collagen over types II and III collagen (Mallya *et al.*, 1990) . Comparison of the cDNA and amino acid sequences of mouse and rat MMP-1 to the other species, showed only a 49-55% homology, while MMP-1 from human, rabbit, porcine and bovine shared 85-88% homology at the cDNA and amino acid level. Some speculated that the rodent MMP-1 was not the same enzyme as the other mammalian MMP-1 (Henriet *et al.*, 1992) and others argued that it was the same but the low percentage of homology was due to species differences. Recently, a novel collagenase was cloned from breast carcinomas (Freije *et al.*, 1994). This collagenase shared 86% homology with the rat and mouse MMP-1 and only 50% homology to human MMP-1. It

preferentially cleaved type II collagen over types I and II (Knäuper *et al.,1996*; Mitchell *et al.*, 1996). This new collagenase has been designated collagenase-3 (MMP-13). Hence, the rodent MMP-1 has now been designated MMP-13. In comparison to human MMP-1, which is expressed widely in all tissues, human MMP-13 has been shown to be present in breast carcinomas (Freije *et al.*, 1994), normal and osteoarthritic cartilage (Borden *et al.*, 1996; Mitchell *et al.*, 1996; Reboul *et al.*, 1996) and osteoblasts (Johansson *et al.*,1997). MMP-13 is the only collagenase found in rodents. Since it is the only collagenase available to rodents, its expression is found in more types of tissues. Although, during murine embryonic and fetal development , it is expressed mainly in the centers of ossification (Mattot *et al.*,1994; Gack, *et al.*, 1995). MMP-8, which was thought to be present only in neutrophils, has been shown recently to be present in human adult articular cartilage (Chubinskaya *et al.*, 1996; Cole *et al.*, 1996). It has also yet to be identified in other species other than man. The basic structural elements of the three human collagenase are shown in Fig 1.3.

1.3.6 Cleavage of interstitial collagens.

Interstitial collagens are cleaved in their triple helical domains by collagenases. Types I, II, and III collagens can be cleaved by MMP-1, 8, 13 (Wu *et al.*, 1990; Devarajan *et al.*, 1992; Mitchell *et al.*, 1996; Billinghurst *et al.*, 1997). Recently it has been shown that MMP-2 (Aimes and Quigley, 1995) and a truncated form of MT1-MMP (Ohuchi *et al.*, 1997) can also cleave interstitial collagens, although these observations have yet to be repeated. MMP-2 was



- MMP-1, -8, and -13 are collagenases that belong to the matrix metalloproteinase family of proteolytic enzymes
- They are secreted as proenzymes into the extracellular matrix and require activation for activity.
- They are also the only enzymes that can cleave triple helical collagen into 3/4 and 1/4 fragments.

Fig. 1.3 Structure and functional domains of human collagenases.

shown to able to cleave type I collagen only if it was TIMP free (Aimes and Quigley, 1995). MT1-MMP was able to cleave types I, II, and III collagens (Ohuchi *et al.*, 1997). MMP-1, 8, 13 and MT1-MMP cleave the triple helical structure at the Gly_{775}-Ile_{776} or Gly_{775}-Leu_{776} bonds of the α-chains of the interstitial collagens. This cleavage generates two cleavage products, one 3/4 fragment (TC^A) and one 1/4 fragment (TC^B). It is believed that collagenases can cleave at this site due to "opening" or relaxation in the triple helix. Site-directed mutagenesis of this site in type I collagen yields uncleavable collagen molecules at the triple helical site (Wu *et al.*, 1990). MMP-13 was shown to cleave type I collagen in the N-telopeptide region in transgenic mice harboring this mutation (Liu *et al.*, 1995). This N-telopeptide cleavage can also be mediated by stromelysin (Wu *et al.*, 1991). Recently, the aminotelopeptidase activity was shown only to be present in MMP-13 and not MMP-1 (Krane *et al.*, 1996). Also, this activity was attributed to sequences found in the catalytic domain of MMP-13 and not in the hemopexin domain (Krane *et al.*, 1996; Lemaitre, *et al.*, 1997).

1.4 Inhibition of Metalloproteinase Activity

1.4.1 Synthetic inhibitors

A number of synthetic inhibitors are available to inhibit MMPs. Chelating agents that interact with the zinc atom in the active site, such as 1,10 ortho-phenanthroline or EDTA are potent inhibitors but have broad biological affects and no specificity. They are therefore not suitable for biological use. Substrate analogue peptides linked to chelating moieties such as hydroxamate, carboxylate,

thiol, phosphonamidate, phosphinate and phosphoramidate groups have been developed and the most potent of these can work in the nanomolar range. These substrates can be engineered to have MMP specificity with high binding affinities (Gray *et al.*, 1987; Lelievre *et al.*,1990; Ewenson *et al.*,1992).

The approach to the design of these inhibitors is based on the incorporation of residues that are normally present in the natural substrates of the target enzyme (Roberts *et al.*, 1987). These inhibitors have high binding energy due to the inclusion of a zinc binding group. Three main strategies have been employed for the incorporation of the zinc group into the substrate peptide: left hand side inhibitors (LHS) that include amino acid residues from the N-terminal side of the cleaved bond; right hand side (RHS) inhibitors that utilize amino acid residues from the C-terminal side of the cleaved bond; and a combined left hand-right hand inhibitors where the zinc ligand is in the middle of the cleaved residues. The RHS inhibitors have been the most successful inhibitors and have resulted in collagenase inhibitors with the greatest activity (Roberts *et al.*, 1987). In the P_1' position, the preferred substituent is a small hydrophobic moeity which mimics the Leu/Ile residues found in collagen. In the P_2' position, most amino acid residues can be used. This is surprising since this position in collagen is a small hydrophobic amino acid. In the P_3' position, which is a glycine in collagen, any residue other than glycine or alanine leads to dramatic loss of activity. The substitution in the P_4' position is usually not important. These classes of inhibitors have been examined for their potency in biological systems involving tissue

degradation (DiPasquale *et al.*,1986; Nixon *et al.*, 1991; Dimartino *et al.*,1991; Mort *et al.*, 1993; Cawston *et al.*, 1995; Chichester *et al.*, 1996; Bottomley *et al.*, 1997; Kozaci *et al.*, 1997). These inhibitors must be stable in biological systems and small enough to penetrate into their target tissues. They must have specific and high levels of inhibitory activity($K_I \leq 10^{-10}$ M). Several of these inhibitors have been designed from crystal structures of MMPs (Blundell, 1994, Dhanaraj *et al.*, 1996). These studies have shown that the S_1' pocket in MMP-1 is shallow but deep in MMP-8, MMP-3 and MMP-13 (Dhanaraj *et al.*, 1996; Welch *et al.*, 1996). This structural property in MMP-8, MMP-3 and MMP-13 allow for design of specific inhibitors that exploit their deep S_1' pocket.

1.4.2 Peptide inhibitors

Synthetic peptides which mimic propeptide sequences (Hanglow *et al.*, 1993), enzyme binding sequences (de Souza and Brentani, 1991) and TIMP sequences have all been shown *in vitro* to have the ability to inhibit MMP cleavage of their substrates. Their IC_{50} are usually in the μM range and their ability to inhibit MMPs *in vivo* has been shown in human tumor cell invasion (Melchiori *et al.*, 1992).

1.4.3 Tissue inhibitors of matrix metalloproteinases (TIMPs)

Tissue inhibitors of metalloproteinases (TIMPs) are natural inhibitors of MMPs found in connective tissues. They form a 1:1 non-covalent bond with activated MMPs and inhibit their activity. Latent MMP-2 can also bind TIMP-2 and form a complex. Four TIMPs have been identified: TIMP-1 (Stricklin, 1986),

TIMP-2 (DeClerck *et al.*, 1991), TIMP-3 (Apte *et al.*, 1995), and TIMP-4 (Greene *et al.*, 1996). Their structure and amino acid sequences are highly conserved among species, indicating an important function for these proteins. TIMP-1 and 3 are glycoproteins with a molecular weight of about 30 kD. TIMP-2 and TIMP-4 are non-glycosylated proteins of 23 and 22kD, respectively. Both TIMP-1 and TIMP-2 have similar affinities for the MMPs with K_d values of about 10^{-10} M. But TIMP-1 is more effective against MMP-1 whereas TIMP-2 is more effective against MMP-2. TIMP-4 has been shown recently to bind MMP-2 in a similar manner to TIMP-2 (Bigg *et al.*, 1997). The TIMPs are highly stable due to six conserved disulfide bonds which are responsible for it's double looped, two domain structure. The N-terminal 134 residues form a domain that is responsible for inhibiting the MMP while the C-terminal region mediates binding to the MMPs (Docherty *et al.*, 1992).

1.4.4 Regulation of TIMPs and Genomic Location

The different TIMP genes are regulated differently. TIMP-1 can be induced by a number of cytokines, growth factors and phorbol esters while TIMP-2 cannot. TIMP-3's regulation is related to the cell cycle (Wick *et al.*, 1995) and it is highly expressed in development during embryogenesis (Apte *et al.*, 1995). TIMP-3 has been implicated in patients with Sorsby's fundus dystrophy (Weber *et al.*, 1994). Patients with this condition harbor a number of different point mutations in their TIMP-3 gene which leads to a loss of central vision from subretinal neovascularization and atrophy of their ocular tissues (Felbor *et al.*,

1995). The newly cloned TIMP-4 is highly expressed only in the adult heart (Greene *et al.*. 1996).

TIMP-1 is located on the X chromosome (Willard *et al.*, 1989), TIMP-2 is on chromosome 17 (DeClerck *et al.*, 1992), and TIMP-3 is on chromosome 22 in humans (Apte *et al.*, 1994). TIMPs 1, 2, 3 all contain two or more transcriptional start sites which give rise to mRNAs of different size (Birkedal-Hansen *et al.*, 1990; Greene *et al.*, 1996).

TIMP has been shown to be present in the rat growth plates (Dean *et al.*. 1989, 1990). The amount of TIMP was found to be deficient in the hypertrophic zone. This observation was also made in rabbit growth plates using immunofluorescent antibodies (Brown *et al.*. 1989). TIMP was localized in the resting and proliferative zone chondrocytes but was absent in the hypertrophic chondrocytes (Brown *et al.*. 1989). This decrease in TIMP levels would favor increased collagenase activity and leading to collagen removal in the hypertrophic zone (Dean *et al.*. 1989, 1990).

1.5 Hypothesis

The mammalian growth plate undergoes rapid remodeling that involves synthesis, resorption and vascularization of its extracellular matrix. These processes are all highly regulated during endochondral bone formation and long bone growth. The growth plate chondrocytes that make up the cartilaginous growth plate follow a highly regulated sequence of maturation events during their life span. These chondrocytes rapidly synthesize an extracellular matrix when they are in their proliferative and resting stages. They then degrade it when they become hypertrophic. Since their extracellular matrix is composed mainly of type II collagen, it is hypothesized that they must produce a collagenase during their differentiation from proliferative chondrocyte to hypertrophic chondrocyte. The specific aim of this thesis is to identify the collagenase(s) and its regulation *in vitro* during chondrocyte maturation using cultures of isolated bovine fetal growth plate chondrocytes.

2. MATERIALS AND METHOD

2.1 Preparation of Samples

2.1.1 Source of Tissue

Bovine fetuses were obtained from a local abbatoir (Colbex, St.Cyrille, QC.) and transported to the laboratory. Within 1.5 hr. of death, femora, tibiae, and humeri were removed. Fetal age was determined by measurement of tibial length as previously described (Pal *et al.*, 1981). Ages of bovine fetuses used in this thesis ranged from 190-221 days. The mouse chondrocyte cell line, MC615 (Mallein-Gerin and Olsen, 1993) was provided by Dr. Bjorn R. Olsen (Harvard Medical School, Boston, MA).

2.1.2 Cell Isolation and Culture

Bovine fetal growth plate chondrocytes were isolated and fractionated into subpopulations, as previously described (Lee *et al.*, 1990; Alini *et al.*, 1994, 1996) with some modifications. Briefly, isolated growth plate tissue slices were washed twice (15 min each) in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Burlington, ON), 20 mM HEPES, and 45 mM $NaHCO_3$, pH 7.4, containing (per ml) 0.15 mg gentamicin (Sigma Chemical Co., St. Louis, MO), 100 μg benzylpenicillin (Gibco), 100 U streptomycin (Gibco), and 0.25 μg Fungizone (Gibco) (medium A). This was followed with two washes (15 min each) in medium A with 10X antibiotics. The slices were then digested with 0.25% (w/v) trypsin in medium A for 30 min at 37^0C. After trypsin digestion and inactivation with 10% fetal calf serum (FCS), the tissue slices were digested with 500 μg/ml

collagenase (type IA, Sigma) , 300 μg/ml hyaluronidase (ovine testicular type V, Sigma) and 50 μg/ml DNAase I (bovine pancreas. Sigma) in medium A overnight at 37^0C with gentle agitation on a gyrotary shaker. Undigested cartilage was removed by filtration through a nylon mesh and cells were recovered with centrifugation (10 min. 400 x g). Cells were washed twice in medium A at room temperature and once in citrate buffer (125 mM NaCl. 18 mM citric acid, and 10 mM K_2HPO_4, pH 6.0) to dissolve residual mineral. The cells were washed in medium A, counted with a hemocytometer and loaded (2.5 x 10^7 cells/ml) onto Percoll (Pharmacia, Uppsala, Sweden) discontinuous density gradients (1.01 to 1.07 g/ml) and centrifuged at 400g x 30 min (Fig. 2.1) Five subpopulations identified as distinct bands (A to E) were carefully removed for analysis (Table 2.1). Chondrocyte subpopulations were seeded on gelatin-coated 12 well flat bottomed plates (Falcon, Becton Dickinson, NJ) at a density of 1 x 10^6 cells/ml. Cells were cultured in 2 ml of DMEM supplemented with 50 μg/ml ascorbic acid , 5 mM sodium β-glycerophosphate (both were freshly prepared at each medium change) 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml sodium selenite (ITS), 1 mg/ml of hydrolyzed BSA and 10 nM tri-iodothyronine (T_3) (Alini *et al.*, 1996). Medium was changed every 2 days. For MMP-13 inhibition studies, C subpopulation growth plate chondrocytes were cultured as above except with the addition of 1, 10 or 50 nM of RS 102, 481 inhibitor (Roche Biosciences, Palo Alto, CA). Bovine fetal epiphyseal chondrocytes were isolated as above, except they were not fractionated on Percoll gradients. Cells were cultured in DMEM

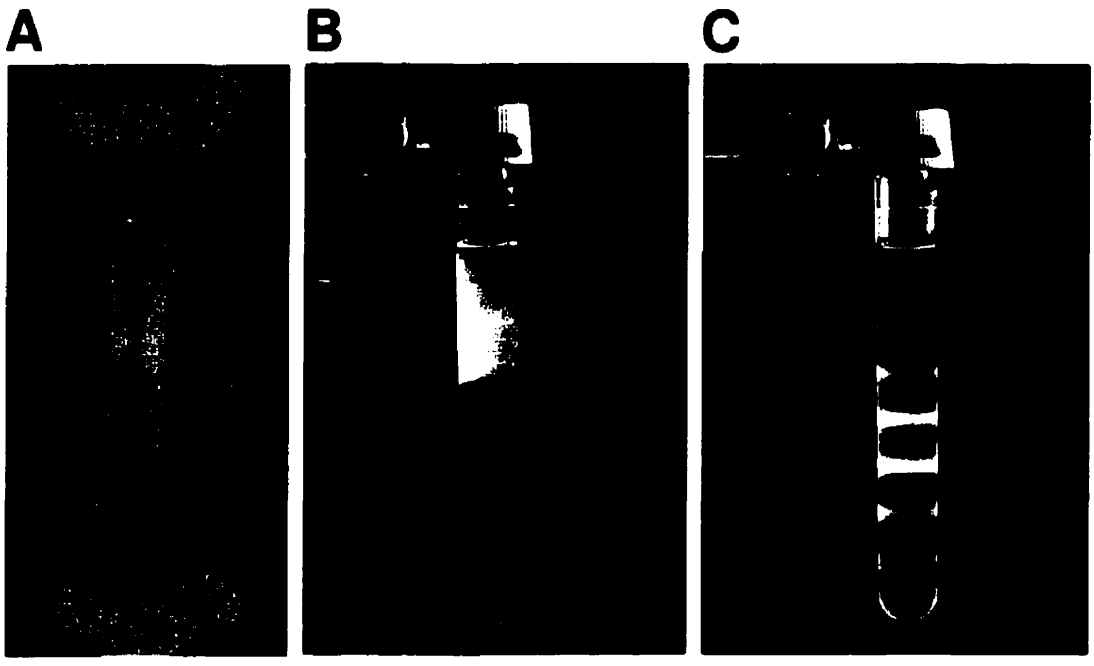


Fig. 2.1 Percoll gradient used in isolation of bovine fetal growth plate chondrocyte subpopulations.
Bovine fetal growth plates were dissected from long bones **(A)**. Percoll gradient loaded with isolated fetal bovine growth plate chondrocytes **(B)**. Five growth plate chondrocyte subpopulations (A-E*) in Percoll gradient after centrifugation **(C)**. *Amount of E subpopulation (lowest band) is faint due to low amount of cells.

Table 2.1 Morphological characteristics of isolated populations of fetal growth plate chondrocytes

Cell populations	Cell Diameter* (μm), (n=3)	(%) of cells in each population after Percoll gradient (n=14)	Viability of each populaton (n=14)
A	25.7 ± 5.9	1.60 ± 1.10	70 - 80%
B	20.2 ± 4.6	20.4 ± 12.3	> 80%
C	17.9 ± 4.3	43.8 ± 10.9	> 95%
D	15.7 ± 3.2	30.1 ± 12.9	> 95%
E	13.9 ± 2.9	4.10 ± 3.90	> 95%

- Total number of cells isolated per g of tissue : $2.8 \times 10^7 \pm 1.4 \times 10^7$ (n=14)
- Viability : 69% ± 7% (n=14)
- Cell recovery after Percoll gradient centrifugation : 64% ± 14% (n=14)
- 15g to 40g of cartilage growth plate was collected per fetus according to age (200-250 days).

***The above data for cellular diameters are taken from a previous publication. (Lee et al., J. Histochem. Cytochem., 38(5):659-673, 1990)**

supplemented with 50 μg/ml ascorbic acid and 10% fetal calf serum (FCS). Bovine fetal skin fibroblasts were isolated from explants (Freshney, 1987) and allowed to grow to confluency in DMEM containing 10% FCS and passaged. Medium was changed every 2 days. Mouse chondrocytes (MC615) were plated onto 12 well tissue culture plates and grown in DMEM containing 10% FCS. These mouse chondrocytes have been immortalized by transfection of the large T antigen of SV40 (Mallein-Gerin and Olsen, 1993). These cells are fibroblastic-like when cultured at low density. When they reach confluency, they adopt the hexagonal shape of chondrocytes and begin to synthesize type II collagen and aggrecan (Mallein-Gerin and Olssen, 1993). Medium was changed every 2 days.

For maximal MMP-1 induction, chondrocytes and fibroblasts were grown in DMEM supplemented with 1 or 10 U/ml of human recombinant Il-1α (R&D Systems, Minneapolis, MN) for 24 hr. For detection of *de novo* protein synthesis, chondrocytes and fibroblasts were cultured in methionine-free DMEM (Gibco, BRL.) supplemented with ^{35}S-methionine (Amersham, Oakville, ON) for 48 hr.

2.2 Total RNA extraction and isolation

Total RNA was isolated from chondrocytes using the alcohol/proteinase K method (Fig 2.2). Briefly, chondrocyte cell layers or cartilage were solubilized in solution D (4 M guanidine isothiocyanate, 20 mM sodium acetate, pH 5.2, 0.1 M 2-mercaptoethanol and 0.5% N-lauroylsarcosine) (Chomczynski and Sacchi, 1989). One volume of isopropanol was added to this mixture and all proteins and nucleic acids were precipitated at 20^0 C overnight. After centrifugation, the pellet

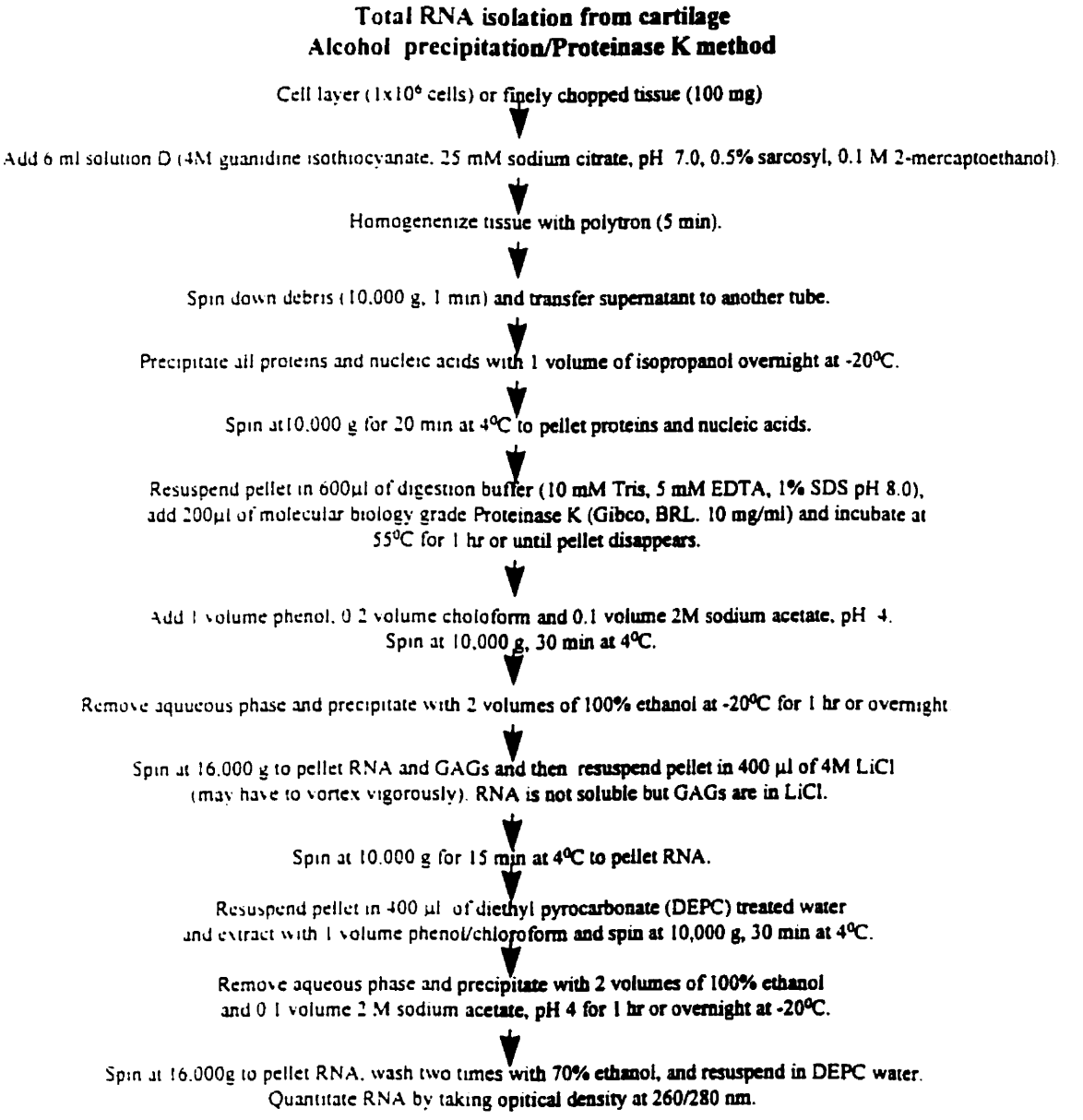


Fig. 2.2 Flow diagram of RNA isolation from cartilage: alcohol precipitation/ Proteinase K method.

containing the proteins and nucleic acid was digested with 1 mg/ ml proteinase K (molecular biology grade; Gibco) for 2 hr. at 65^0 C. After digestion, the mixture was then extracted with 1 volume of phenol and 0.1 volume of chloroform/ alcohol (48:1). The aqueous phase was recovered after centrifugation and precipitated with 1 volume of isopropanol. The RNA and remaining contaminating GAGs were recovered by centrifugation. This pellet was washed in 4 M LiCl (Puissant and Hondebine, 1990) which solubilizes the GAGs but not the RNA. The RNA is recovered by centrifugation, resuspended in solution D and extracted with phenol/chloroform. Pure total RNA is recovered by precipitating the aqueous phase and washing with 70% ethanol to remove any excess salt. The total RNA pellet is then resuspended in diethyl pyrocarbonate-treated (DEPC) water and the optical density (O.D.) 260/280 nm is taken to quantitate (1 O.D. 260 = 40 μg of RNA) and assess the purity of the preparation (260/280 nM ≥ 1.8). Total RNA was isolated from fibroblasts using the guanidine/acid/phenol/chloroform method (Chomczynski and Sacchi, 1989).

2.3 Reverse transcription

First strand cDNA was prepared by incubating 20 μg of total RNA (from fetal fibroblasts or chondrocytes) with a reaction mixture of 50 μl containing 2.5 μg of oligo-dT or random hexamer primers (Pharmacia), 50 units of RNAsin ribonuclease inhibitor (Gibco), 100 units of Moloney murine leukemia virus (MMLV) reverse transcriptase (Gibco), 1 mM deoxynucleotide triphosphates (dNTPs) (Pharmacia), 50 mM Tris-HCl pH 8.5, 8 mM $MgCl_2$, 30 mM KCl, and

10 mM dithiothreitol (DTT) at 42^0C for 2 hr in a thermal cycler.

2.3.1 Polymerase chain reaction (PCR)

10 µl of reverse transcribed total RNA was incubated with 2.5 units of Taq polymerase (Gibco, BRL) in 50 mM KCl, 2 or 4 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.5, 100 µM dNTPs and 100 pmol each of upstream and downstream oligonucleotide primers in 100 µl reaction tubes. PCR amplifications were carried out in a thermal cycler. Generally, the PCR protocol was 30-40 cycles at 95^0C for 1 min, 55^0C or 60^0C for 1 min and 72^0C for 1 min followed by a 5 minute incubation at 72^0C. If the cDNA fragment was larger than 1200 bp, the first incubation step at 72^0C during the PCR was increased to 3 min, instead of 1 min. The oligonucleotides and PCR optimal annealing conditions for each cDNA are described in Tables 2.2 and 2.3. All oligonucleotide primers were synthesized on an ABI 381A DNA synthesizer (Applied Biosystems, Foster City, CA), purified on OPC columns (Applied Biosystems), and resuspended in 10 mM Tris-HCl, pH 8.5. PCR product sizes where verified by electrophoresis in 1% agarose gels in TAE (TAE = 40 mM Tris, 20 mM acetic acid/ 1 mM EDTA) containing 3 µl of a 1 mg/ml solution of ethidium bromide in water.

2.3.2 Cloning of bovine MMP-1 and MMP-13

cDNAs of bovine MMP-1 and MMP-13 were obtained by PCR using oligonucleotide primers found in Table 2.2. and conditions found in Table 2.3. MMP-1 primer sequences were selected by sequence alignment of the published human (Whitham *et al.*, 1986) porcine (Clarke *et al.*, 1990) and bovine (Tamura *et*

al., 1994) MMP-1 cDNAs (Fig. 2.3.). MMP-13 primer sequences were selected by utilizing conserved regions of the human (Freije *et al.*, 1994), rat (Quinn *et al.*, 1990) and mouse (Henriet *et al.*, 1992) MMP-13 (Fig. 2.4.). Additional bovine 5' MMP-13 sequence was kindly provided by Dr. Karen Hasty (University of Tennessee, Memphis, TN).

2.4 DNA sequencing

cDNA products were purified by a phenol/chloroform extraction to remove the Taq polymerase, followed by a molecular cutoff spin column (Centricon 3000, Amicon Inc., Beverly, MA) to remove unused oligonucleotide primers. Purified cDNAs were subcloned into pCR-Script SK(+) vector (Stratagene, La Jolla, CA) according to manufacturers instructions. Purified plasmids containing the cDNA insert of interest were purified from mini-preps (Sambrook *et al.*, 1986) from *E. Coli* bacteria. Plasmid DNA were sequenced by the Sanger dideoxy chain termination method (Sanger, and Coulson, 1975) , using ^{35}S dATP (Amersham) and a commercial kit (Sequenase II sequencing kit; U.S. Biochemicals Corp. Cleveland, OH). The primers used in the sequencing reactions were either specific oligonucleotides found in the cDNA of interest or the M13 universal sequencing primer (5' GTAAAACGACGGCCAGT 3'). Samples were denatured by boiling for 5 min, and electrophoresed on a 6 or 8% polyacrylamide/7 M urea gel. Gels were transferred to Watman paper, dried for autoradiography and exposed to Kodak X-OMAT film (Eastman Kodak Co., Rochester, NY).

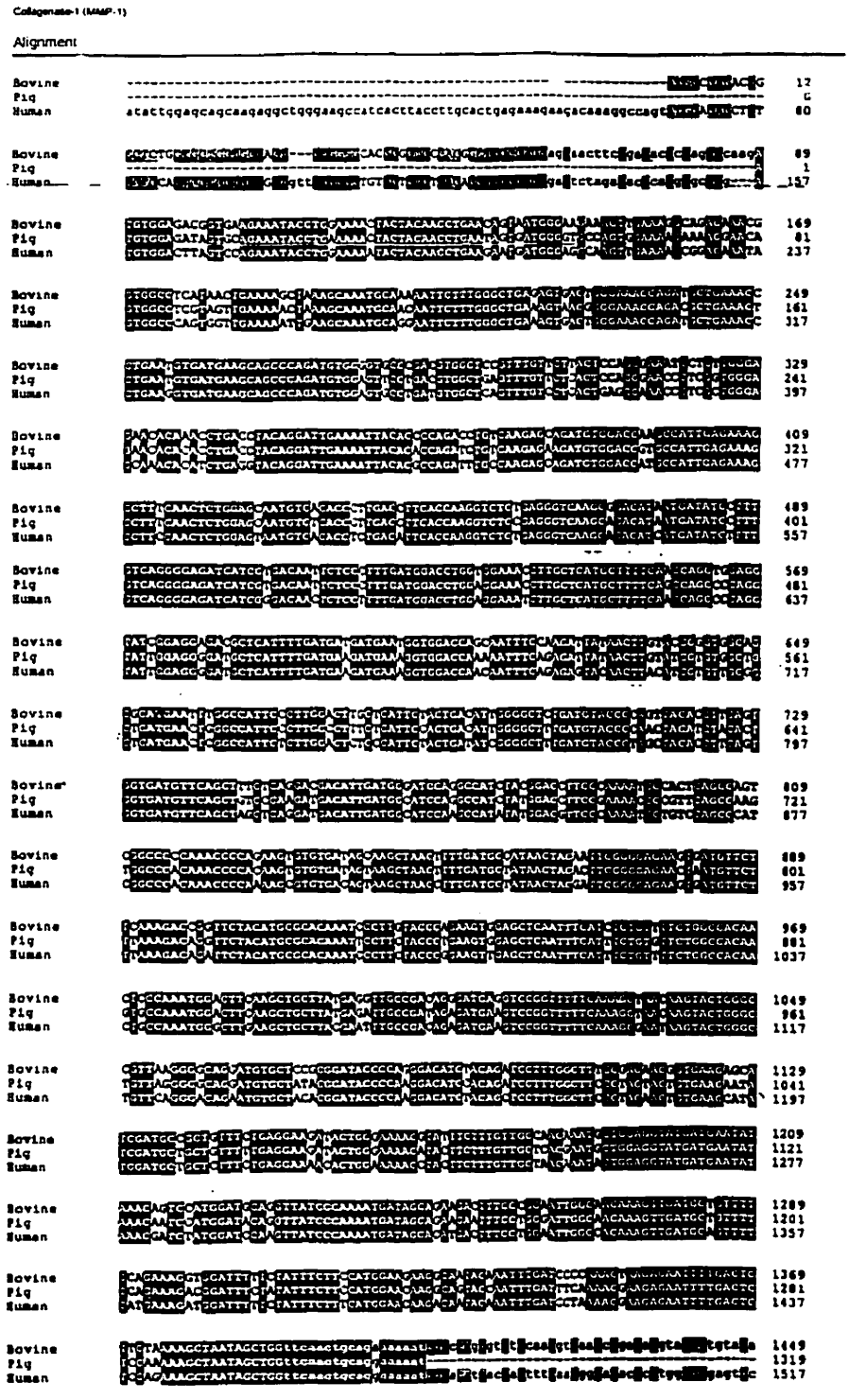


Fig. 2.3 Sequence alignments of bovine, pig and human MMP-1 cDNAs.

Uppercase letters represent region of homology. Shaded area are identical nucleotides. The sequences are from Tamura *et al.*, 1994 for bovine MMP-1; Clarke *et al.*, 1990 for pig; and Whitham *et al.*, 1986 for human MMP-1.

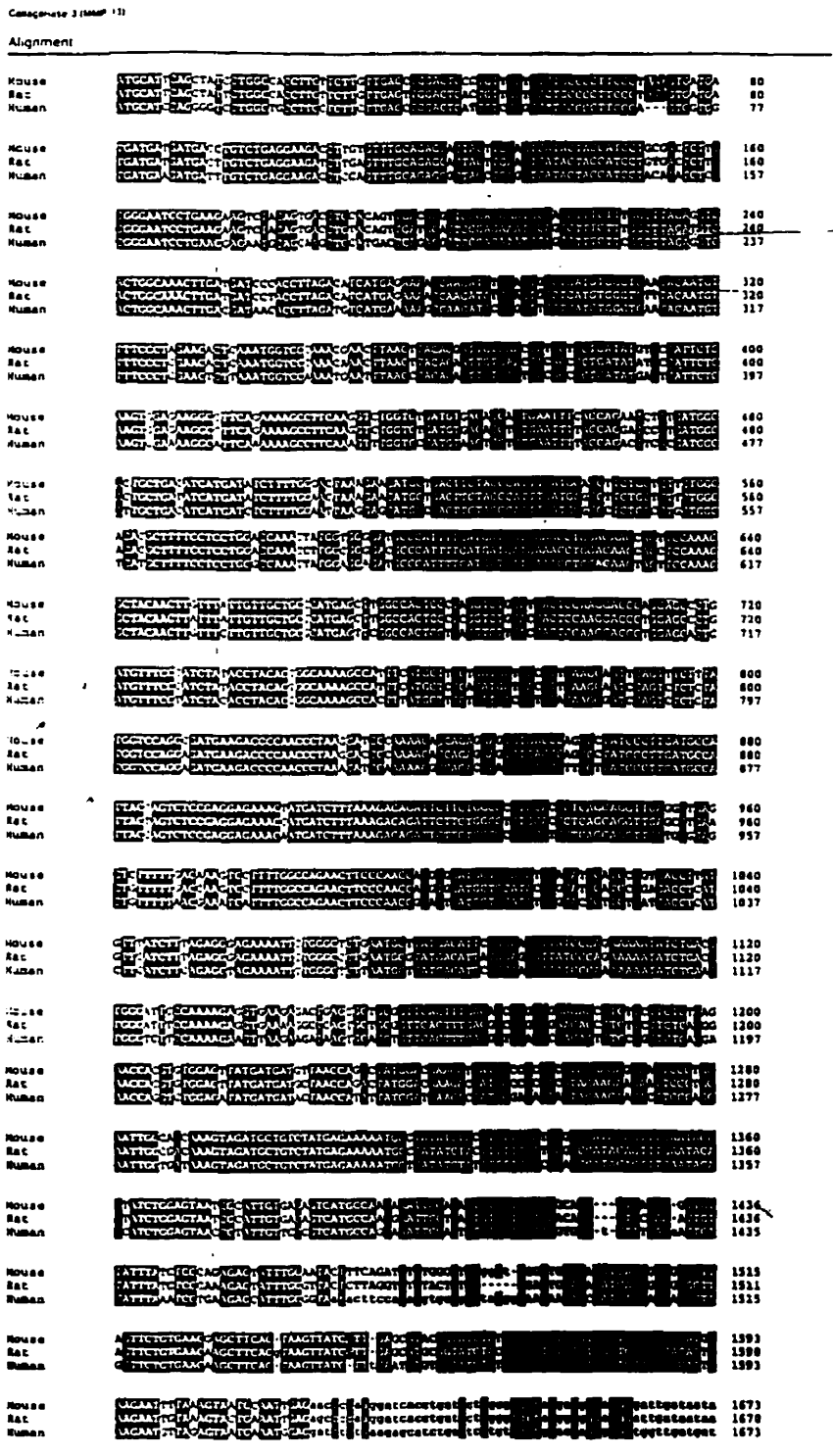


Fig. 2.4 Sequence alignment of human, rat, and mouse MMP-13 cDNAs.

Uppercase letters represent region of homology. Shaded area are identical nucleotides

The sequences are from Freije *et al.*, 1994 for human MMP-13; Quinn *et al.*, 1990 for rat MMP-13; and Henriet *et al.*, 1992 for mouse MMP-13.

Table 2.2 Summary of the oligonucleotide primers used for PCR.

Primer	Sequence (5'-3')	Gene	Starting bp
HGAPDH5	CTCTCCAGAACATCATCCCTGCC	Human GAPDH	606
HGAPDH3	CGTTGTCATACCAGGAAATGAGCTT	Human GAPDH	926
BTTYPEIIC5	ACCTGGATGCCATCAAGTT	Bovine type II collagen	3712
BTTYPEIIC3	TACAGAGGTGTTGACAGA	Bovine type II collagen	4700
BTTYPEX5	ACAGGAATGCCTGTGTCTGCTTTCAC	Bovine type X collagen	1768
BTTYPEX3	AGCCTGCATTGGGCAGCTGGAGCCA	Bovine type X collagen	2080
BTMMP1S5	TTCTTCAAAGACCGCTTCTACATGCGC	Bovine MMP-1	897
BTMMP1S3	CCCAACAAAGAAATACGTTTTCCCAGT	Bovine MMP-1	1167
BTMMP1M5	GGAAAACTACTACAACCTGAA	Bovine MMP-1	125
BTMMP1M3	AAGGGTTTGTGCGCATGTACAA	Bovine MMP-1	901
BTMMP1L5	GCGCGAATTCACCATGCCGAGACTGCCTCTGCTGCTG	Bovine MMP-1	1
BTMMP1L3	GCGCAAGCTTCAATTTTTTCTGCAGTTGAACCA	Bovine MMP-1	1386
BTMMP13L25	GGGCGGATCCACCATGACCCAAAGGTCCTGGCTGGC	Bovine MMP-13	1
BTMMP13L23	GCGCAAGCTTCAACACCACCATAAGGGAATTTGTTGTCAT	Bovine MMP-13	1389
BTMMP13S5	CTTCCTCTTCTTCAGCTGGAC	Bovine MMP-13	24
BTMMP13S3	ATGTATTCACCCACATCAGG	Bovine MMP-13	296
BTMMP13M5	GAGTTATGATGATGCTAACCA	Bovine MMP-13	1210
BTMMP13M3	AAATGCTCTTCAGGATTTAAATAACAA	Bovine MMP-13	1433
BTMMP13L3	ATGTGGTTCCAGCCACGCATAGTCAT	Bovine MMP-13	1601

Table 2.3 Summary of the PCR conditions and the sizes of the amplified products for each primer pair

Primer Pair	Annealing Termperature (^{0}C)	Size of Amplified cDNA fragment (bp)
HGAPDH5/HGAPDH3	55	344
BTTYPEIIC5/BTTYPEIIC3	55	909
BTTYPEXC55/BTTYPEXC3	55	337
BTMMP1L5/BTMMP1L3	55	295
BTMMP1S5/BTMMP1S3	60	899
BTMMP1M5/BTMMP1M3	55	1435
BTMMP13L25/BTMMP13L23	55	1428
BTMMP13S5/BTMMP13S3	55	291
BTMMP13S5/BTMMP13L13	60	1577
BTMMP13M5/BTMMP13M3	55	250

2.5 Northern blot analysis

Total RNA was fractionated on a 1% agarose/2.2 M formaldehyde gel (Sambrook *et al.*, 1989). Even loading of the RNA was verified by ethidium bromide staining. After electrophoresis, the gel was washed in deionized water to remove excess formaldehyde. RNA was transferred onto Hybond-N (Amersham) or N+ (Amersham) membrane by capillary blotting overnight with 20X SSC (1X SSC= 0.15 M sodium acetate, 0.15 M sodium citrate) . The membrane was air-dried and then the RNA was cross-linked onto the membrane by ultraviolet radiation (Stratagene crosslinker). cDNA probes were labeled with ^{32}P-dCTP using a random priming kit (Stratagene) and unincorporated radionucleotides were removed by using a gel filtration column (Nick columns, Pharmacia). The specific activity of the probe was determined by counting in a Beckman scintillation spectrometer (Packard, Meridan, CT). The membranes were prehybridized in a commercially available hybridization buffer (Quickhyb, Stratagene) for 30 min, and then hybridized for 2 hr at 65^{0}C in hybridization buffer containing 200 µl/ml of denatured salmon sperm DNA and heat-denatured ^{32}P-labeled probe (specific activity >1x10^8 cpm/µg) in a hybridization oven (Robbins Scientific, Sunnyvale, CA). The membranes were washed sequentially twice in 2X SSC at room temperature for 5 min, twice in 2X SSC and 1% SDS at room temperature for 15 min, and twice in 0.1X SSC and 0.1% SDS at 65^{0}C for 15 min. The membranes were dried, and exposed to Kodak X-AR film (Eastman Kodak) at -70^{0}C with an intensifying screen (Eastman Kodak) for varying time periods.

2.6 Antibody production and characterization

2.6.1 Identification and synthesis of collagenase specific epitopes

For determination of specific epitopes for generation of polyclonal antisera against MMP-1 and MMP-13, the amino acid sequences of all the known collagenases (MMP-1, 8 and 13) from all known species were aligned and compared using a computer program. MACAW (National Center for Biotechnology Information. Washington D.C.). One specific epitope was chosen for MMP-1 (AIEKAFQLWSNV, peptide 305) corresponding to amino acids 133-144 and MMP-13 (PNPKHPKTPEK, peptide 364), corresponding to amino acids 275-285 (Fig. 2.5.). Each of these epitopes are unique to each MMP and highly conserved among different species. The specificity of the epitope was further compared to all of the Swiss protein database using the BLAST search program that is available from the National Center for Biotechnology Information. A cysteine residue was added to the amino terminal to both peptides to allow for conjugation to ovalbumin.

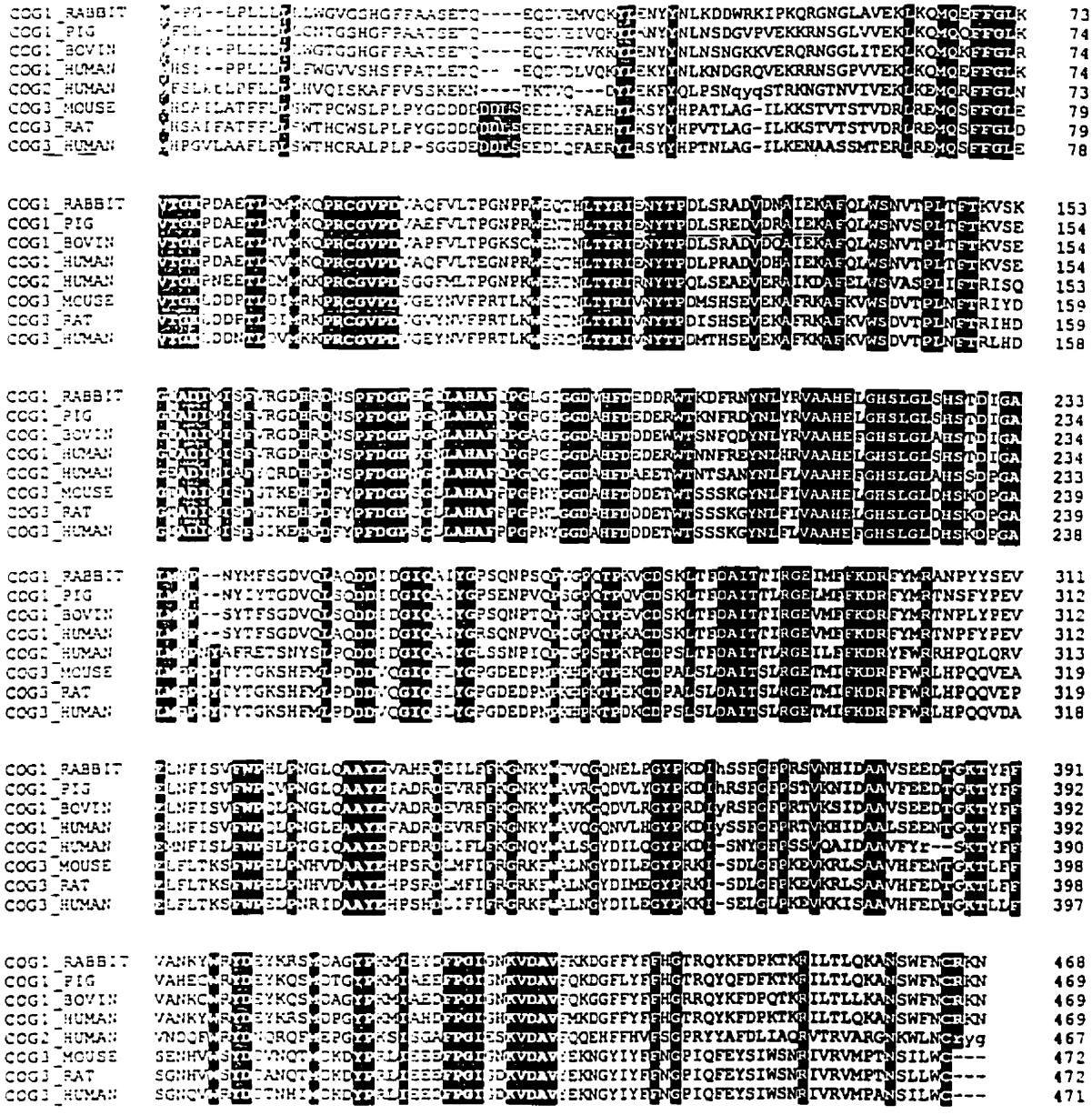
2.6.2 Preparation of polyclonal antibodies from rabbit antisera

For anti-MMP-1 and MMP-13 antisera production, female New Zealand White rabbits weighing 2.5-3.0 kg (Ferme des Chenes Bleus Inc.; Montreal, QC) were initially immunized intramuscularly with 0.5 mg of peptide conjugated to ovalbumin (OVA, Sigma) in 0.25 ml of phosphate buffered saline (PBS) and emulsified in 0.25 ml of Freund's complete adjuvant (Difco, Detroit, MI.) Booster injections were given intramuscularly in Freund's incomplete adjuvant (Difco,

Fig. 2.5 Sequence alignment of collagenases-1,-2,-3, protein of different species.

Specific epitopes for generation of polyclonal sera against MMP-1 and MMP-13 were selected from this alignment. The epitopes were chosen with the following criteria: they should be unique to each MMP and but highly conserved among different species. The MMP-1 epitope that was chosen corresponded to amino acids 133-144 (AIEKAFQLWSNV). The MMP-13 epitope that was chosen corresponded to amino acids 275-285 (PNPKHPKTPEK). COG=collagenase. Uppercase characters represent regions of homology. Shaded areas represent identical amino acid residues. The sequences are from Fini *et al.*, 1987 for rabbit MMP-1; Clarke *et al.*, 1990 for pig MMP-1; Tamura *et al.*, 1994 for bovine MMP-1; Whitham *et al.*, 1986 for human MMP-1; Hasty *et al.*, 1990 for human MMP-8; Henriet *et al.*, 1992 for mouse MMP-13; Quinn *et al.*, 1990 for rat MMP-13 and Freije *et al.*, 1994 for human MMP-13.

Alignment



Detroit, MI.) every two weeks. After the second booster, test bleeds were performed and antibody titers were determined by enzyme-linked immunosorbent assay (ELISA), as described below. After two boosters, the antisera produced good titers and the rabbits were exsanguinated by cardiac puncture and approximately 80 ml of serum was obtained. All animal work was done at the McGill animal resource center (McGill University, Montreal, QC).

2.6.3 Immunoanalyses for determining antibody titer and specificity

2.6.3.1 Preparation of recombinant collagenases and TIMP

Purified recombinant human MMP-1 and MMP-13 were kind gifts of Drs. Peter Mitchell and John Hambor (Pfizer Inc., Groton, CT). Both enzymes were expressed in Sf9 insect cells that were infected with recombinant baculovirus (Mitchell *et al.*, 1996). MMP-1 was affinity purified using a monoclonal antibody column and MMP-13 was purified using a heparin-agarose and SP-Sepharose Fast Flow columns. Both enzymes were >95% pure as determined by Coomassie blue staining of SDS-PAGE gels. Recombinant human MMP-8 was a kind gift of Dr. Harold van Wart (Roche Bioscience, Palo Alto, CA). MMP-8 was also expressed with recombinant baculovirus. Recombinant human TIMP-1 was a kind gift of Dr. John Mort (Joint Diseases Lab, Shriner's Hospital, Montreal, QC). TIMP-1 was expressed using the *Pichia pastoris* yeast system (Invitrogen, San Diego, CA). To activate pro MMPs, 2 mM APMA was added to the enzymes followed by incubation at 37^0C for 2 hr.

2.6.3.2 Direct-binding ELISA

The immunizing peptides alone were diluted to 20 μg/ml in 0.1M carbonate buffer, pH 9.2, and 50 μl was added to each well (1 μg/well) of 96 well Immulon-2 flat bottom microtiter plates (Dyantech Laboratories, Inc., Chantilly, VA). In other cases, plates were coated with 100 ng/well of recombinant human pro-MMP-1 or activated MMP-1 or activated MMP-1 with TIMP-1 (1:1 molar ratio) or TIMP-1 or recombinant human pro-MMP-13 or activated MMP-13 or activated MMP-13 with TIMP-1 (1:1 molar ratio). After 24 hr at 4^0C, the plates were washed three times with PBS containing 0.1% v/v Tween 20 (Sigma). Non-coated binding sites were blocked with 150 μl of 1% w/v BSA in PBS(PBS-1% BSA) for 30 min at room temperature. The plates were then washed once with PBS-Tween and 50 μl of serial dilutions of the appropriate polyclonal antiserum were added to individual wells. After 1 hr at 37^0C, the plates were washed three times with PBS-Tween. Alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma) diluted 1:30 000 in PBS-1% BSA-Tween was added at 50 μl/well. After 1 hr at 37^0C, the plates were washed three times with PBS-Tween and once with distilled water. Finally, 50 μl of freshly prepared alkaline phosphatase substrate, disodium p-nitrophenyl phosphate (Sigma) at 0.5 mg/ml in 8.9 mM diethanolamine, 0.25 mM $MgCl_2$, pH 9.8 was added to each well for at least 30 min at 37^0C. The absorbencies were measured at 405 nm on a ELx808 microplate reader (Bio-Tek Instruments, Inc., Winooski, VT).

2.6.3.2 Inhibition ELISA

Linbro 96 well round bottom microtiter plates (ICN Flow, McLean,VA) were pre-coated with 100 µl/well of PBS-1% BSA for 30 min at room temperature and washed once with PBS-Tween. Four non-specific binding wells each contained 100 µl of 50 µl PBS-1% BSA and 50 µl PBS-1% BSA-Tween. Each polyclonal antiserum preparation was diluted 1:200 (as determined by checkerboard analyses of antisera and peptide titrations, data not shown) in PBS-1% BSA-Tween and 50 µl was added to each of the remaining wells of these pre-incubation plates. Four wells with antisera were mixed with 50 µl/well of PBS-1% BSA to determine maximum binding in the absence of the antibody reactive peptides or proteins. To the remaining test wells containing 50 µl of diluted antiserum were added 50 µl of appropriate dilutions of the standard peptides 305 or 364: pro. activated or TIMP-1 bound MMP-1 (1:1 molar ratio); pro. activated or TIMP-1 bound MMP-13 (1:1 molar ratio). After incubation for 1 hr at 37^0C, 50 µl of each pre-incubated sample was transferred to the equivalent wells of Immulon-2 ELISA plates that were precoated with 100 ng/well of either peptide 305 (MMP-1) or peptide 364 (MMP-13) peptide in PBS, pH 7.2. These plates were incubated for 30 min at 4^0C and then washed three times in PBS-Tween. 50 µl of goat anti rabbit IgG conjugated to alkaline phosphatase (Sigma) that was diluted to 1:20,000 in PBS-1% Tween were added to these plates and incubated for 1 hr at 37^0C. The plates were washed three times with PBS-Tween and three times with distilled water. An ELISA amplification system kit (Gibco BRL,

Gaithersburg, MD) was used to increase sensitivity. Amplifier substrate (50 µl) (NADPH) was added to each well and after 15 min at room temperature, 50 µl of amplifier (alcohol dehydrogenase and diaphorase) solution was added to each well according to the manufacturer instructions. After a final 15 min at room temperature, the color development was halted with 50 µl of 0.3 M H_2S0_4 and the absorbance was measured at 490 nm. For each plate the mean absorbance from the four non-specific binding wells was subtracted from the absorbance values of each of the other wells. The percentage inhibition of binding by standards or samples was calculated relative to the mean absorbance from the four maximum binding wells which represented 0% inhibition (100% binding). Results were expressed on a molar basis using the calculated molecular weight for MMP-1 at 53.361 D and for peptide 305 at 1486 D. MMP-13's calculated molecular weight was 53,927 D and for peptide 364 at 1337 D.

2.6.3.4 Electrophoresis and immunoblotting

For Western blots, 500 µl of conditioned medium from chondrocytes or fibroblasts was precipitated with 2 volumes 100% ethanol. The proteins were separated by SDS-PAGE as previously described (Laemmli, 1970) under denaturing conditions using 10%, 1 mm thick, 7 cm x 8 cm mini Protean gels (Bio-Rad Laboratories, Mississauga, ON). Gels were either stained with Commassie Blue R-250 (Bio-Rad) or electrophoretically blotted onto nitrocellulose (Bio-Rad) in methanol-free buffer containing 25 mM Tris, 0.2 M glycine, pH 8.3 overnight at 4^0C. Nitrocellulose membranes were blocked with

PBS-3% BSA and incubated either overnight or for 1 hr with the appropriate dilution of affinity purified IgG at 4^0C. Affinity purified IgG was obtained by using a peptide affinity column. MMP-1 or MMP-13 affinity columns were prepared by coupling of the free sulfhydryl group on the amino terminal cysteine on peptide 305 (MMP-1) or 364 (MMP-13) to Sulpholink gel (Pierce Chemical Company, Rockford, IL) according to manufacturer's instructions. After three 10 min washes in PBS- 1% BSA-1%Tween, the membranes were incubated for 1 hr at room temperature with alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma) diluted 1:30,000 with PBS-3% BSA-1%Tween. The membranes were washed again three times for 10 min each in PBS-1% BSA-1%Tween and then rinsed with distilled water. Alkaline phosphatase substrate solution (Bio-Rad) was added to visualize the immune complex. After optimal color development (10-20 min) the reaction was stopped by washing off the substrate solution with distilled water.

2.7 Immunoprecipitation

For immunoprecipitation, ^{35}S-methionine-labeled conditioned medium was incubated with anti-MMP-1 or anti-MMP-13 affinity purified IgG in 150 mM NaCl, 1% Nonidet P-40 (Bio-Rad), 0.1% SDS, 50 mM Tris, pH7.5 overnight at 4^0C. The immune complex was precipitated with Protein A-Sepharose (Pharmacia). After washing three times in 150 mM NaCl and 50 mM Tris, pH 7.5 at room temperature, the sample was boiled and the immunoprecipitate was separated by SDS-PAGE. The gel was then dried and exposed to Kodak X-AR

film (Eastman Kodak) at -70^{0}C for varying time periods.

2.8 Substrate gel enzyme zymography

Gelatin zymography was used to assay for gelatinolytic activity as previously described (Banda *et al.*, 1987). Briefly, porcine gelatin (Sigma, Type I) was allowed to form a gel with SDS and polyacrylamide. Growth plate chondrocyte conditioned medium (20 µl) was separated in this gel using standard SDS-PAGE procedures under non-reducing conditions at 4^0 C. The gel was washed twice at room temperature in 2.5% Triton X-100 with agitation. It was then incubated in 50mM Tris-HCl (pH. 8), 2mM $CaCl_2$ overnight at 37^{0}C with agitation on a rotary shaker. The gel was then fixed and stained in 25% methanol, 10% acetic acid and 0.2% Coomassie brilliant blue (Bio-Rad) and destained in H_20.

2.9 Cell proliferation

DNA was determined using a modification of the method described (LaBarca and Paigen, 1980). Briefly, chondrocyte cell layers were digested with proteinase K (0.5 mg/ml) for 24 hr at 56^{0}C in 0.1 M sodium phosphate buffer, pH 6.5, containing 0.01% EDTA. DNA standard curves were prepared from calf thymus DNA (0.1-1.0 mg) in phosphate/EDTA buffer. To a 100 µl sample of tissue digest, were added 2 ml of a solution containing 1.0 mg/ml bisbenzimide (Hoechst H 33248, Sigma) in 50 mM sodium phosphate, pH 7.4 and 2 M NaCl. Fluorescence was recorded with an excitation at 356 nm and emission at 458 nm, using a fluorescence spectrophotometer (model 650-IOS, Perkin-Elmer

Corporation, Norwalk, CT).

2.10 Collagen purification

Human fetal cartilage was collected from therapeutic abortions. Human and bovine type II collagen were prepared by pepsin digestion and differential salt precipitation, as previously described (Miller *et al.*, 1971). The purification steps involved are shown in Figure 2.6.

2.11 Collagenase assay

Acid-soluble telopeptide-free human type II collagen was acetylated using ^{14}C-acetic anhydride as described by Dean and Woessner (1985). Collagenase activity was assayed as previously described (Dean and Woessner, 1985) with some modifications (Fig 2.7). Briefly, enzyme samples (up to 100 µl in assay buffer) were incubated with 10 µl of ^{14}C-telopeptide-free collagen substrate (specific activity 3.7 x 10^6 cpm/mg collagen at 3.1 mg/ml. in 50 mM Tris/HCl. pH 7.6, 0.3 M NaCl) in 1.5 ml microfuge tubes. 0.5 mM APMA was added to activate procollagenase. 1 mM 1,10-phenanthroline was added to inhibit collagenase and served as a blank control. After 18 hr incubation at 30^{0}C, the reaction was terminated by the addition of 0.5 mM EDTA. Collagenase cleavage products were further digested by 35 µg trypsin (Sigma) and 35 µg α-chymotrypsin (Sigma) in the presence of 200 µg bovine type II collagen as a carrier protein for 2 hr at 30^{0}C. The undigested ^{14}C-telopeptide type II collagen was precipitated in 10% tricholoroacetic acid (TCA) at 4^{0}C. Following centrifugation, 100 µl of supernatant was added to 1 ml of Aquasol scintillation

Fig. 2.6 Purification of type II collagen from cartilage.

1. Mince cartilage with scalpel.
2. Make up 4 M guanidine-HCl (20 volume/ gram of cartilage) in 0.15 mM potassium acetate, pH 6.3 to remove proteoglycans.
3. Add 1 μg/ml pepstatin, 1mM PMSF, 1 mM iodoacetamide and 1 mM EDTA.
4. Add 4 M guanidine-HCl to cartilage and stir 24-48 hr at 4^0C.
5. Pour solution in centrifuge tubes and spin at 10,000 g for 10 min 4^0C.
6. Remove supernatant and pour pellet into beaker. Repeat steps 4 and 5.
7. Pour off supernatant and rinse four times with water. Centrifuge after each wash at 10,000g for 10 min at 4^0C.
8. Prepare pepsin digestion solution (1 mg/ml, Sigma) in 0.5 M acetic acid.
9. Add pellet to digestion solution and stir overnight at 4^0C.
10. Spin at 15,000 g for 20 min at 4^0C and remove supernatant.
11. Adjust NaCl concentration to 0.9 M by using 3.6 M NaCl in 0.5 M acetic acid dropwise (usually 250 ml NaCl + 750 ml supernatant).
12. Stir overnight at 4^0C.
13. Centrifuge at 15,000 g for 20 min at 4^0C.
14. Collect precipitate and pour supernatant into a beaker.
15. Prepare 8 volume of 0.05 M Tris-HCl, pH 7.5 with 1 M NaCl.
16. Centrifuge at 12,000 g for 5 min at 4^0C.
17. Make up 300 volume 0.5 M acetic acid for dialysis.
18. Dialyze overnight at 4^0C.
19. Transfer to 20 mM Na_2HPO_4 buffer and dialyze at 4^0C until pH of buffer is < 8.
20. Centrifuge collagen suspension at 15,000 g for 30 min at 4^0C.
21. Discard supernatant and redissolve precipitate in 5 volume of 0.5 M acetic acid in beaker and mix at room temperature for 10 min.
22. Centrifuge solution at 10,000 g for 5 min at room temperature.
23. Collect supernatant and discard pellet.
24. Prepare 4.4 M NaCl in 0.5 M acetic acid.
25. Add 4.4 M NaCl solution until 2.2 M NaCl final concentration.
26. Repeat step 25 four times.
27. Centrifuge at 15,000 g for 30 min at room temperature.
28. Dialyze against 0.5 M acetic acid
29. Transfer collagen into large lyophilization tube.
30. Weigh lyophilized collagen.
31. Check purity by SDS-PAGE.
32. Store at -20^0C.

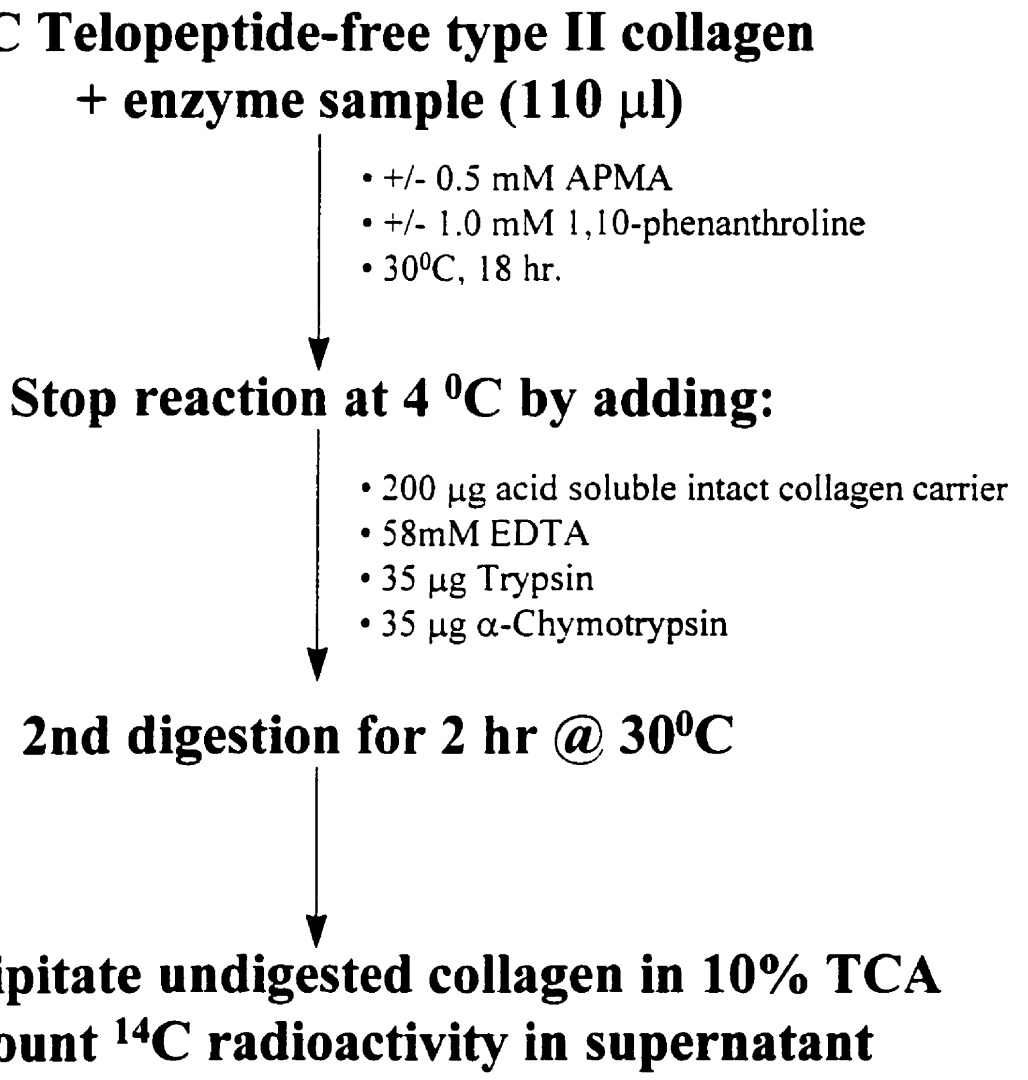


Fig. 2.7 Flow diagram of collagenase assay.

fluid (New England Nuclear, Boston, MA) and counted in a Beckman scintillation spectrometer (Packard). The percent digestion was calculated as the total cpm in the supernatant minus cpm in the 1,10 phenanthroline blank divided by original counts in the ^{14}C-telopeptide-free type II collagen x 100. One unit of collagenase is defined as the amount of collagenase that digests 1 μg of ^{14}C-telopeptide-free type II collagen in 1 min at 30^{0}C.

TIMP in conditioned media was inactivated by reduction of its six disulfide bridges in 2mM dithioethreitol at 37^{0}C for 30 min. To prevent spontaneous re-activation of TIMP, the reduced TIMP was alkylated with 5 mM iodoacetamide at 37^{0}C for 30 min.

2.12 Preparation of synthetic MMP-13 inhibitor and determination of inhibition constants

Enzyme kinetics were carried out by Dr. Harold van Wart (Roche Biosciences) with catalytic domains of human MMP-1, MMP-2 and MMP-3 (Agouron Pharmaceuticals, La Jolla, CA), and MMP-13 (Roche Biosciences), which were all expressed in *E.coli*. MMP-8 and MMP-9 were isolated and purified from neutrophils. The substrate used for the MMP assays was MCA-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 (Bachem, Torrance, CA). Mca is 7-methoxycoumarin-4-yl) acetyl and Dpa is N-3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl. Initial rates of hydrolysis (v) were measured florometrically at 37^{0}C using λ_{ex}325 nm; λ_{em}393 nm with a Perkin Elmer LS-50B luminescence spectrometer. Values of v were determined from varying concentrations of the

synthetic carboxylate inhibitor RS 102,481. The K_I values are shown in Table 2.4. They were obtained by the following equation: $v = -(K_I + [I]_0 - [E]_0) + \{(K_I + [I]_0 - [E]_0)^2 + 4 K_I [E]_0^{1/2} / 2[E]_0$ where $[I]_0$ = initial inhibitor concentration and $[E]_0$ = initial enzyme concentration. All assays were done in 50 mM Tris-HCl, 200 nM NaCl, 10 nM $CaCl_2$, 2.5% DMSO and 0.005% Brij 35, pH 7.5. RS 102, 481 was synthesized in the chemistry department of Roche Biosciences. 102,481 belongs to the carboxylate family of synthetic peptide inhibitors which contain a carboxylic acid group as it's active moiety. It was designed to bind the deep S_1' pocket of MMP-13. In these studies, we have assumed that this preferential inhibitor of human MMP-13 has similar activity against bovine MMP-13. Comparison of bovine MMP-13 and human MMP-13 catalytic domains show that they are virtually identical. Also, the catalytic domains of bovine MMP-1, MMP-2 and MMP-3 show a high degree of similarity to their human counterparts (Bottomley *et al.*, 1997).

2.12.1 Collagenase digestion of ^{14}C- labeled type II collagen

One µg of human recombinant MMP-1 and MMP-8 or 100 ng of human recombinant MMP-13 were activated with 2 mM APMA at 37^{0}C for 2 hr. in 50mM Tris, 10mM $CaCl_2$, 600mM NaCl, 01% BRIJ. 35, and 0.2% NaN_3 at ph.7.0. Two hundred µg of ^{14}C-labeled human type II collagen was added to the activated MMPs with or without selective MMP-13 inhibitor RS 102,481 and incubated in 50mM Tris, 10mM $CaCl_2$, 600mM NaCl, 01% BRIJ. 35, and 0.2% NaN_3 at pH 7.0 at 30^{0}C. Samples with inhibitor RS 102,481 were incubated for

Table 2.4 Inhibitory profile of MMP-13 selective inhibitor used in bovine fetal growth plate chondrocyte cultures

Compound	K_I (nM)					
	MMP-1	MMP-8	MMP-13	MMP-3	MMP-2	MMP-7
RS 102,481	1,100	18	0.080	19	32	31,000

1hr. Samples without inhibitor were incubated for 1.2 4, 12 24, 48 and 72 hr. Fifty mM EDTA was added to stop the reaction. The cleavage products were visualized by SDS-PAGE.

2.13 Determination of matrix mineralization utilizing calcium ($^{45}Ca^{2+}$) incorporation

Chondrocyte cell layers were labeled at every medium change (every 2 days) with 1.25 μCi/ml of $^{45}Ca^{2+}$ (Amersham). Upon completion of the culturing period, the cell layers were collected, washed twice in DMEM alone and dried under vacuum for 1 hr. The cell layers were then solubilized at 70^{0}C by the addition of 1ml /well 90% formic acid for 45 min before liquid scintillation counting.

2.14 Collagen biosynthesis

To assess collagen biosynthesis in chondrocyte cell layers, ^{3}H-proline (25 μCi/ml, Amersham) was added to cultures at every medium change (every 2 days) in the presence of 70 μg/ml of β-aminoproprionitrile. Collagens from the media were precipitated by the addition of $(NH_4)_2SO_4$ to a final concentration of 33% (of saturation) for 24 hr at 4^{0}C. After centrifugation, pellets were washed twice with 70% ethanol. The dry pellets were analyzed directly by SDS-PAGE using 10% gels followed by fluorography (Laskey and Hills, 1975).

2.15 Determination of collagen degradation

Type II collagen degradation in bovine fetal growth plate chondrocyte cell layers was measured using the CB11B ELISA (Hollander *et al.*, 1994). This assay

measures an intra-chain epitope that arises from the unwinding of the triple helical collagen. Chondrocyte cell layers were digested with 1 mg/ml α-chymotrypsin (Sigma) in 50 mM Tris-HCl, pH 7.6 with inhibitors (1 mM EDTA, 1 mM iodoacetamide, 10 μg/ml pepstatin A) overnight at 37^0C. Chymotrypsin was inhibited by 160 μg/ml N-tosyl-L-phenylalanine-chlormethyl ketone (TPCK). The supernatant was removed and assayed for denatured collagen. The residual cell layer was further digested with 1 mg/ml Proteinase K (Sigma) with inhibitors (as above) overnight at 56^0C. Proteinase K was inactivated by boiling for 15 min and this fraction was also assayed with CB11B. The α-chymotrypsin fraction represents total denatured collagen in the tissue while the addition of the α–chymotrypsin digest and the Proteinase-K represent total collagen in the tissue. Epitope was expressed as pmole of peptide/μg of DNA.

2.16 Determination of proteoglycan degradation

To measure the release of sulphated glycosaminoglycans (sGAGs) into the conditioned medium, the dimethylmethylene blue (DMMB) dye binding assay was employed (Farndale *et al.*, 1986). Briefly, 10 μl of samples were added to 190 μl of the 0.1 M dye reagent (BDH Chemicals, Montreal, QC) in a 96 well microtiter plate. The absorbance was read at 525 nm within 10 min. Proteoglycan release was plotted as absorbance 525 nm vs. time in culture.

2.17 Histological analysis of chondrocyte cell layers

Chondrocyte cell layers were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.2, overnight at 4^0C. Tissue was dehydrated with graded ethanols

and embedded in Spurr resin (Polysciences Inc., Washington, PA). Semi-thin sections (1-2 μm) were stained with Von Kossa's reagent (for mineral) or with toluidine blue.

3. RESULTS

3.1 Isolation of bovine fetal growth plate chondrocytes and serum-free culture system

Bovine fetal growth plate chondrocytes were isolated and separated into different subpopulations by Percoll discontinuous gradient. Chondrocytes were cultured at high density cultures in serum-free medium. These cells do not undergo significant cell division (Alini *et al.*, 1996). Chondrocytes show typical hexagonal shape and calcify their matrix with time (Fig 3.1).

3.2 Isolation of total RNA from bovine fetal cartilage and chondrocyte cell layers

Total RNA was isolated from bovine fetal growth plate cartilage and chondrocyte cell layers using the alcohol/Proteinase K method (Fig. 3.2.). In comparison, the yield of total RNA isolated from fetal bovine growth plate cartilage and chondrocyte cell layers using Chomczynski and Sacchi's method was very low (Fig 3.2). Also the RNA pellet was insoluble and contained contaminating matrix proteins. Total RNA isolated directly from cartilage using the Chomczynski and Sacchi's method could not be resolved by formaldehyde/agarose electrophoresis. GAGs interfere with ethidium bromide staining and migration of total RNA in agarose/formaldehyde gels. This results in false quantitation of loading. It also interfered with signal intensity of GAPDH on Northern blots (Fig. 3.3c) but not of MMP-1 (Fig. 3.3d).

3.1 Light micrographs of bovine fetal growth plate chondrocyte subpopulation C cells in culture with time.

Bovine fetal growth plate C subpopulation chondrocytes (fetal age = 206 days) were plated out in high density cultures (1 x 10^6 cells/ml). 2 days in culture (A). 4 days in culture (B). 12 days in culture (C). 20 days in culture (D). 100X magnification

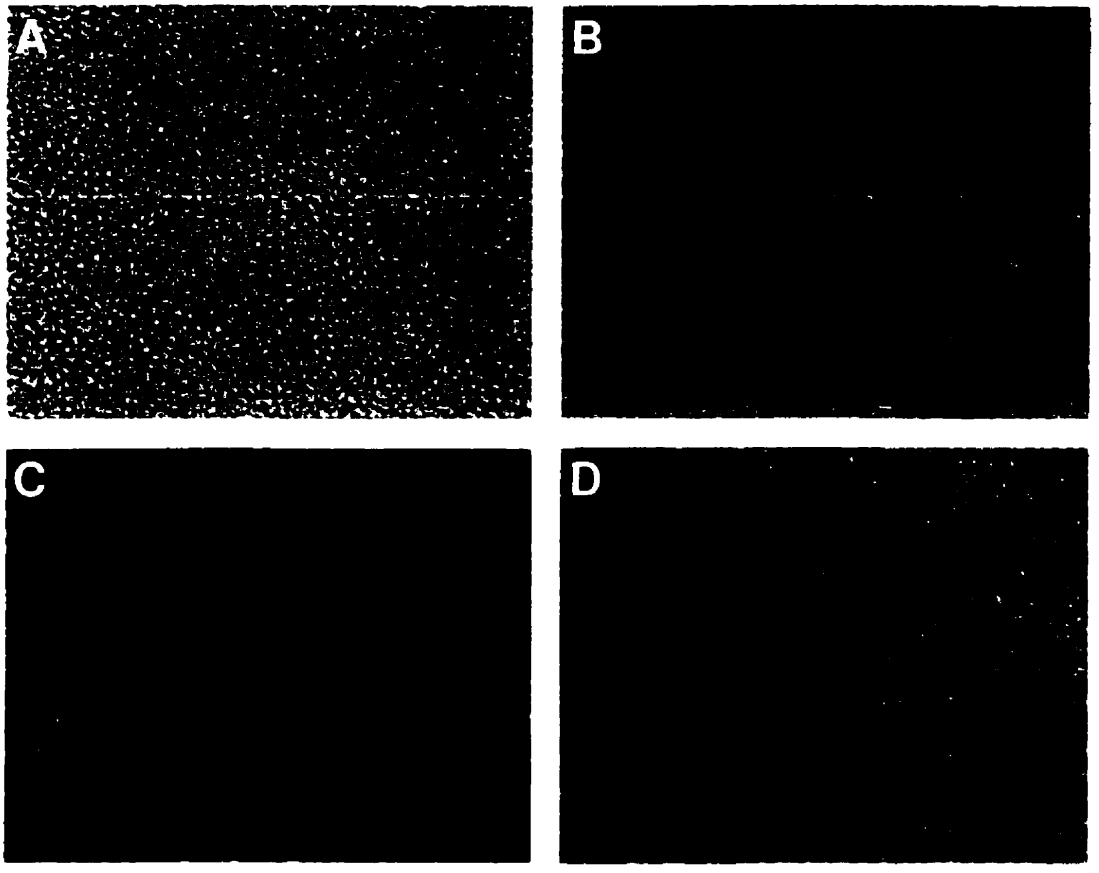

A
B
C
D

Fig. 3.2 Comparison of total RNA isolated from bovine fetal growth plate cartilage and chondrocytes.

Total RNA was isolated using equal amounts of cell layer ($1x10^6$ cells) or 100 mg of tissue by the alcohol/Proteinase K method or by Chomczynski and Sacci method. RNA was electrophoresed on a fomaldehyde/1% agarose gel and stained with 3 µl (10 mg/ml) of ethidium bromide. Total RNA isolated from tissue using the alcohol/Proteinase K method (lane 1). Total RNA isolated from cell layer using the alcohol/Proteinase K method (lane 2). Total RNA isolated from tissue using Chomczynski and Sacci method (lane 3). Total RNA isolated from cell layers using Chomczynski and Sacci method (lane 4). Total RNA isolated using the alcohol/Proteinase K method results in high yields and pure RNA (lane 1 and 2) while Chomczynski and Sacci method results in low yields and matrix contaminated RNA. Total RNA from cartilage cannot even enter the gel due to matrix contamination.

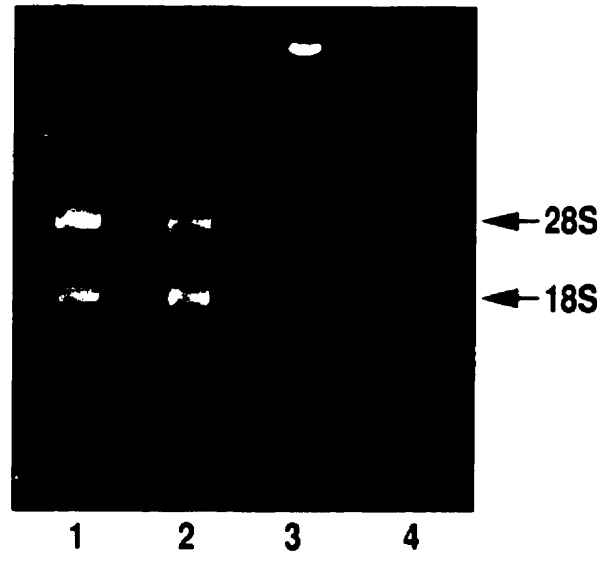
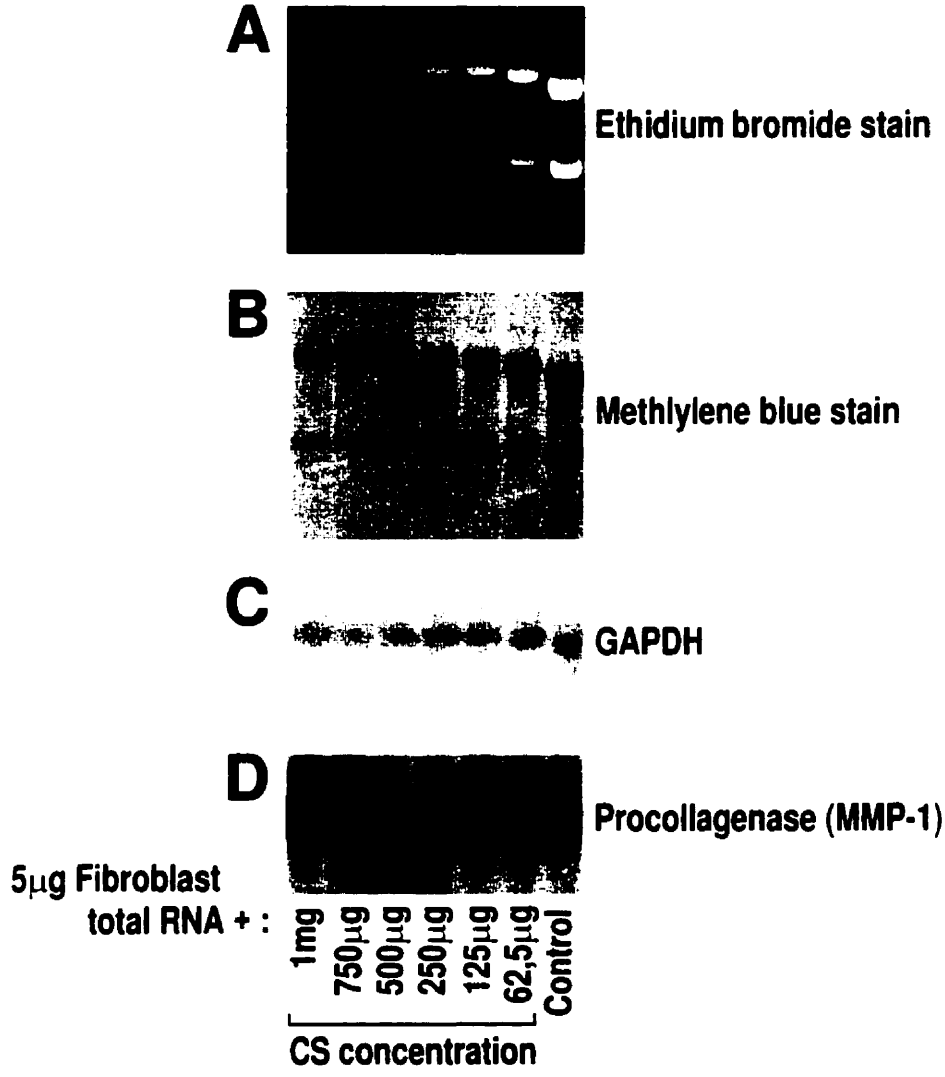

28S
18S
1
2
3
4

Fig. 3.3 Effect of glycosaminoglycans on mRNA quantitation.

Increasing concentrations of shark chondroitin sulfate (CS) were added to 5 μg of fibroblast total cellular RNA. Total cellular RNA was electrophoresed in a formaldehyde/1% agarose gel and stained with 3 μl (10 mg/ml) of ethidium bromide. Note, inhibition of ethidium bromide staining with increased CS concentration. Migration of RNA is also affected. RNA was transferred onto a nylon membrane and stained with methlylene blue stain. CS does not affect methylene blue staining of RNA. The nylon membrane was probed with GAPDH and procollagenase (MMP-1) cDNAs. Signal intensity for GAPDH decreased with increasing amounts of CS while the MMP-1 signal remained constant.


A
Ethidium bromide stain
B
Methlylene blue stain
C
GAPDH
D
Procollagenase (MMP-1)
5μg Fibroblast
total RNA + :
1mg
750μg
500μg
250μg
125μg
62,5μg
Control
CS concentration

3.3 Generation of specific cDNA probes for analysis of gene expression

Specific cDNA probes for bovine type II and X collagens, GAPDH, MMP-1 and MMP-13 were obtained by PCR. All probes specificities were confirmed by DNA sequencing (data not shown).

3.4 Expression of type X collagen mRNA and protein with cellular hypertrophy

To monitor cellular hypertrophy, type X collagen mRNA and protein expression were followed in culture. Type X collagen mRNA expression was observed first in isolated hypertrophic cells (A subpopulation) while expression appears later in less mature cells in culture with time (B and C subpopulations) (Fig. 3.4). C subpopulation chondrocytes synthesize type X collagen protein during maturation as seen by analysis of ^{3}H-proline labeled culture medium (Fig. 3.5).

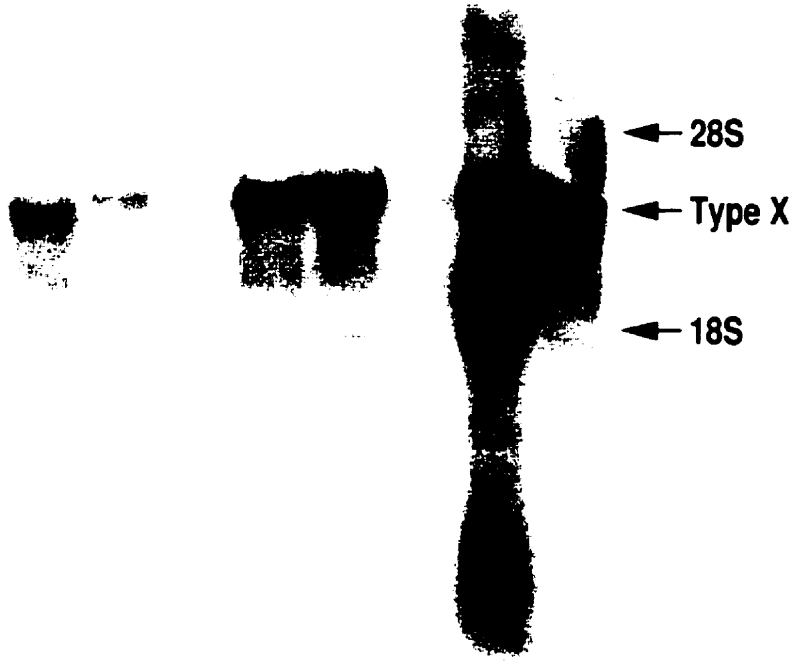
3.5 MMP-1 mRNA expression in bovine fetal skin fibroblasts and fetal growth plate chondrocyte subpopulations

MMP-1 mRNA expression was analyzed by Northern blotting of freshly isolated bovine fetal growth plate chondrocyte subpopulations (Fig.3.6) The most mature subpopulation at isolation (A) showed expression of type X collagen, while other subpopulation were negative. MMP-1 mRNA expression was only seen in IL-1α stimulated bovine fetal skin fibroblast cultures, not in unstimulated cultures. Type X collagen is not expressed in bovine fetal skin fibroblast cultures.

Fig. 3.4 Type X collagen mRNA expression in bovine fetal growth plate chondrocyte subpopulations.

Northern blot analysis of type X collagen mRNA expression during maturation of bovine fetal growth plate chondrocyte subpopulations. 15 μg of total RNA was loaded per lane. A= hypertrophic cells at isolation: B = prehypertrophic but more mature than: C= prehypertrophic (fetal age = 211 days). Numbers denote days in culture.

Northern blot



A0 A4 B0 B8 B12 C0 C12 C16

SUBPOPULATIONS

(4h exposure)

Fig. 3.5 Collagen synthesis in bovine fetal growth plate chondrocyte C subpopulation.

C subpopulation growth plate chondrocytes (fetal age = 206 days) were labeled with ^{3}H-proline and conditioned media were collected every 2 days. Labeled collagens were analyzed by SDS-PAGE. Types II and X collagens are the major collagens synthesized by these chondrocytes. C subpopulation chondrocytes (1 X 10^{6} cells/ml) progress from prehypertrophic cells to hypertrophic cells in serum-free conditions.

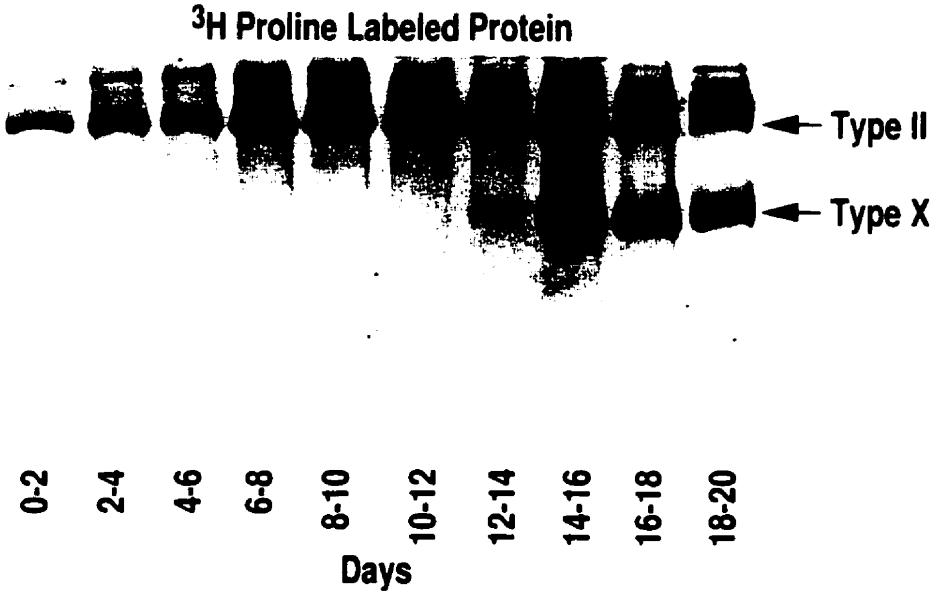

^{3}H Proline Labeled Protein
Type II
Type X
0-2
2-4
4-6
6-8
8-10
10-12
12-14
14-16
16-18
18-20
Days

Fig. 3.6 Type X collagen and MMP-1 expression in freshly isolated bovine fetal growth plate chondrocyte subpopulations and bovine skin fibroblasts.

Subpopulation A (lane 1). Subpopulation B (lane 2). Subpopulation C (lane 3). Subpopulation D (lane 4). Skin fibroblasts (lane 5). 15 μg of total RNA was loaded per lane. Blots were probed with bovine MMP-1 cDNA and exposed for 48 hr. (overexposed). Type X collagen mRNA expression is restricted to bovine growth plate chondrocyte subpopulation A. (A) MMP-1 mRNA expression is restricted to IL-1α (1 ng/ml) stimulated (24 hr) bovine fetal skin fibroblasts (B).

Northern blot

A Type X collagen

B MMP-1

1 2 3 4 5

Type II and X collagen expressions were analyzed by Northern blotting of cultured B and C subpopulation growth plate chondrocytes (Fig.3.7). Type II collagen mRNA increased with time and decreased as cells became hypertrophic. Type X collagen mRNA in the B subpopulation increased with time as cells became hypertrophic in culture. Since the B subpopulation is more mature than C, type X mRNA was not detectable in C subpopulation at these times. MMP-1 mRNA was always undetectable in these cultures.

MMP-1 mRNA was analyzed by Northern blotting of cultured bovine fetal growth plate and articular chondrocytes (Fig. 3.8). Chondrocytes were also stimulated with 1 or 10 ng/ml IL-1α for 24 hr. None of the fetal chondrocyte cultures expressed MMP-1. Only IL-1α stimulated fetal skin fibroblasts showed MMP-1 mRNA expression (Fig. 3.8).

3.6 Polyclonal antibodies to mammalian MMP-1 and MMP-13

3.6.1 Direct-binding ELISAs for determination of antibody titers

Sera obtained from rabbits immunized with peptide-OVA conjugates of MMP-1 and MMP-13 epitopes were initially characterized by direct binding ELISAs. Each of the anti-MMP antibodies reacted with its own immunizing peptide (Fig. 3.9) None of the antisera reacted with non-specific peptide or BSA (Fig. 3.9). Sera were also tested by direct binding ELISA on human recombinant pro MMP-1 or MMP-13, active MMP-1 or 13, active MMP-1+TIMP-1 or active MMP-13 + TIMP-1 (Fig. 3.10). Both MMP-1 and MMP-13 antisera recognized pro, active and TIMP complexed enzyme similarly. None of the antisera cross

Fig. 3.7 Type II collagen, type X collagen and MMP-1 expression in bovine fetal growth plate chondrocyte subpopulations with time.

B and C subpopulation cells were cultured from 2-12 days. Type II collagen, type X collagen and MMP-1 mRNA expression were examined by Northern blot. Type II collagen mRNA was expressed in both subpopulations. Type X collagen was first expressed in the more mature B subpopulation. MMP-1 mRNA was not detectable in either subpopulation at any of the days. Blots were probed with bovine cDNAs for type II collagen, type X collagen, and MMP-1. 5 μg of total RNA was loaded per lane. Blots were exposed for type II collagen: 12 hr, type X collagen: 12 hr and MMP-1: 3 days.

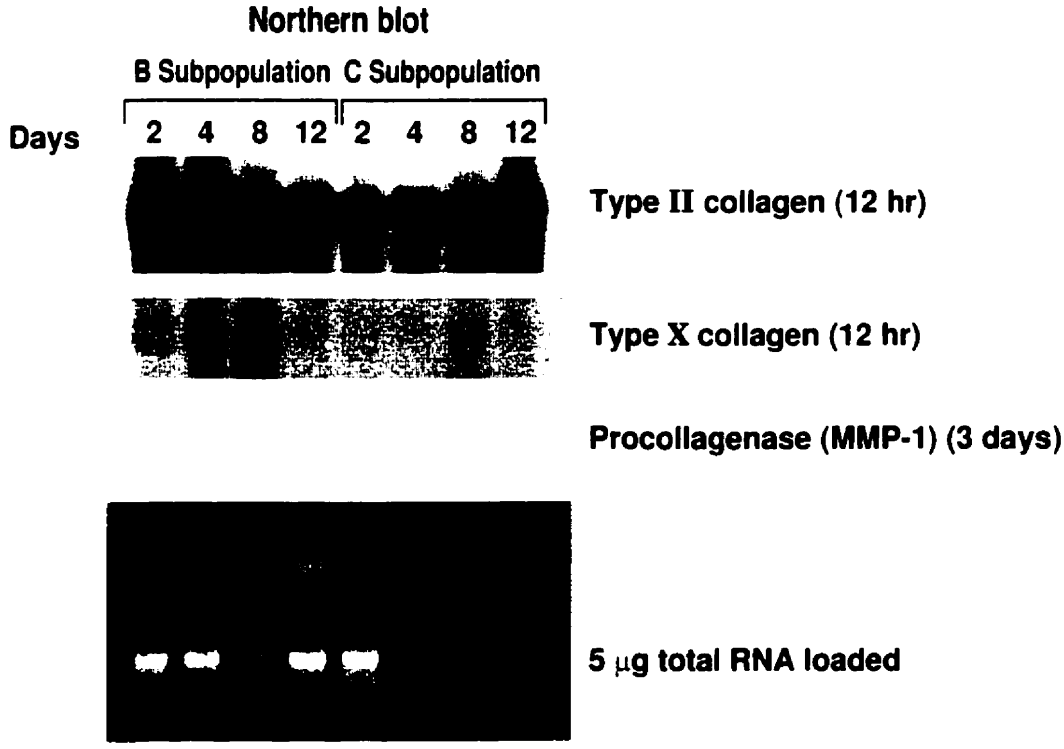
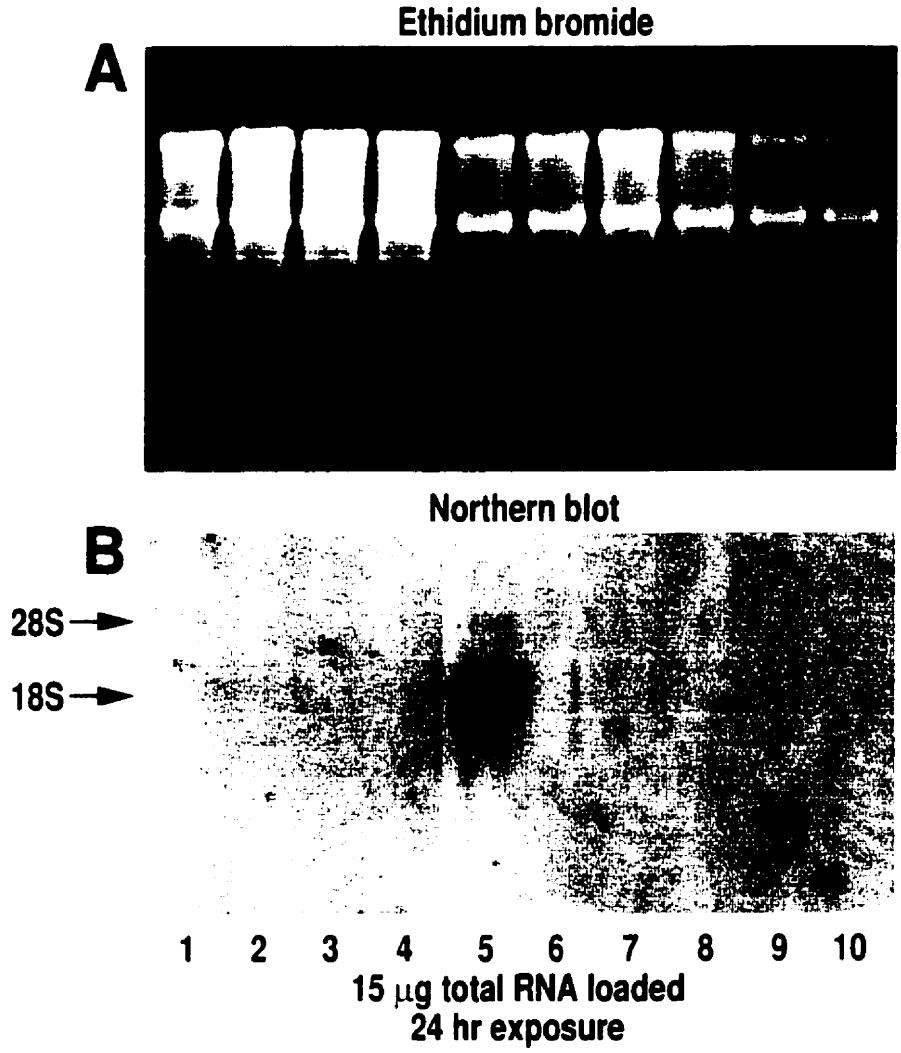

Northern blot
B Subpopulation
C Subpopulation
Days
2 4 8 12 2 4 8 12
Type II collagen (12 hr)
Type X collagen (12 hr)
Procollagenase (MMP-1) (3 days)
5 μg total RNA loaded

Fig. 3.8 MMP-1 expression in bovine fetal growth plate and articular chondrocytes with IL-1α stimulation.

MMP-1 mRNA expression in bovine fetal growth plate and articular chondrocytes was examined by Northern blot. MMP-1 mRNA is only detectable in IL-1α stimulated fetal fibroblasts. MMP-1 mRNA is absent in fetal growth plate and articular chondrocytes that are stimulated with IL-1α (1 ng/ml or 10 ng/ml). Chondrocytes and fibroblasts were stimulated for 24 hr. 15 μg of total RNA was loaded per lane. Blot was exposed for 24 hr. A subpopulation (lane 1), B subpopulation (lane 2), C subpopulation (lane 3), D subpopulation (lane 4). Chondrocytes were at time=0 unless otherwise indicated.

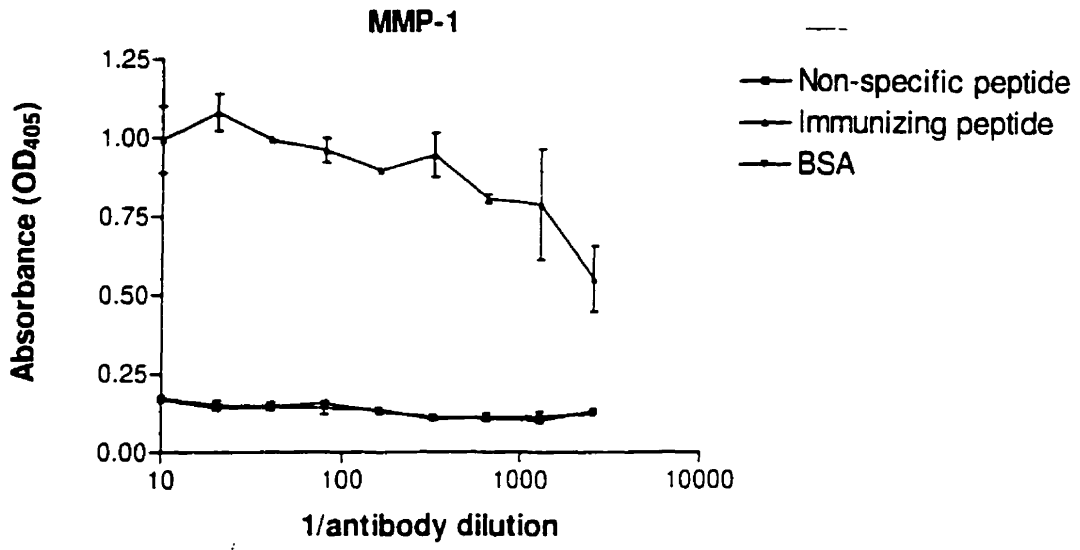


Lanes 1-4: Uncultured bovine fetal growth plate chondrocytes
Lane 5: IL-1α (1 ng/ml) 24 hr stimulated bovine fetal skin fibroblasts
Lane 6: Control bovine fetal articular condrocytes
Lane 7: IL-1α (1 ng/ml) 24 hr stimulated bovine fetal articular chondrocytes
Lane 8: Control bovine fetal growth plate chondrocytes
Lane 9: IL-1α (1 ng/ml) 24 hr stimulated bovine fetal growth plate chondrocytes
Lane 10: IL-1α (10 ng/ml) 24 hr stimulated bovine fetal growth plate chondrocytes

Lanes 6-10: Chondrocytes were grown 6 days in culture before stimulation

Fig. 3.9 Immunoreactivities of MMP-1 and MMP-13 antiserum with immunizing peptides, as determined by direct-binding ELISA.

MMP-1 and MMP-13 antisera were tested against immunizing peptides, BSA and non-specific peptides. Both antisera were specific for their respective immunizing peptide. No reactivity was seen with non-specific peptide or BSA. Non specific peptide for MMP-1 was AFRKAFKVWSDV and for MMP-13 was TQPVGPQTPEV.


MMP-1
Absorbance (OD_{405})
1.25
1.00
0.75
0.50
0.25
0.00
10
100
1000
10000
1/antibody dilution
Non-specific peptide
Immunizing peptide
BSA

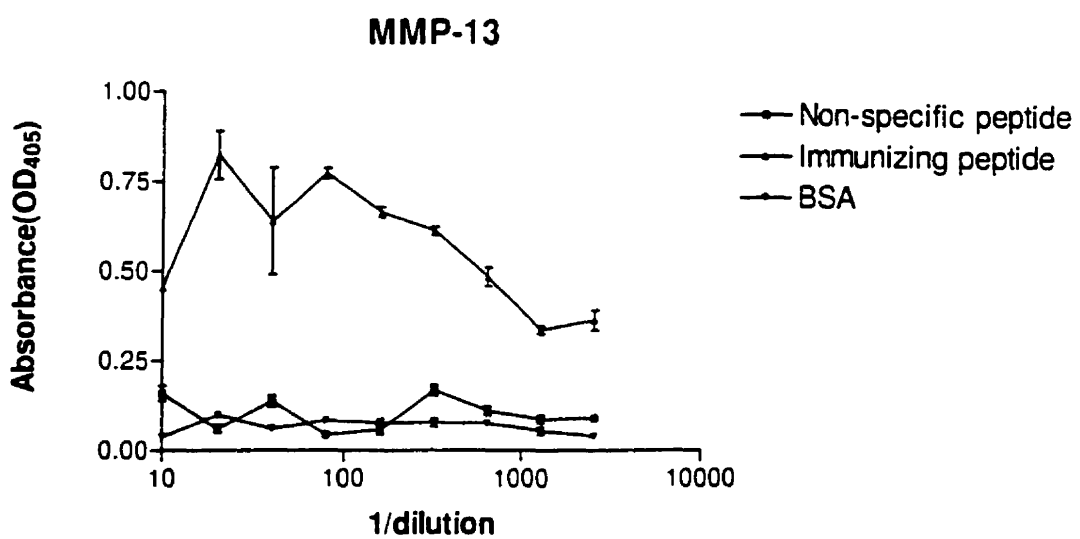
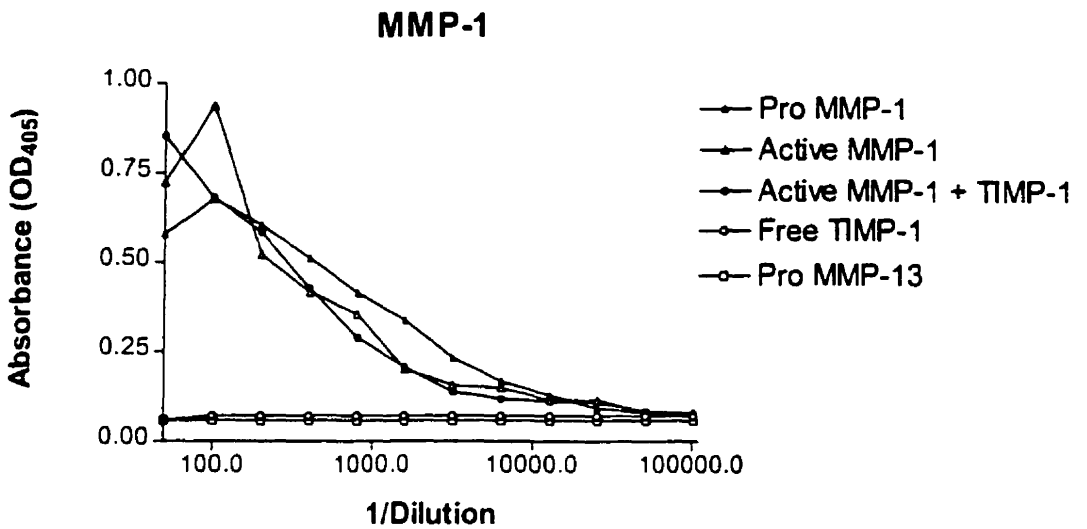
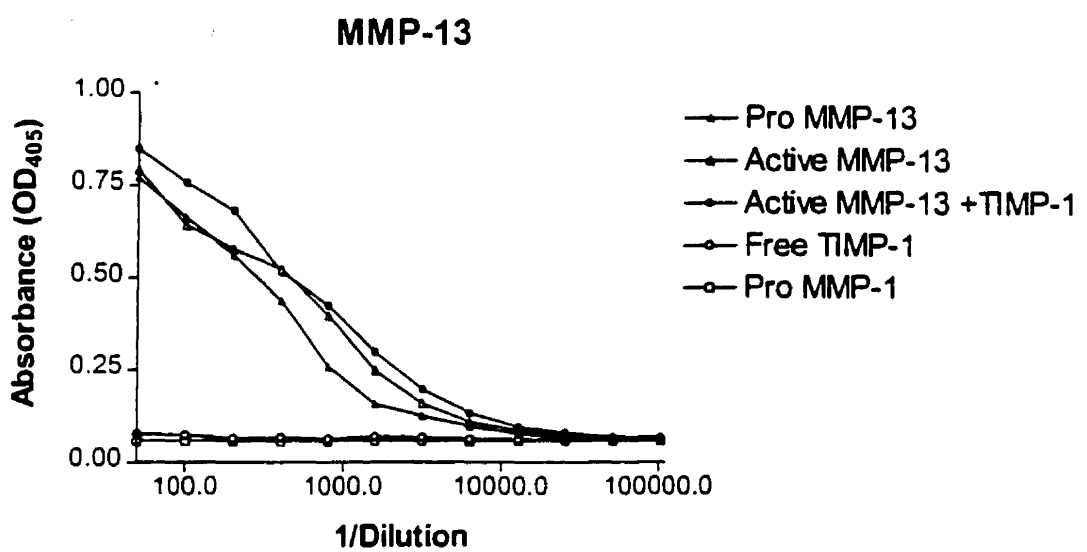

MMP-13
Absorbance(OD_{405})
1.00
0.75
0.50
0.25
0.00
10
100
1000
10000
1/dilution
Non-specific peptide
Immunizing peptide
BSA

FIG. 3.10 Immunoreactivities of MMP-1 and MMP-13 antiserum with human recombinant MMP-1 and MMP-13, as determined by direct-binding ELISA.

MMP-1 and MMP-13 antisera were tested against 100 ng/well of pro, actived or TIMP-1 complex forms (1:1 molar ratio, MMP: TIMP-1) of their respective human enzymes. All forms gave similar binding. Both antisera failed to recognize free TIMP-1 or the other MMP.


MMP-1
Absorbance (OD405)
1.00
0.75
0.50
0.25
0.00
100.0
1000.0
10000.0
100000.0
1/Dilution
Pro MMP-1
Active MMP-1
Active MMP-1 + TIMP-1
Free TIMP-1
Pro MMP-13


MMP-13
Absorbance (OD405)
1.00
0.75
0.50
0.25
0.00
100.0
1000.0
10000.0
100000.0
1/Dilution
Pro MMP-13
Active MMP-13
Active MMP-13 +TIMP-1
Free TIMP-1
Pro MMP-1

reacted with the other MMP and free TIMP-1 (Fig. 3.10).

3.6.2 Inhibition ELISAs for MMP-1 and MMP-13

Inhibition ELISAs for MMP-1 and MMP-13 were set up to test antigen recognition in solution. Both antisera recognized their respective pro, active and TIMP-1 complexed forms similarly (Fig 3.11a. and .Fig 3.12a). Both antisera failed to cross react with the other MMP.

3.6.2.1 Comparative reactivity of peptides and recombinant collagenases

Comparison of reactivity of peptides and recombinant collagenases on a molar basis, resulted in a similar inhibition curve (Fig. 3.11b and Fig. 3.12b) for both antisera.

3.6.3 Western blot analysis on MMP-1 and MMP-13 antisera

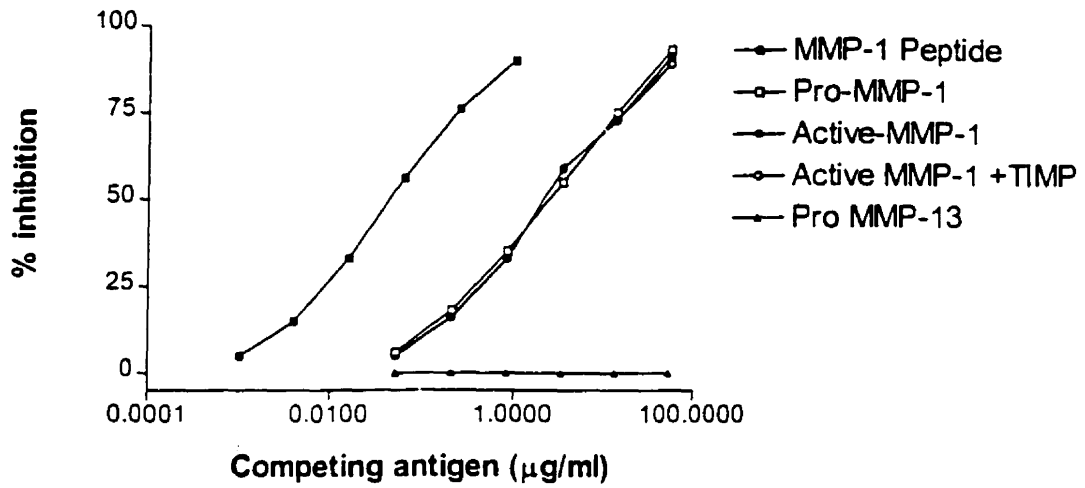
To test antisera specificities on Western blots, 100 ng of human recombinant pro MMP-1, pro MMP-8 and pro MMP-13 were electrophoresed on SDS-PAGE and transferred to nitrocellulose. Blots were probed with either MMP-1 or MMP-13 antisera (Fig. 3.13). Both antisera reacted only with their respective antigens. Note, both pro-MMP-1 and pro-MMP-13 undergo autolysis during storage, and both antisera reacted to active and autolytic fragments of these enzymes.

MMP-1 antisera were tested with conditioned medium from control and stimulated IL-1α bovine fetal skin fibroblasts cultures (Fig. 3.14) Antisera reacted to bovine pro-MMP-1 (60 kD) in the conditioned media from both the 1 and 10 ng IL-1α stimulated bovine fetal skin fibroblasts. Control fibroblasts were negative.

Fig. 3.11 Inhibition ELISA to show specificity of MMP-1 antiserum for different forms of MMP-1.

MMP-1 antiserum was tested against the immunizing peptide, pro, actived, or TIMP-1 complexed MMP-1 and pro MMP-13 (1:1 molar ratio, MMP:TIMP-1). All antigens exhibited similar inhibition curves except pro-MMP-13 which did not inhibit the antisera. Graph A: % inhibition vs competing antigen, μg/ml, Graph B: % inhibition vs competing antigen, μM.

A


100
75
50
25
0
% inhibition
0.0001
0.0100
1.0000
100.0000
Competing antigen (μg/ml)
MMP-1 Peptide
Pro-MMP-1
Active-MMP-1
Active MMP-1 +TIMP
Pro MMP-13

B

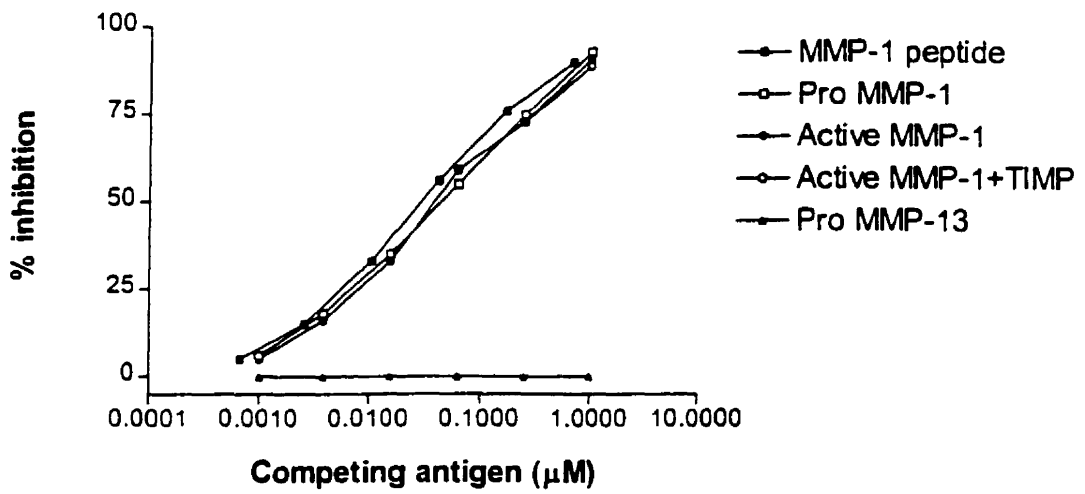
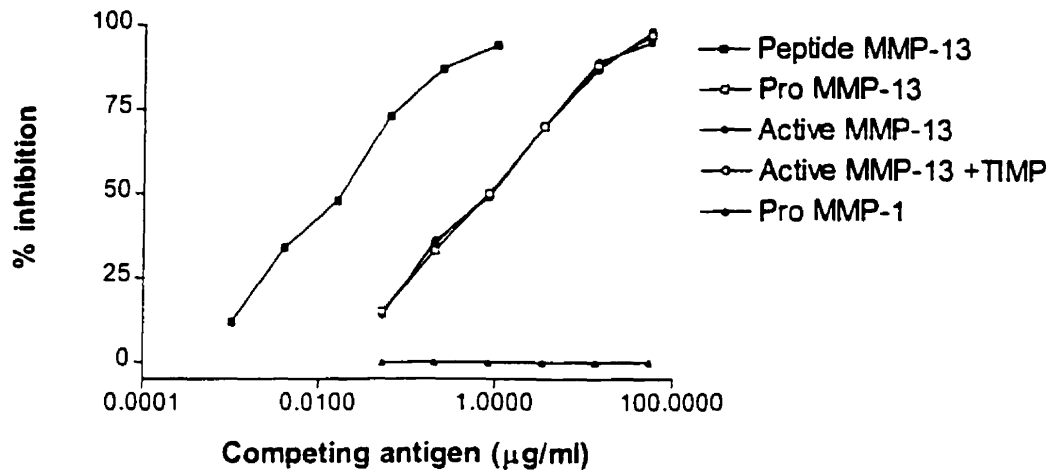

100
75
50
25
0
% inhibition
0.0001
0.0010
0.0100
0.1000
1.0000
10.0000
Competing antigen (μM)
MMP-1 peptide
Pro MMP-1
Active MMP-1
Active MMP-1+TIMP
Pro MMP-13

Fig. 3.12 Inhibition ELISA to show specificity of MMP-13 antiserum for different forms of MMP-13.

MMP-13 antiserum was tested against the immunizing peptide, pro, active, or TIMP-1 complexed MMP-13 and pro-MMP-1 (1:1 molar ratio, MMP:TIMP). All antigens exhibited similar inhibition curves except pro-MMP-1 which did not inhibit the antiserum. Graph A: % inhibition vs competing antigen, μg/ml, Graph B: % inhibition vs competing antigen, μM.

A


100
75
50
25
0
% inhibition
0.0001
0.0100
1.0000
100.0000
Competing antigen (μg/ml)
Peptide MMP-13
Pro MMP-13
Active MMP-13
Active MMP-13 +TIMP
Pro MMP-1

B

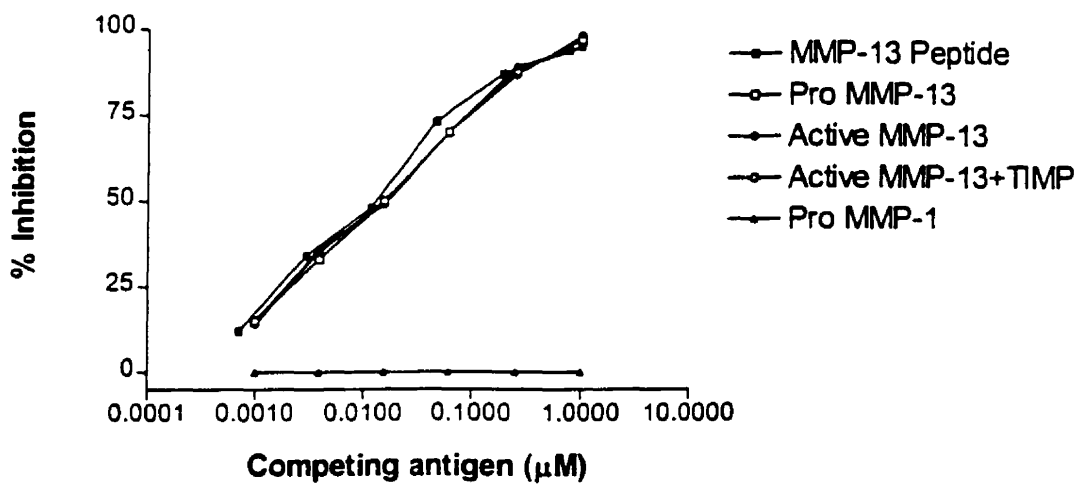
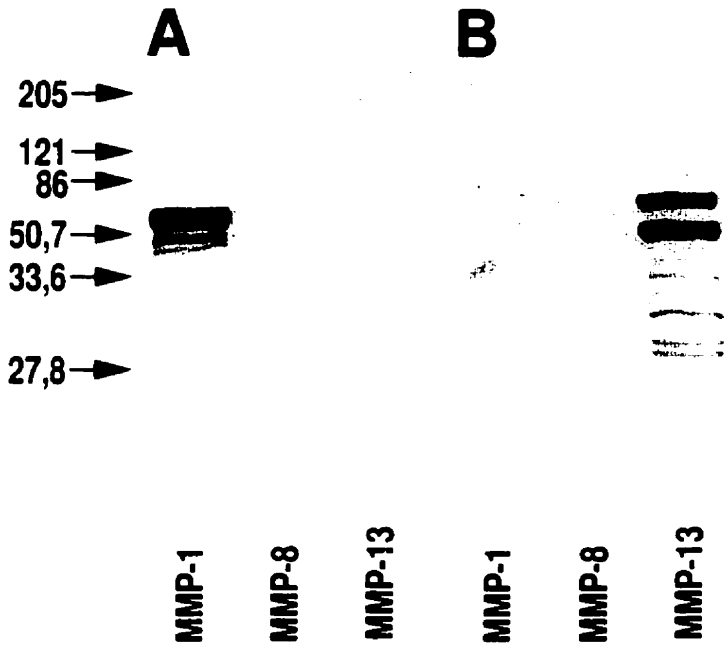

100
75
50
25
0
% Inhibition
0.0001
0.0010
0.0100
0.1000
1.0000
10.0000
Competing antigen (μM)
MMP-13 Peptide
Pro MMP-13
Active MMP-13
Active MMP-13+TIMP
Pro MMP-1

Fig. 3.13 Western blot analysis to show specificity of MMP-1 and MMP-13 antibodies

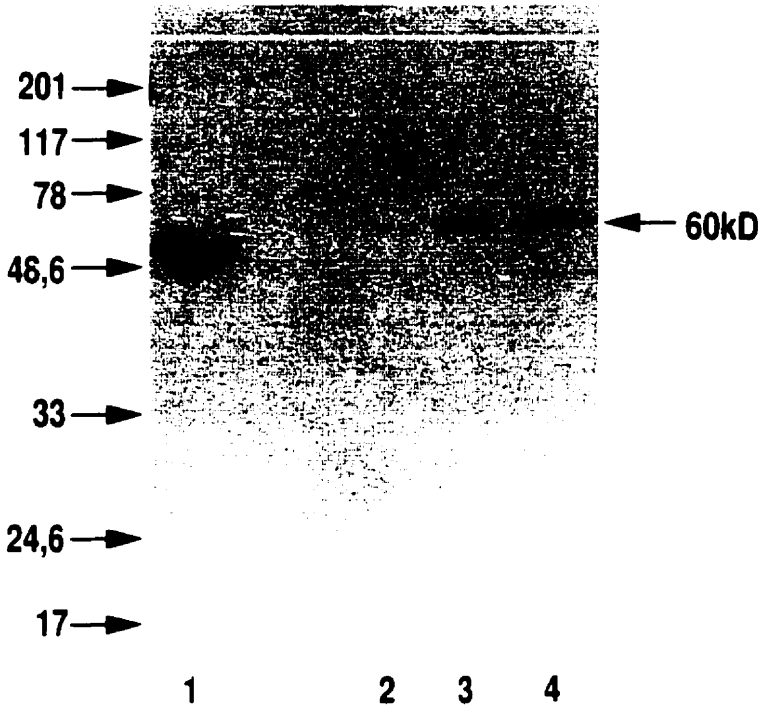
Human recombinant pro MMP-1, pro MMP-8, and pro MMP-13 were probed with either anti-MMP-1 (A) or anti-MMP-13 (B) antiserum. Both antisera bound only to their respective antigens. Both enzymes underwent autolysis during storage and therefore there are immunoreactive bands of these products. These recombinant enzymes are slightly smaller (pro MMP-1 = 55 kD, pro MMP-13 = 60 kD) in molecular weight than their native forms due to different glycosylation pattern in insect cells versus mammalian cells.



- 100ng of Human recombinant MMP-1, 8, 13 were loaded.
- Dilution of Antisera = 1:250

Fig. 3.14 Western blot analysis of MMP-1 in conditioned medium from IL-1α stimulated bovine fetal skin fibroblasts.

MMP-1 antiserum (1:250 dilution) was used to probe conditioned media from control (lane 2), 1 ng/ml IL-1α stimulated (lane 3), and 10 ng/ml IL-1α stimulated (lane 4) fetal bovine skin fibroblasts. Fibrobasts were stimulated for 24 hr. Lane 1 contains 100 ng of recombinant human pro MMP-1. Native enzyme is larger than recombinant due to differences in glycosylation in insect cells versus mammalian cells.


201
117
78
46,6
33
24,6
17
60kD
1
2
3
4

3.7 Cloning of bovine MMP-13 (Collagenase-3)

Full length bovine MMP-1 and MMP-13 cDNAs were obtained by PCR (Fig. 3.15). cDNAs were cloned into a commercial cloning kit (pCR-Script SK (+) which allowed quick sub-cloning and sequencing. The complete coding sequence was obtained by DNA sequencing using specific primers as shown in Table 2.2 or the M13 universal primers.

3.7.1 Sequence analysis of bovine MMP-13 cDNA and protein

The complete cDNA sequence obtained by sequencing of PCR products is shown in Fig. 3.16. The deduced protein sequence is also shown in Fig. 3.16. Sequence alignment of MMP-13 cDNAs (Fig 3.17) showed bovine MMP-13 cDNA to have 92% sequence homology to human MMP-13 cDNA and 82% homology to mouse and rat MMP-13 cDNAs. Sequence alignment of MMP-13 protein sequences (Fig 3.18) showed bovine MMP-13 protein to have 90% sequence homology to human MMP-13 protein and 85% homology to mouse and rat MMP-13 protein.

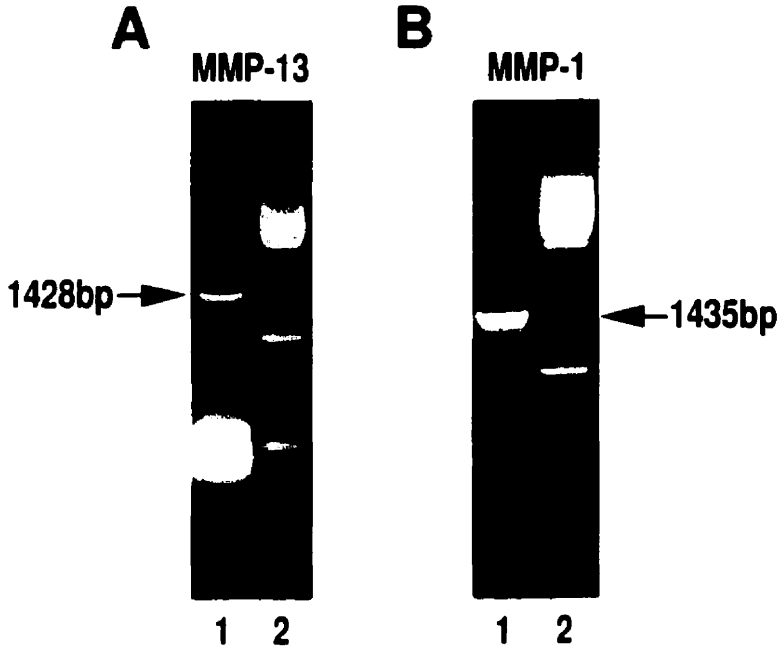
Comparison of bovine MMP-1 and MMP-13 cDNA sequences showed only a 28% homology (Fig. 3.19). Comparison of bovine MMP-1 and MMP-13 protein sequences showed a homology of 48% (Fig. 3.20).

3.8 Analysis of MMP-1 and MMP-13 mRNA and protein expression in fetal growth plate chondrocyte cultures during maturation

3.8.1 PCR analysis

Type X collagen, MMP-1 and MMP-13 mRNA expression were examined

Fig. 3.15 Agarose gel of PCR products for full length bovine MMP-1 and MMP-13. cDNAs of MMP-1 and MMP-13 that were generated by PCR (conditions from tables 2.2 and 2.3) were electrophoresed on a 1% Tris/acetic acid/EDTA (TAE) agarose gel. Lane 1 (PCR product). Lane 2 (100 bp ladder). MMP-13 (A). MMP-1 (B)


A
MMP-13
1428bp
1 2
B
MMP-1
1435bp
1 2

```
ATGCACCCAAGGGTCCTGGCTGGCTTCCTCTTCTTCAGCTGGACGGCTTGTTGGTCTCTG
M  H  P  R  V  L  A  G  F  L  F  F  S  W  T  A  C  W  S  L
CCCCTTCCCAGTGATGGAGATTCTGAAGACTTGTCCGAGGAAGACTTCCAGTTTGCAGAG
P  L  P  S  D  G  D  S  E  D  L  S  E  E  D  F  Q  F  A  E
AGCTACCTGAAATCATACTACTATCCTCAGAATCCTGCTGGAATCCTGAAGAAAACTGCA
S  Y  L  K  S  Y  Y  Y  P  Q  N  P  A  G  I  L  K  K  T  A
GCAAGCTCTGTGATTGACAGGCTTAGAGAAATGCAGTCATTTTTTGGCTTAGAGGTGACT
A  S  S  V  I  D  R  L  R  E  M  Q  S  F  F  G  L  E  V  T
GGCAGACTTGATGATAACACCTTAGACATCATGAAAAAACCAAGATGTGGGGTCCCTGAT
G  R  L  D  D  N  T  L  D  I  M  K  K  P  R  C  G  V  P  D
GTGGGTGAATACAATGTTTTTCCTCGAACTCTCAAGTGGTCCAAAATGAACTTAACCTAC
V  G  E  Y  N  V  F  P  R  T  L  K  W  S  K  M  N  L  T  Y
AGAATTGTGAATTATACACCTGATCTGACTCATTCTGAAGTGGAAAAGGCCTTCAGAAAA
R  I  V  N  Y  T  P  D  L  T  H  S  E  V  E  K  A  F  R  K
GCCTTCAAGGTGTGGTCTGATGTGACCCCTCTGAATTTTACCAGAATTCACAATGGCACT
A  F  K  V  W  S  D  V  T  P  L  N  F  T  R  I  H  N  G  T
GCTGATATCATGATCTCTTTTTGGAACTAAAGAGCATGGTGACTTCTACCCATTTGATGGA
A  D  I  M  I  S  F  G  T  K  E  H  G  D  F  Y  P  F  D  G
CCCTCTGGTCTGTTGGCTCACGCTTTCCCTCCTGGACCAAATTATGGAGGAGATGCTCAT
P  S  G  L  L  A  H  A  F  P  P  G  P  N  Y  G  G  D  A  H
TTTGATGATGATGAAACCTGGACAAGTAGTTCCAAAGGCTACAACTTGTTTCTTGTTGCT
F  D  D  D  E  T  W  T  S  S  K  G  Y  N  L  F  L  V  A
GCCCATGAGTTTGGCCATTCCTTAGGATTGGACCACTCCAAGGACCCAGGAGCACTCATG
A  H  E  F  G  H  S  L  G  L  D  H  S  K  D  P  G  A  L  M
TTTCCCATCTACACCTACACTGGCAAAAGCCACTTTATGCTTCCTGATGATGATGTCCAA
F  P  I  Y  T  Y  T  G  K  S  H  F  M  L  P  D  D  D  V  Q
GGGATTCAGTCTCCTCTATGGTCCAGGAGATGAAGACCCCTACTCTAAACATCCCAAAACG
G  I  Q  S  L  Y  G  P  G  D  E  D  P  Y  S  K  H  P  K  T
CCAGACAAATGTGACCCTTCCTTATCCCTTGATGCCATAACCAGTCTCCGCGGAGAAACA
P  D  K  C  D  P  S  L  S  L  D  A  I  T  S  L  R  G  E  T
CTGATCTTTAAAGACAGATTCTTCTGGCGGCTGCATCCTCAGCAGGTTGAAGCAGAGCTG
L  I  F  K  D  R  F  F  W  R  L  H  P  Q  Q  V  E  A  E  L
TTTTTAACAAAATCGTTTGGGCCAGAACTTCCCAACCGTATTGATGCCGCCTATGAGCAC
F  L  T  K  S  F  W  P  E  L  P  N  R  I  D  A  A  Y  E  H
CCTTCCCATGACCTTATCTTCATCTTTAGAGGCAGAAAATTTTGGGCACTTAGTGGTTAT
P  S  H  D  L  I  F  I  F  R  G  R  K  F  W  A  L  S  G  Y
GATATTCTGGAAGATTACCCAAAAAAAAATATCTGAACTGGGATTTCCAAAACACGTTAAA
D  I  L  E  D  Y  P  K  K  I  S  E  L  G  F  P  K  H  V  K
AAGATCAGCGCAGCCCTTCACTTTGAGGATTCAGGGAAGACGCTCTTCTTTTCAGAAAAC
K  I  S  A  A  L  H  F  E  D  S  G  K  T  L  F  F  S  E  N
CAAGTCTGGAGCTATGATGACACTAACCATGTGATGGATAAAGACTACCCGAGACTCATT
Q  V  W  S  Y  D  D  T  N  H  V  M  D  K  D  Y  P  R  L  I
GAAGAGGTCTTCCCAGGAATTGGTGATAAAGTAGATGCTGTCTACCAGAAAAATGGTTAT
E  E  V  F  P  G  I  G  D  K  V  D  A  V  Y  Q  K  N  G  Y
ATCTATTTTTTCAATGGACCCATACAGTTTGAATACAGCATCTGGAGTAACCGTATTGTT
I  Y  F  F  N  G  P  I  Q  F  E  Y  S  I  W  S  N  R  I  V
CGAGTCATGACAACAAATTCCTTATTGTGGTGTTAAAAGTGTTTTTAAAATATTGTTATT
R  V  M  T  T  N  S  L  L  W  C
TAAATCCTGGAAAGCATTTGAGATAACACTTCCAGACATTTGGGGGGTGAGTTATGGGAG

AAGGAGAGATATCGGGGGTTCTGTGAACAAGCTTCAGTAAGTTATCTTTTGAATATGCAG

TATCTATATGACTATGCGTGGCTGGAACCACAT
```

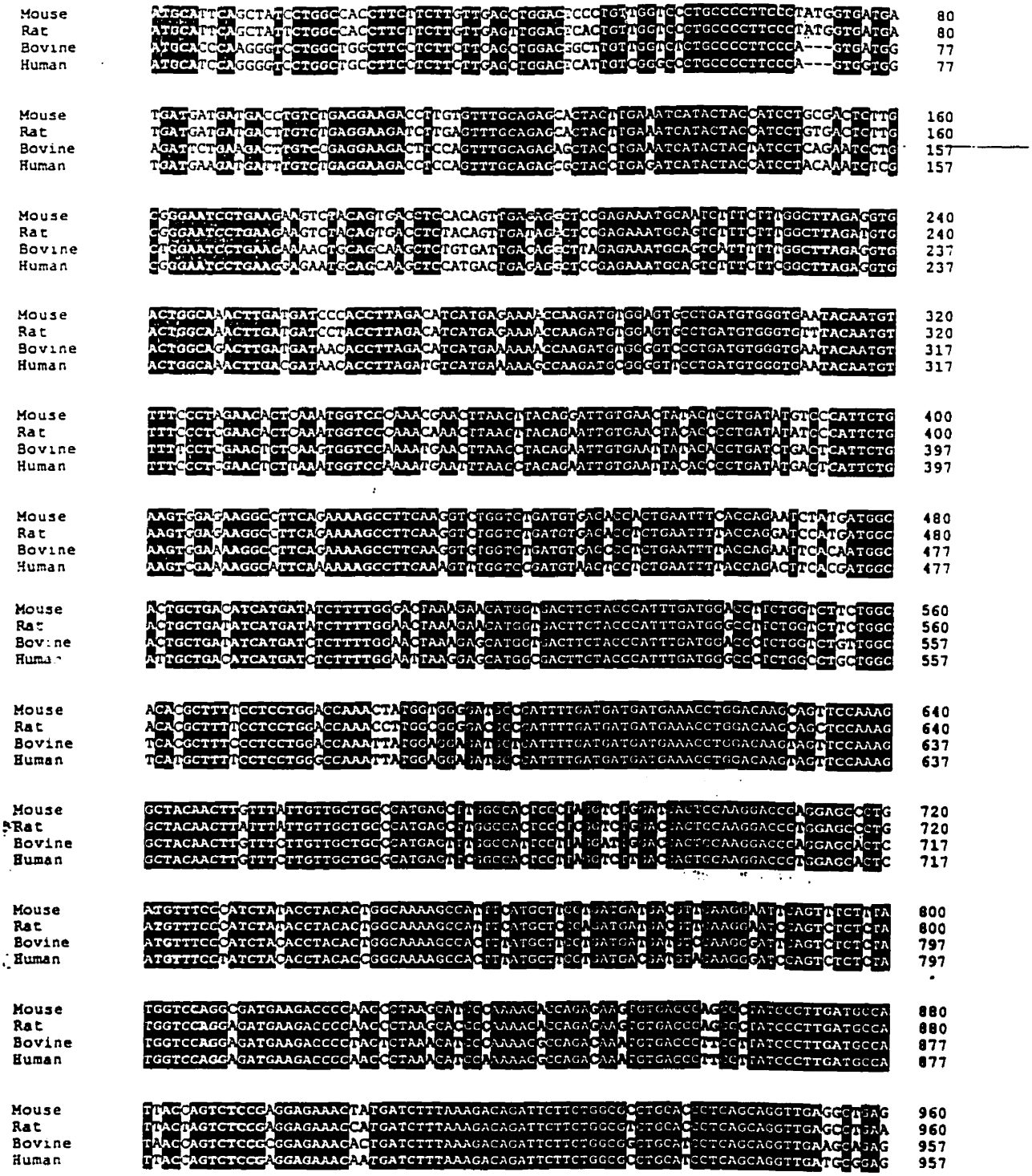
Fig. 3.16 Complete coding sequence and deduced amino acid sequence for bovine MMP-13.

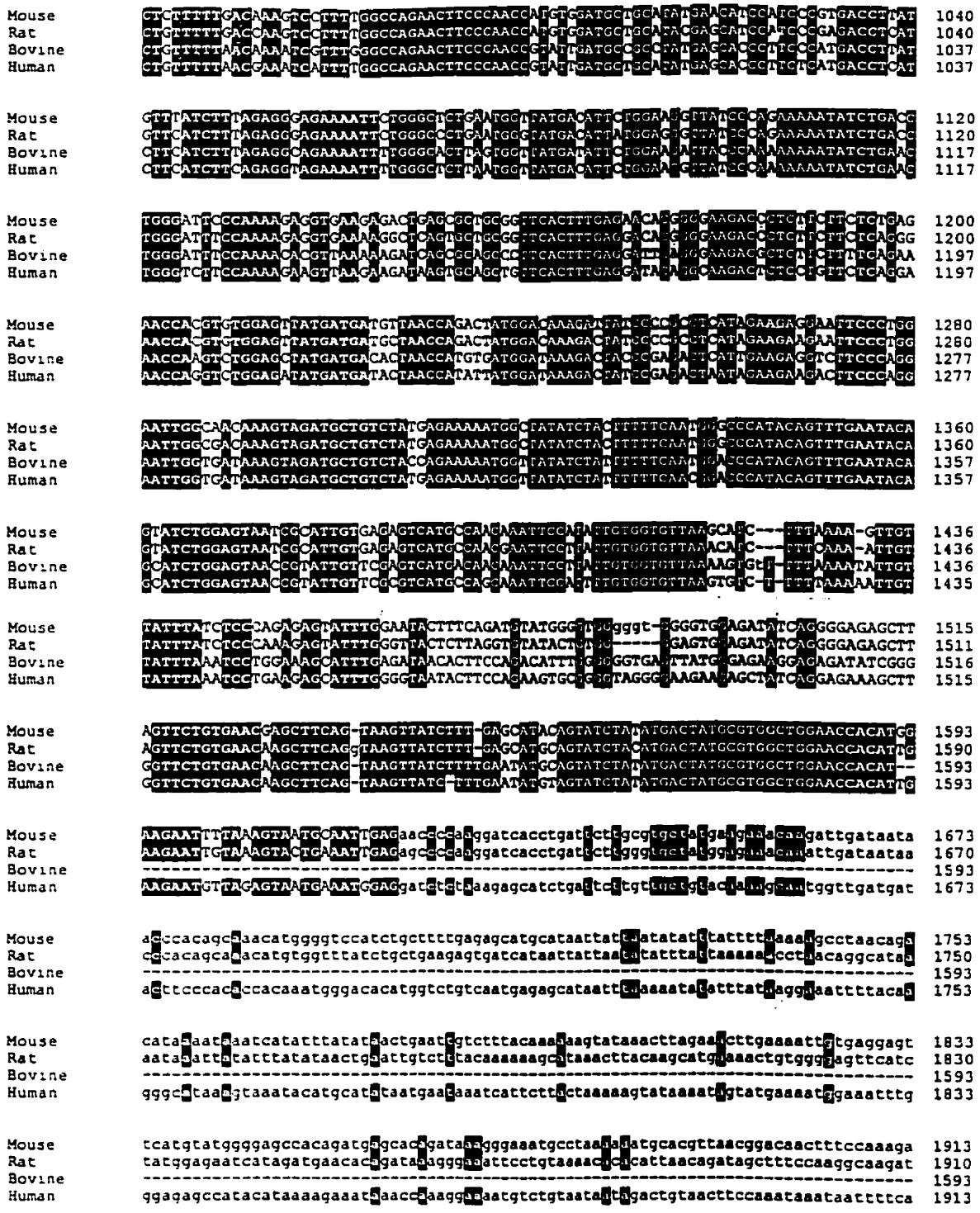
Fig. 3.17 Alignment of mammalian MMP-13 cDNA sequences.

Uppercase letters represent regions of homology. Shaded areas are identical nucleotides. The sequences are from Freije *et al.*, 1994 for human MMP-13; Quinn *et al.*, 1990 for rat MMP-13; and Henriet *et al.*, 1992 for mouse MMP-13.

Collagenase-3 (MMP-13)

Alignment




Mouse
Rat
Bovine
Human

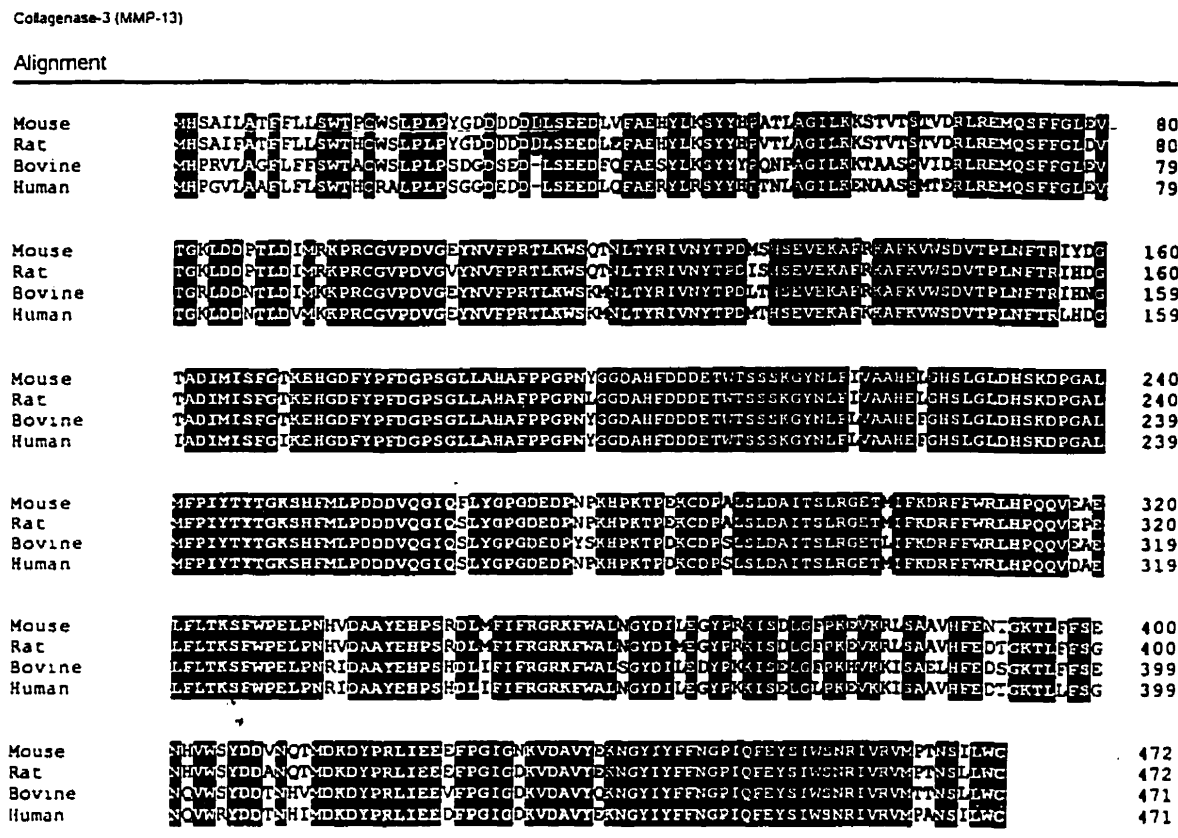


Fig. 3.18 Alignments of mammalian MMP-13 amino acid sequences.

Uppercase letters represent region of homology. Shaded area are identical amino acids. The sequences are from Henriet *et al.*, 1992 for mouse MMP-13; Quinn *et al.*, 1990 for rat MMP-13 and Freije *et al.*, 1994 for human MMP-13.

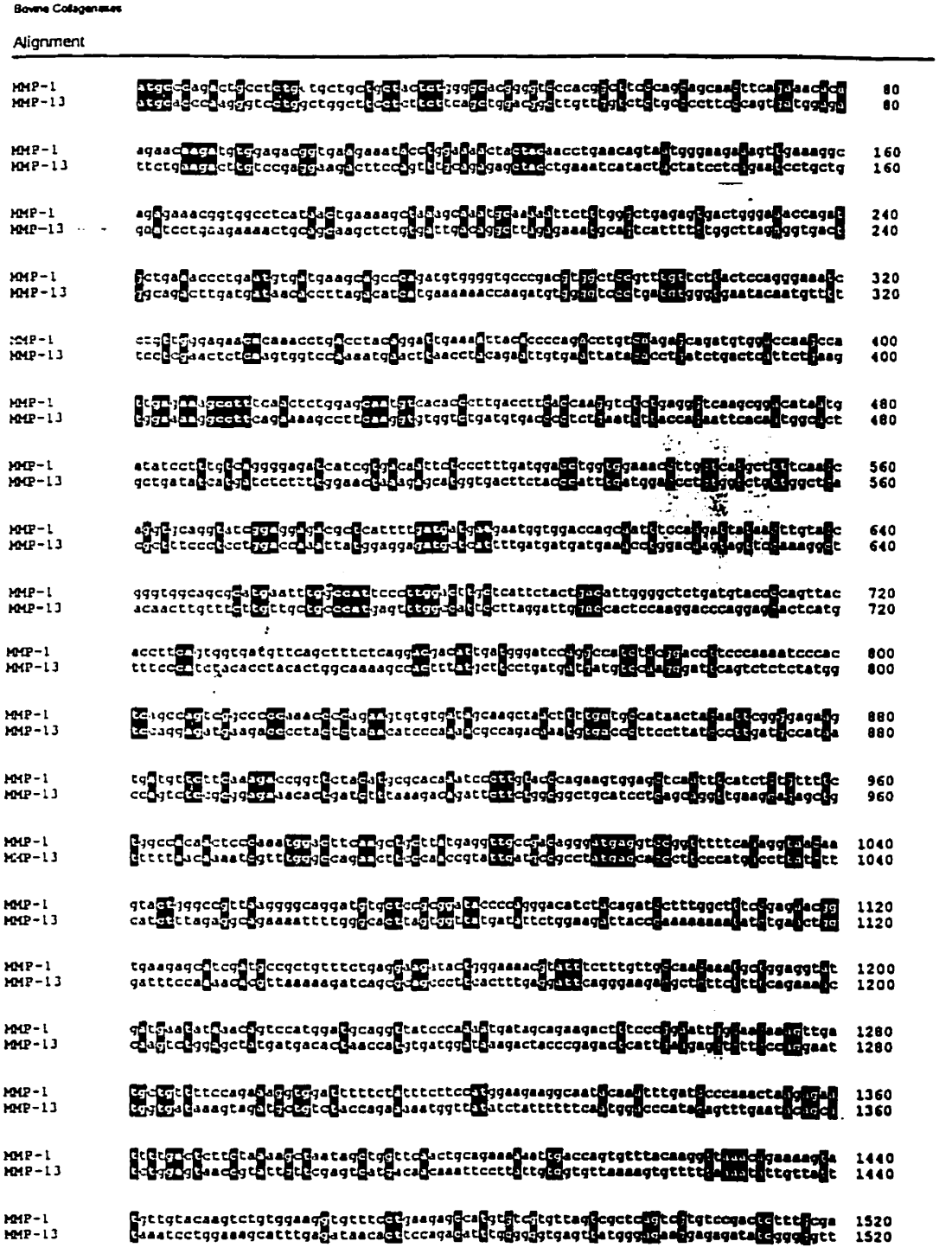


Fig. 3.19 Alignments of bovine MMP-1 and MMP-13 cDNA sequences.

Shaded area represent identical nucleotides. The bovine MMP-1 sequence is from Tamura *et al.*, 1994.

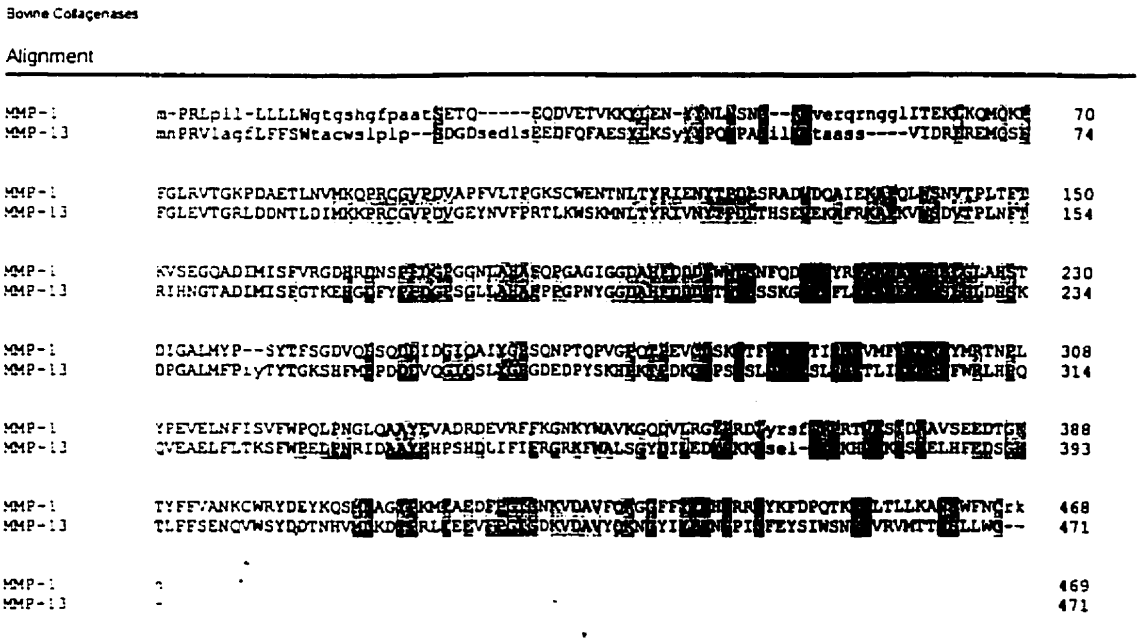


Fig. 3.20. Alignment of bovine MMP-1 and MMP-13 protein sequences.

Uppercase characters represent regions of homology. Shaded areas represent identical amino acid residues. The bovine MMP-1 sequence is from Tamura *et al.*, 1994.

by PCR using reversed transcribed total RNA from hypertrophic fetal bovine growth plate chondrocytes and IL-1α stimulated fetal bovine skin fibroblasts (Fig. 3.21). MMP-13 mRNA was only expressed in type X collagen expressing chondrocytes (C subpopulation, fetal age = 206 days). MMP-1 was expressed only in IL-1α stimulated fetal bovine skin fibroblasts.

3.8.2 Northern blot analysis

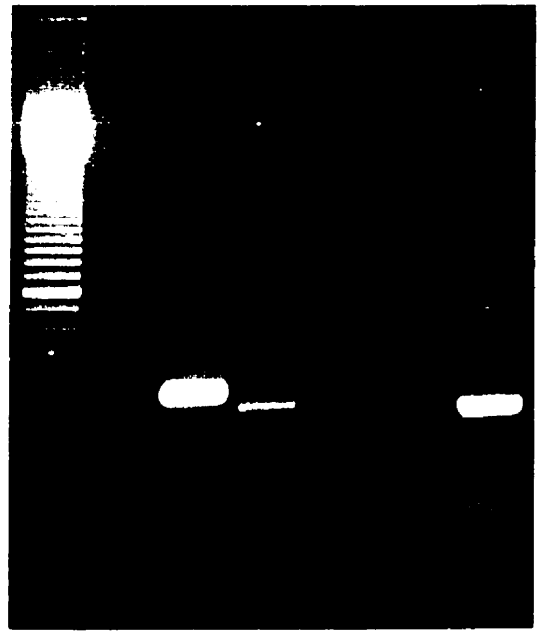
Total RNA from cultured bovine growth plate C subpopulation chondrocytes (fetal age = 206 days) was probed with MMP-1 and MMP-13 cDNAs (Fig. 3.22). MMP-13 mRNA expression was only seen when cells synthesized type X collagen, days 12-14, and increased till days 20-22 . MMP-1 was expressed only in IL-1α stimulated fetal bovine skin fibroblasts. MMP-13 mRNA showed two transcript sizes of 2.8 and 2.0 kb. This is consistent with human MMP-13 mRNA seen in human adult cartilage (Mitchell *et al.*, 1996; Reboul *et al.*, 1996).

3.8.3 Western blot and immunoprecipitation

Conditioned media (500 µl) from cultured bovine growth plate C subpopulation chondrocytes (fetal age = 206 days) were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were probed with either anti-MMP-1 or anti-MMP-13 affinity purified IgG (1:250 dilution) (Fig. 3.23). Only anti-MMP-13 affinity purified IgG showed a positive reaction to a band of 65 kD that corresponds to pro MMP-13 at 16-18 and 18-20 days of culture. Cells first synthesized type X collagen at 12-14 days. There was also a band for MMP-

Fig. 3.21 PCR analysis of MMP-1, MMP-13 and type II collagen mRNA in bovine fetal growth plate chondrocytes and fibroblasts.

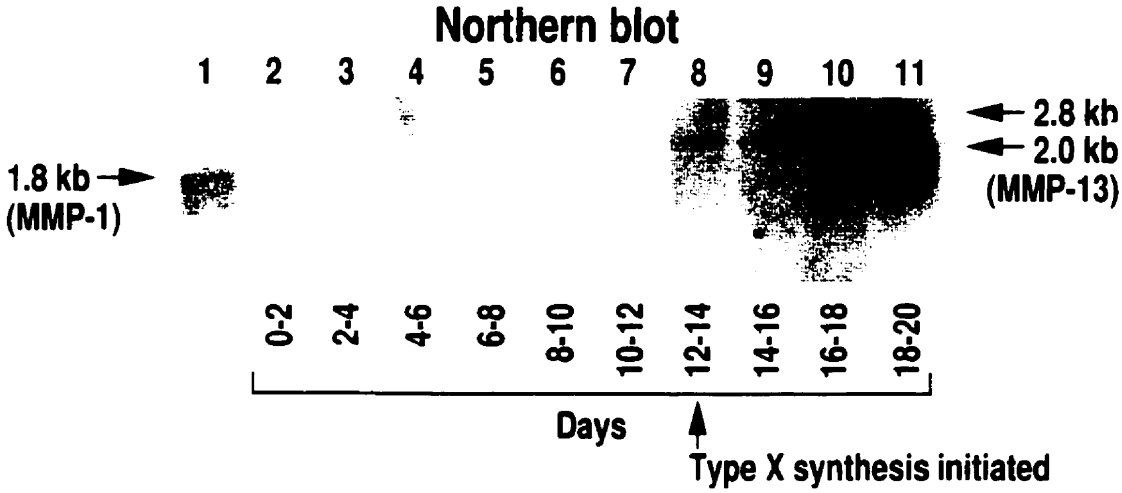
PCR products were analyzed on a Tris/acetic acid/EDTA (TAE) 1% agarose gel. MMP-1 mRNA expression is only detectable in IL-1α stimulated fibroblasts (1 ng/ml for 24 hr). Type X collagen and MMP-13 mRNA are expressed only in hypertrophic chondrocytes (C subpopulation, days 16-18 in culture, fetal age = 206 days).



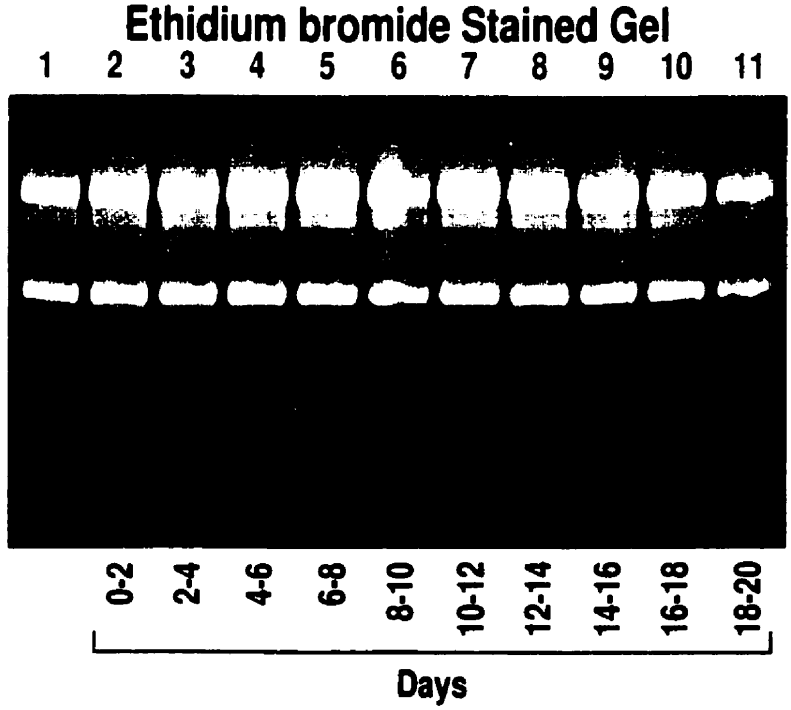
Lane 1: 100 b.p. DNA ladder
Lanes 2-3: Type X collagen 337 bp
Lanes 4-5: MMP-1 295 bp
Lanes 6-7: MMP-13 291 bp
Lanes 2, 4, 6: (IL-1α stimulated fetal bovine skin fibroblasts)
Lanes 3, 5, 7: (Fetal bovine growth plate chondrocyte C subpopulation, days 16-18)

Fig. 3.22 Northern blot of MMP-1 and MMP-13 expression during maturation in culture of bovine fetal growth plate chondrocyte C subpopulation.

IL-1α (1 ng/ml, 24 hr) stimulated bovine fetal fibroblasts are positive for MMP-1 mRNA. MMP-1 mRNA is undetectable in bovine fetal growth plate chondrocyte cultures during maturation. MMP-13 mRNA expression is first detected on days 12-14 and increases until days 18-20. This is also the time period when cells are synthesizing type X collagen (Fig. 3.5). Bovine MMP-13 mRNA showed two transcript sizes of 2.0 and 2.8 kb. Total RNA was extracted from bovine fetal growth plate chondrocyte C subpopulation every 2 days. 5 μg of RNA was loaded per lane. The membrane was probed with ^{32}P-labeled MMP-1 (295 bp) and MMP-13 (291 bp) cDNAs. The blot was exposed for 24 hr. (Fetal age = 206 days)



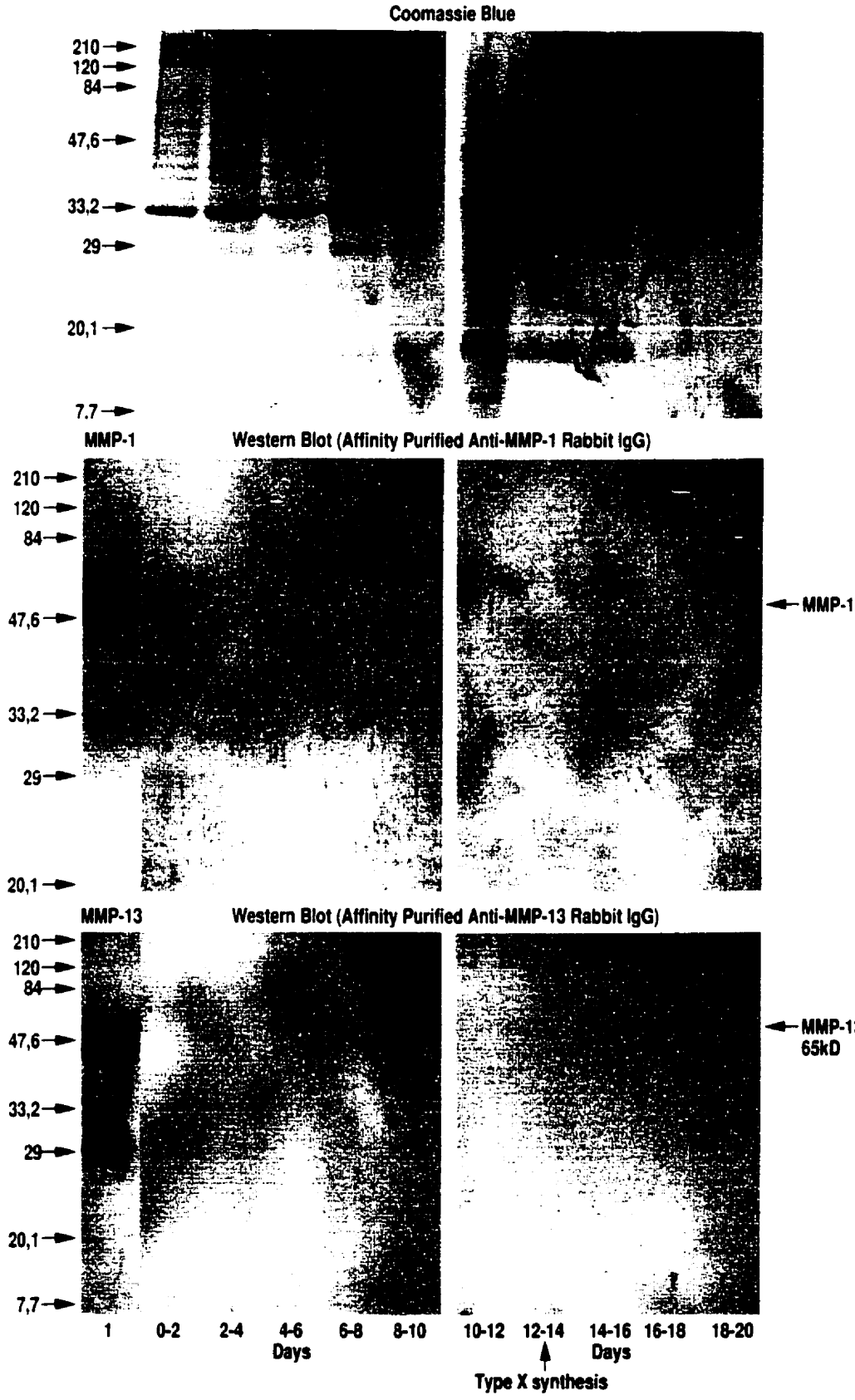
Membrane was probed with cDNAs of bovine MMP-1 + 13
MMP-1 mRNA = 1.8 kb
MMP-13 mRNA = 2.0, 2.8 kb
Exposure = 24 hr



Lane 1: IL-1α (10 U/ml) stimulated bovine fetal skin fibroblasts
Lanes 2-11: C subpopulation fetal growth plate chondrocytes
5 μg total RNA loaded

Fig. 3.23 Western blot of MMP-1 and MMP-13 protein during maturation of bovine fetal growth plate chondrocyte C subpopulation.

Conditioned medium was collected every 2 days from growth plate chondrocyte cultures. Conditioned media (500 μl) were precipitated with 2 volumes of 100% ethanol and analyzed by SDS-PAGE. Proteins were stained with Coomassie blue or blotted onto nitrocellulose and probed with anti-MMP-1 and MMP-13 affinity purified IgG. Recombinant MMP-1 or MMP-13 (Lane 1). MMP-1 protein is undetectable throughout the culture period in bovine fetal growth plate chondrocyte C subpopulation. MMP-13 is detected at days 16-18 and 18-20. Type X synthesis is shown in Fig. 3.5. (Fetal age = 206 days)


Coomassie Blue
210
120
84
47,6
33,2
29
20,1
7,7
MMP-1
Western Blot (Affinity Purified Anti-MMP-1 Rabbit IgG)
210
120
84
47,6
33,2
29
20,1
MMP-1
MMP-13
Western Blot (Affinity Purified Anti-MMP-13 Rabbit IgG)
210
120
84
47,6
33,2
29
20,1
7,7
MMP-13
65kD
1
0-2
2-4
4-6
6-8
8-10
10-12
12-14
14-16
16-18
18-20
Days
Days
Type X synthesis

13 at 30 kD at days 18-20. This band is probably an autolytic fragment of MMP-13.

Immunoprecipitation was performed on conditioned medium from bovine growth plate C subpopulation chondrocytes (fetal age = 206 days) cultured with ^{35}S methionine. pro MMP-13 protein (65 kD) was detected in medium of cells that synthesized type X collagen (Fig. 3.24 b and d). There are also smaller bands around 30 kD at days 12-14, 14-16 and 16-18 which are probably an autolytic fragment of MMP-13 (Fig 3.24b). There is also a 200 kD protein that is precipitated throughout the culturing period (Fig. 3.24 b and c). This is probably a non-specific reaction with fibronectin. MMP-1 protein was only immunoprecipitated from IL-1α stimulated bovine fetal skin fibroblast ^{35}S methionine-labeled conditioned medium.

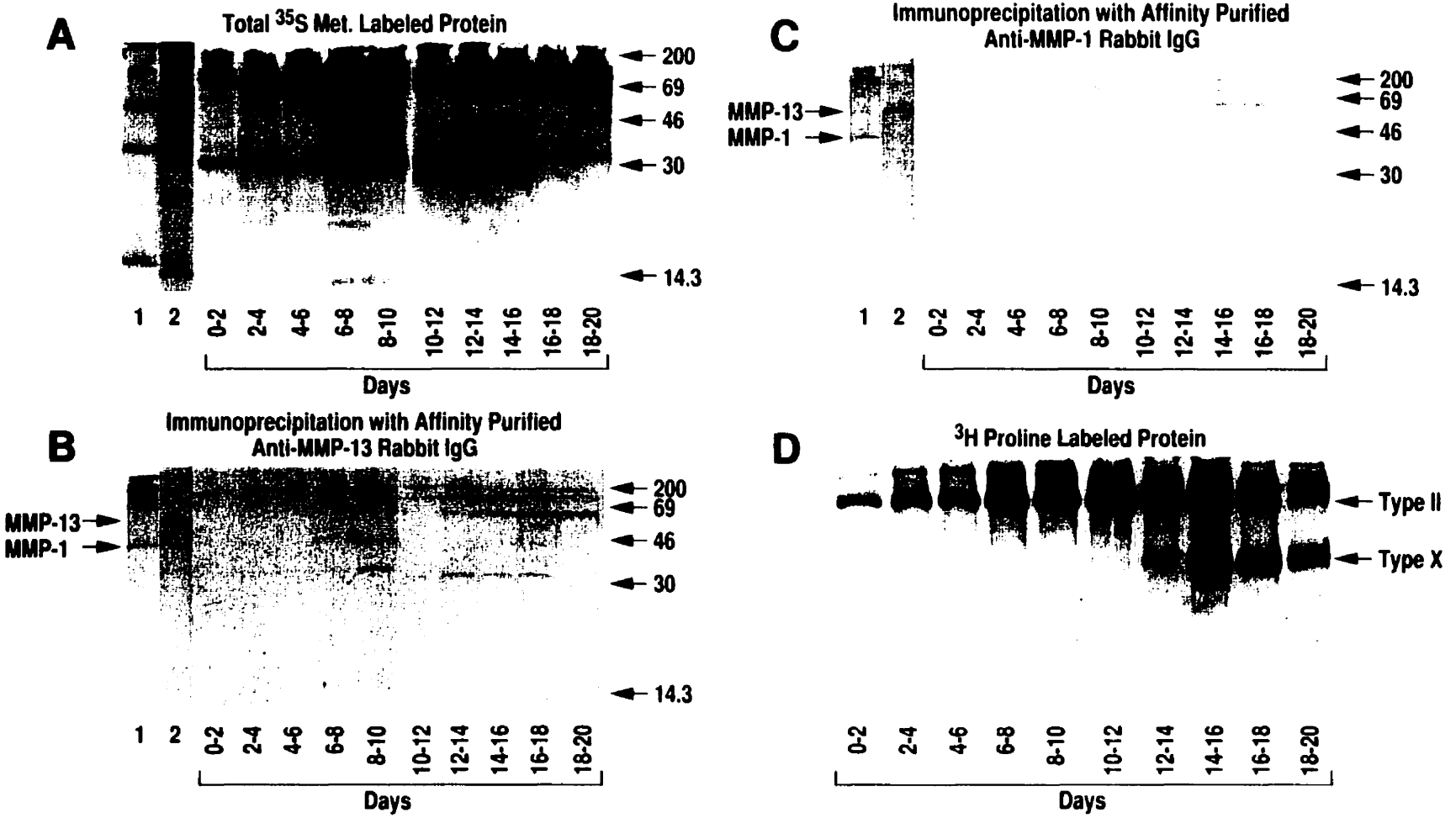
MMP-13 antisera was generated against a conserved epitope found in mouse, rat and human MMP-13. This epitope: PNPKHPKTPEK, is slightly different in bovine, PYSKHPKTPDK. There are 7/11 residues that are identical, which still confer reactivity of antiserum against bovine MMP-13.

3.8.4 Collagenase activity during fetal growth plate chondrocyte maturation

Total and active collagenase activity was measured in media from bovine fetal growth plate C subpopulation chondrocytes (fetal age = 206 days) grown in culture (Fig 3.25). Total and active collagenase activity was first detected when cells synthesized type X collagen (Fig. 3.24d). Both total and active collagenase activity increased as chondrocytes matured. Collagenase activity was present only

Fig. 3.24 Immunoprecipitation of newly synthesized MMP-1 and MMP-13 during maturation of bovine fetal growth plate chondrocyte C subpopulation

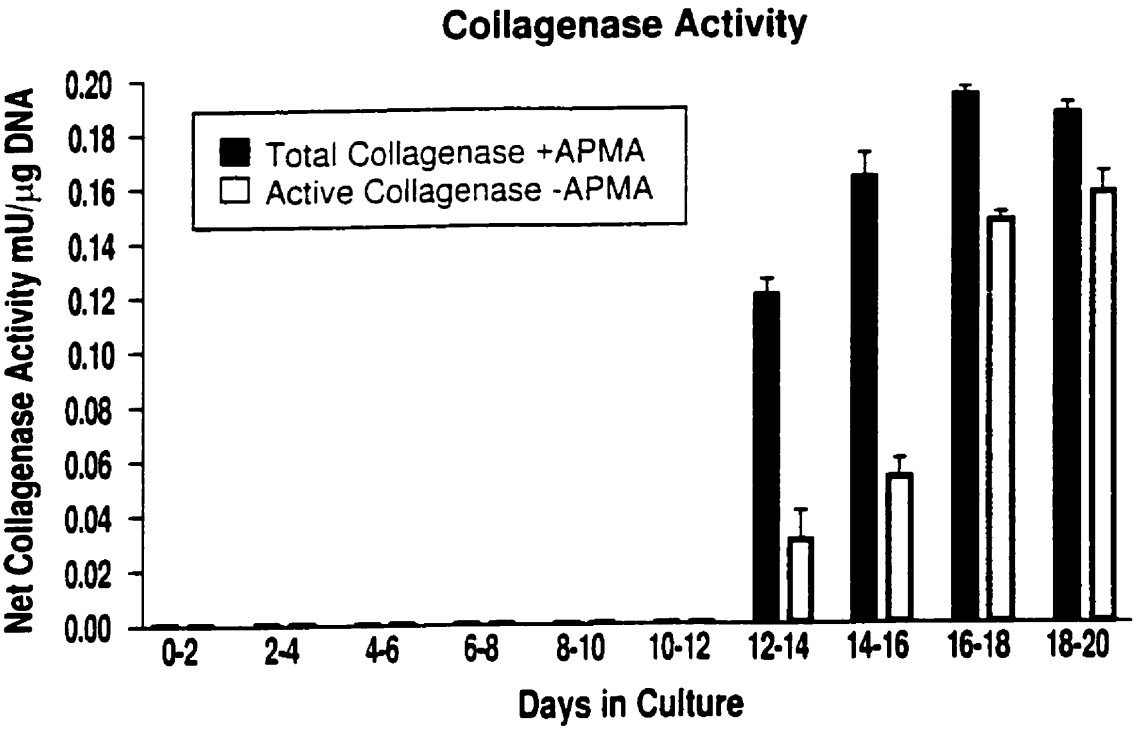
Bovine fetal growth plate chondrocyte C subpopulation conditioned media were labeled with ^{35}S-methionine for 48 hr and changed every 2 days. 500 µl of conditioned medium was used for analysis. Proteins were analyzed by SDS-PAGE and visualized by autoradiography. Total protein synthesis (A). Medium was immunoprecipitated with either affinity purified anti-MMP-13 IgG (B) or MMP-1 (C) IgG. IL-1 α stimulated (1 ng/ml, 24 hr) mouse chondrocyte line MC615 was used as a positive control for MMP-13 IgG (Lane 2, conditioned medium was immnunoprecipitated with anti-MMP-13 IgG only). IL-1 α (1 ng/ml, 24 hr) stimulated fetal bovine skin fibroblasts was used as a positive control for MMP-1 IgG (Lane 1, conditioned medium was immnunoprecipitated with anti-MMP-1 IgG only). ^{3}H proline incorporation into collagens (D) revealed that cells first synthesized type X collagen and became hypertrophic at days 12-14.



Lane 1: IL-1α stimulated bovine fetal skin fibroblasts (10 ng/ml)
Lane 2: IL-1α stimulated mouse chondrocytes (10 ng/ml)
Lanes 3-12: C subpopulation bovine fetal growth plate chondrocytes

Fig. 3.25 Collagenase activity during maturation of bovine fetal growth plate chondrocyte C subpopulation.

Conditioned medium was collected every 2 days from growth plate chondrocyte C subpopulation cultures. Condition media (100 µl) were added to ^{14}C-labeled human type II collagen and assayed for collagenase activity. Collagenase activity was detected at the same time as type X collagen synthesis (fig. 3.24d) and MMP-13 protein synthesis (fig. 3.23 and 3.24b). There was always more pro MMP-13 than active MMP-13 in the conditioned media.



1U collagenase = 1µg ^{14}C collagen digested/minute

in the medias that were first positive for MMP-13 protein as determined by Western blot (Fig 3.23) and immunoprecipitation (Fig. 3.24b). There was always more latent than active MMP-13 in the medium.

3.9 The effects of an inhibitor of MMP-13 activity on fetal growth plate chondrocytes during maturation

3.9.1 Cleavage of ^{14}C-labeled type II collagen by MMP-1, MMP-8 and MMP-13

Activated human recombinant MMP-1 (1 μg), MMP-8 (1 μg), and MMP-13 (100 ng) were incubated with ^{14}C-labeled collagen (Fig. 3.26). 1 nM and 10 nM RS 102,481 was added to some of the reaction mixtures to test inhibition. Only MMP-13 was inhibited by RS 102,481 at 1 nM and 10 nM. MMP-8 was slightly inhibited at 10 nM.. MMP-1 was not inhibited at either concentration. All collagenases produced the typical TC^A and TC^B fragments with time in the absence of the inhibitor.

3.9.2 Effects of inhibitor on type II collagen and type X collagen synthesis

Type X collagen synthesis was inhibited when fetal growth plate C subpopulation C chondrocytes (fetal age = 206 days) were cultured in the presence of 10 nM RS 102,481 (Fig. 3.27). Type X collagen was first synthesized in control cells at days 14-16 to 20-22 (Fig 3.27). In a separate experiment, (fetal age = 221 days) type X collagen synthesis was also shown to be inhibited by 1 nM as well as 10 nM RS 102,481 (Fig. 3.28). Type X collagen was first detected in controls cell conditioned media at days 8-10 and persisted to days 20-22 (Fig 3.28). Type II

Fig. 3.26 Time course of cleavage of triple helical human ^{14}C labeled type II collagen by recombinant human MMP-1, MMP-8 and MMP-13 with or without a synthetic preferential inhibitor of MMP-13, RS 102,481.

Purified ^{14}C-labeled type II collagen was digested with APMA-activated human recombinant MMP-1 (1 μg), MMP-8 (1 μg), and MMP-13 (100 ng), in the absence or presence of 1 or 10 nM concentrations of preferential MMP-13 inhibitor RS 102, 481 (Roche Biosciences). Proteins were analyzed by SDS-PAGE and visualized by autoradiography. Lane 1(+ 1 nM 102, 481, 1 hr). Lane 2 (+ 10 nM 102, 481, 1 hr). Lane 3-9 (without inhibitor) at times, 1 hr, 2 hr, 4 hr, 12 hr, 24 hr, 48 hr, 72 hr.

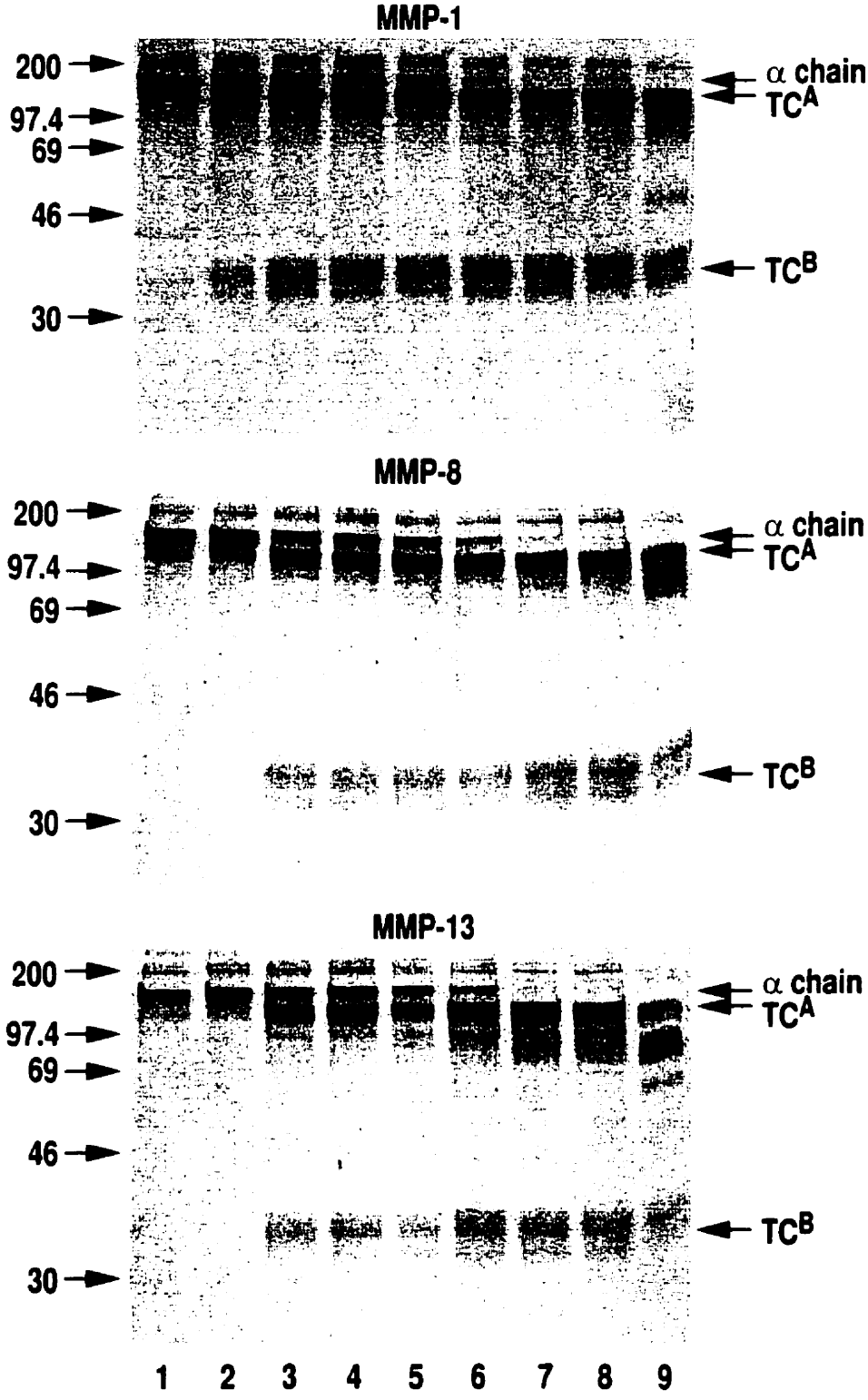

MMP-1
200
97.4
69
46
30
α chain
TC^A
TC^B
MMP-8
200
97.4
69
46
30
α chain
TC^A
TC^B
MMP-13
200
97.4
69
46
30
α chain
TC^A
TC^B
1 2 3 4 5 6 7 8 9

Fig. 3.27 The effect of RS 102,481, a synthetic preferential inhibitor of MMP-13, on collagen synthesis in bovine fetal growth plate chondrocytes C subpopulation during maturation.

^{3}H-proline labeled collagens were precipitated from conditioned medium (500 μl) every 2 days. Collagens were analyzed by SDS-PAGE and visualized by autoradiography. 10 nM of RS 102,481 inhibited type X collagen synthesis in C subpopulation cells as compared with controls. Type II collagen synthesis was unaffected. Inhibitor 102,481 was present throughout culture period. (Fetal age=206 days)

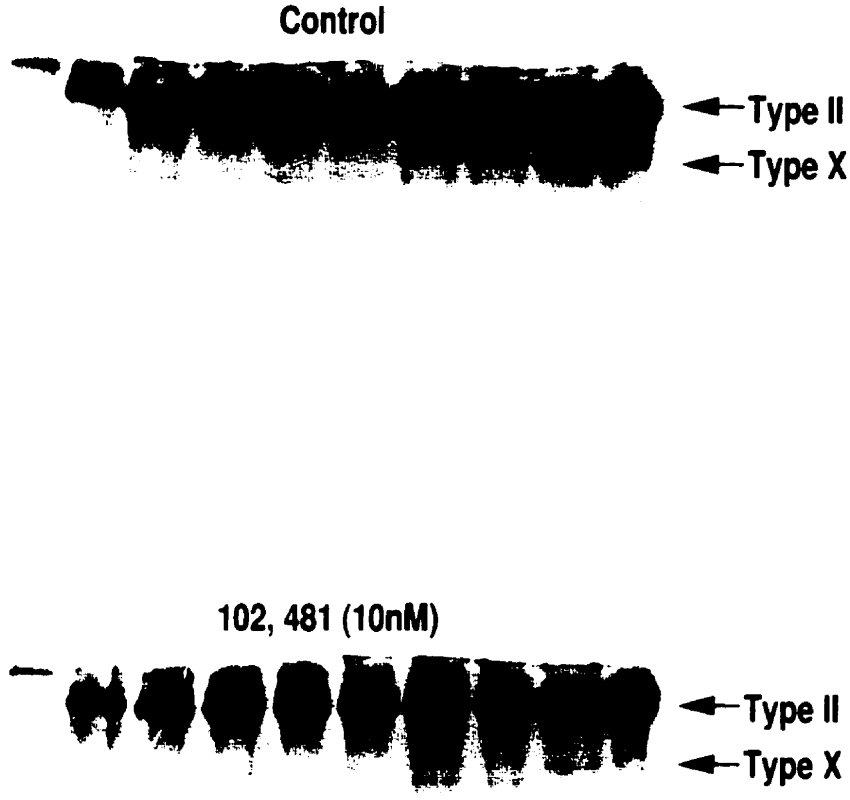

Control
Type II
Type X
102, 481 (10nM)
Type II
Type X
2-4
4-6
6-8
8-10
10-12
12-14
14-16
16-18
18-20
20-22
DAYS

Fig. 3.28 The effect of RS 102,481, a synthetic preferential inhibitor of MMP-13, on collagen synthesis in bovine fetal growth plate chondrocyte C subpopulation during maturation.

^{3}H-proline labeled collagens were precipitated from conditioned medium (500 μl) every 2 days. Collagens were analyzed by SDS-PAGE and visualized by autoradiography. At 1 and 10 nM of inhibitor, RS 102,481, inhibited type X collagen synthesis in C subpopulation cells as compared with control. Type II collagen synthesis was unaffected. 50 nM inhibited both types II and X collagen synthesis. (Fetal age =221 days)

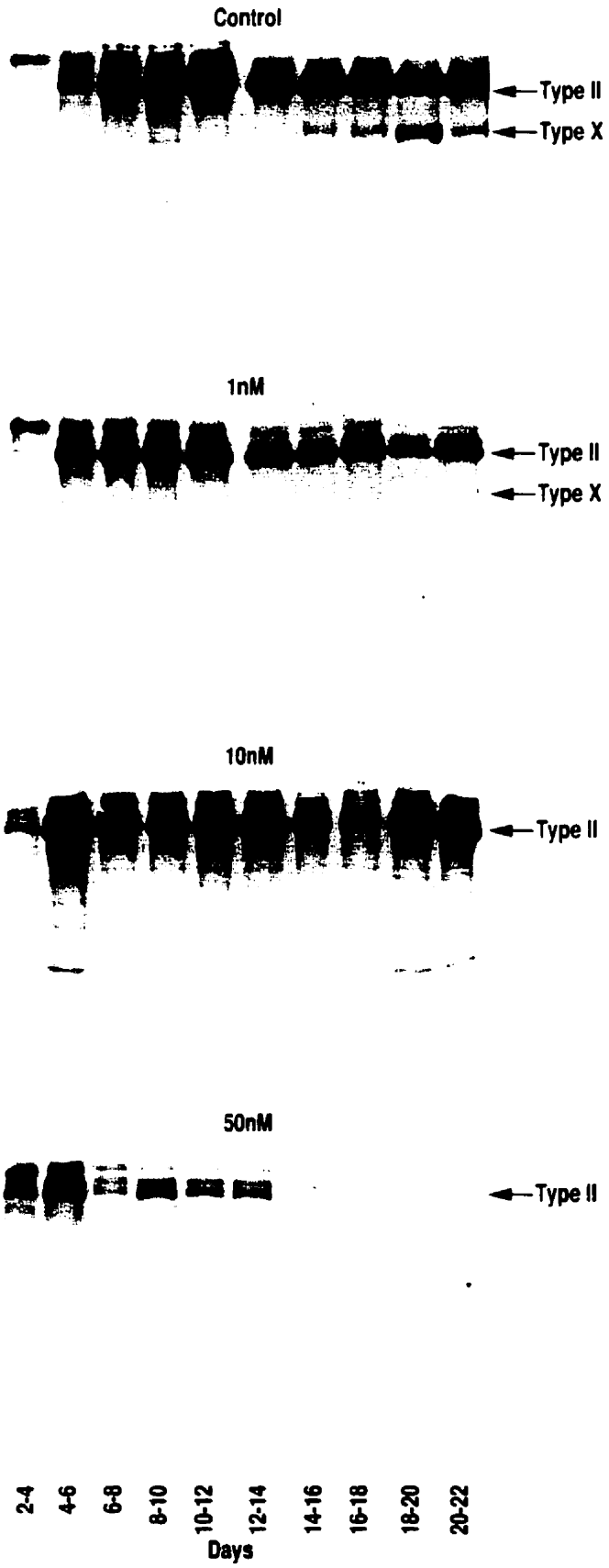
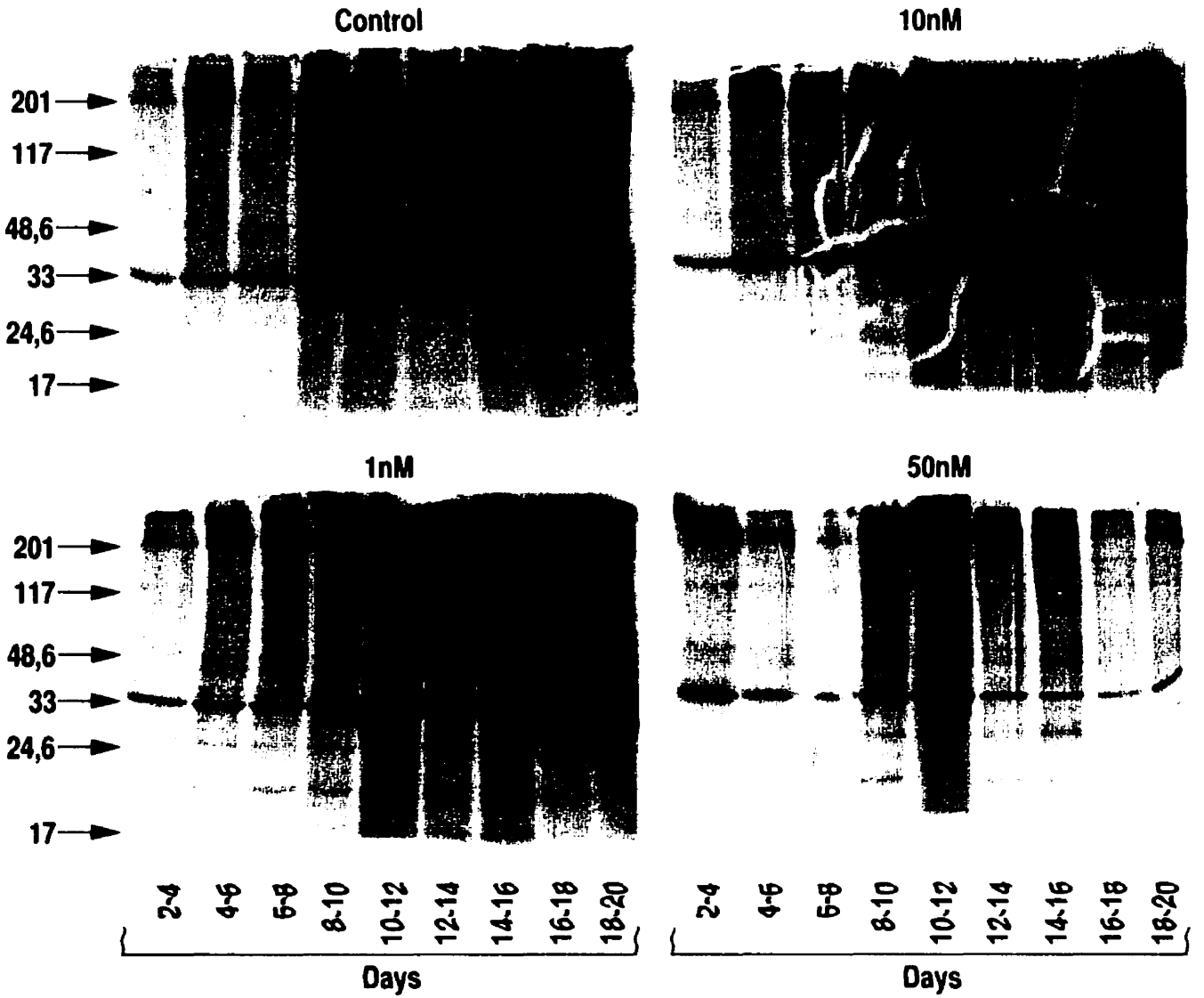

Control
Type II
Type X
1nM
Type II
Type X
10nM
Type II
50nM
Type II
2-4
4-6
6-8
8-10
10-12
12-14
14-16
16-18
18-20
20-22
Days

Fig. 3.29 The effect of RS 102,481, a synthetic preferential inhibitor of MMP-13, on ^{35}S methionine-labeled culture medium from bovine fetal growth plate chondrocyte C subpopulation during maturation.

^{35}S methionine-labeled cultured media (500 µl) were collected every 2 days and analyzed SDS-PAGE and visualized by autoradiography. No effects on newly synthesized proteins can be seen at 1 and 10 nM of RS 102, 481 inhibitor as compared to control. There is however, inhibition of newly synthesized proteins at 50 nM. (Fetal age = 221 days).


Control
10nM
1nM
50nM
201
117
48,6
33
24,6
17
2-4
4-6
6-8
8-10
10-12
12-14
14-16
16-18
18-20
Days
Days

collagen synthesis did not appear to be effected by RS 102, 481 using ^{3}H proline labeled collagen analysis. At 50 nM RS 102,481 may have had a toxic effect on cells resulting in a loss of type II collagen synthesis.

3.9.3 Effects of inhibitor on total protein synthesis

Newly synthesized protein was measured using 500 µl of ^{35}S methionine-labeled conditioned media from bovine fetal growth plate C subpopulation chondrocytes (fetal age = 221 days) (Fig 3.29). Total protein synthesis was visualized by Coomassie blue stained gels of 500 µl of conditioned media from bovine fetal growth plate C subpopulation chondrocytes (fetal age = 221 days) (Fig 3.30). The amount of newly synthesized proteins increased with maturation in the control cultures and cultures grown in the presence of 1 and 10 nM RS 102.481. There was no significant detectable differences in total protein synthesis or newly synthesized proteins with RS 102, 481 except at 50 nM when both newly synthesized and total protein content was inhibited. This inhibition did not occur until days 12-14 and persisted till days 18-20. This is probably due a toxic effect of the inhibitor on the cells.

3.9.4 Effects of inhibitor on gelatinase synthesis

Total gelatinase synthesis in bovine fetal growth plate C subpopulation chondrocytes (fetal age = 221 days) was measured by gelatin substrate gel zymography (Fig. 3.31) using 20 µl of conditioned medium. All cultures produced both the 92 kD and 72 kD gelatinases. There were no significant differences in gelatinase synthesis between control cultures and cultures with 1, 10

Fig. 3.30 The effect of RS 102,481, a synthetic preferential inhibitor of MMP-13, on secreted proteins in culture medium from bovine fetal growth plate chondrocyte C subpopulation during maturation.

Cultured media (500 µl) were collected every 2 days and analyzed by SDS-PAGE. No changes can be seen at 1 and 10 nM of RS 102, 481 inhibitor on secreted proteins as compared to control. There is however, inhibition of secreted proteins 50 nM. (Fetal age =221 days)

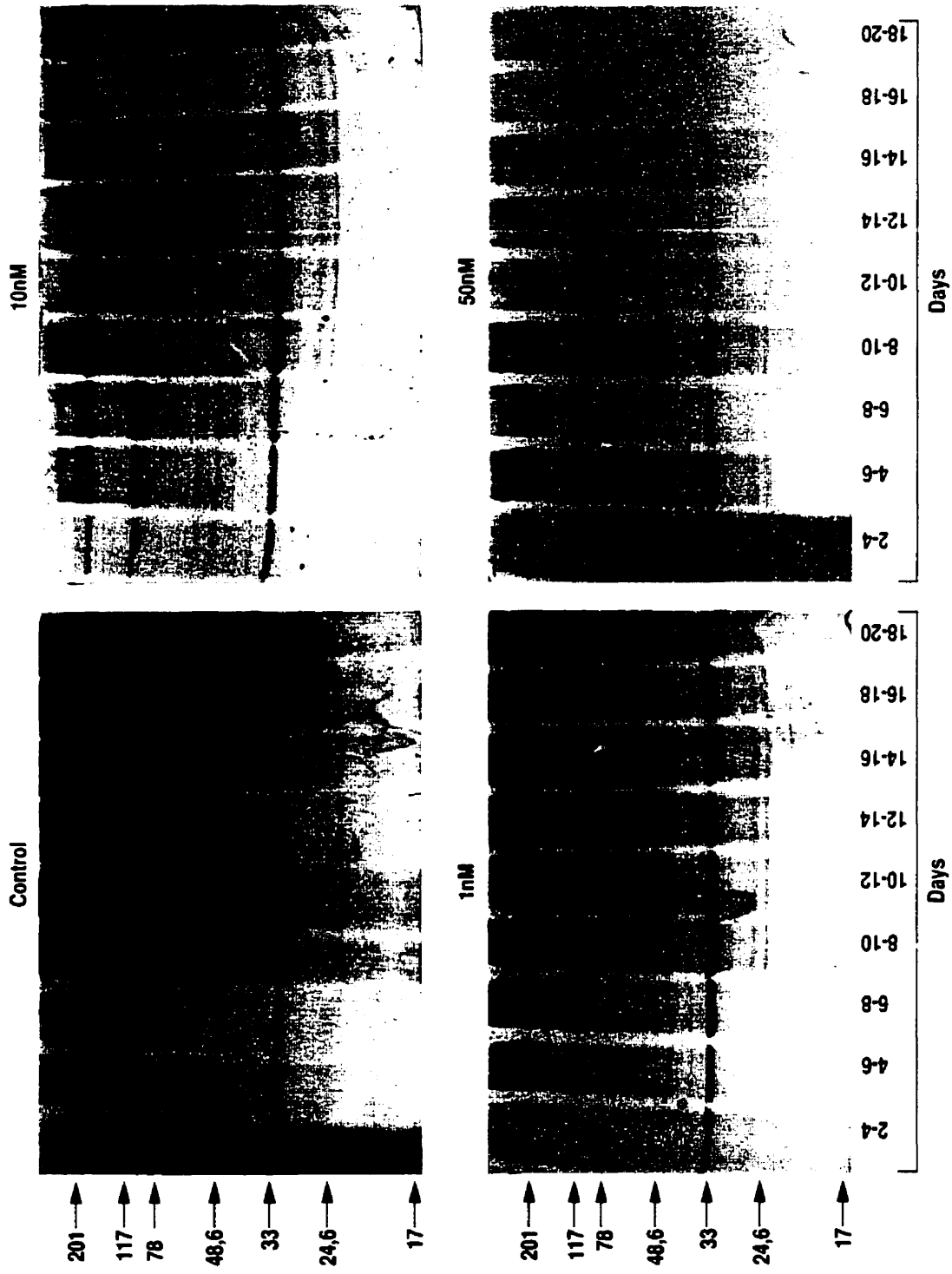
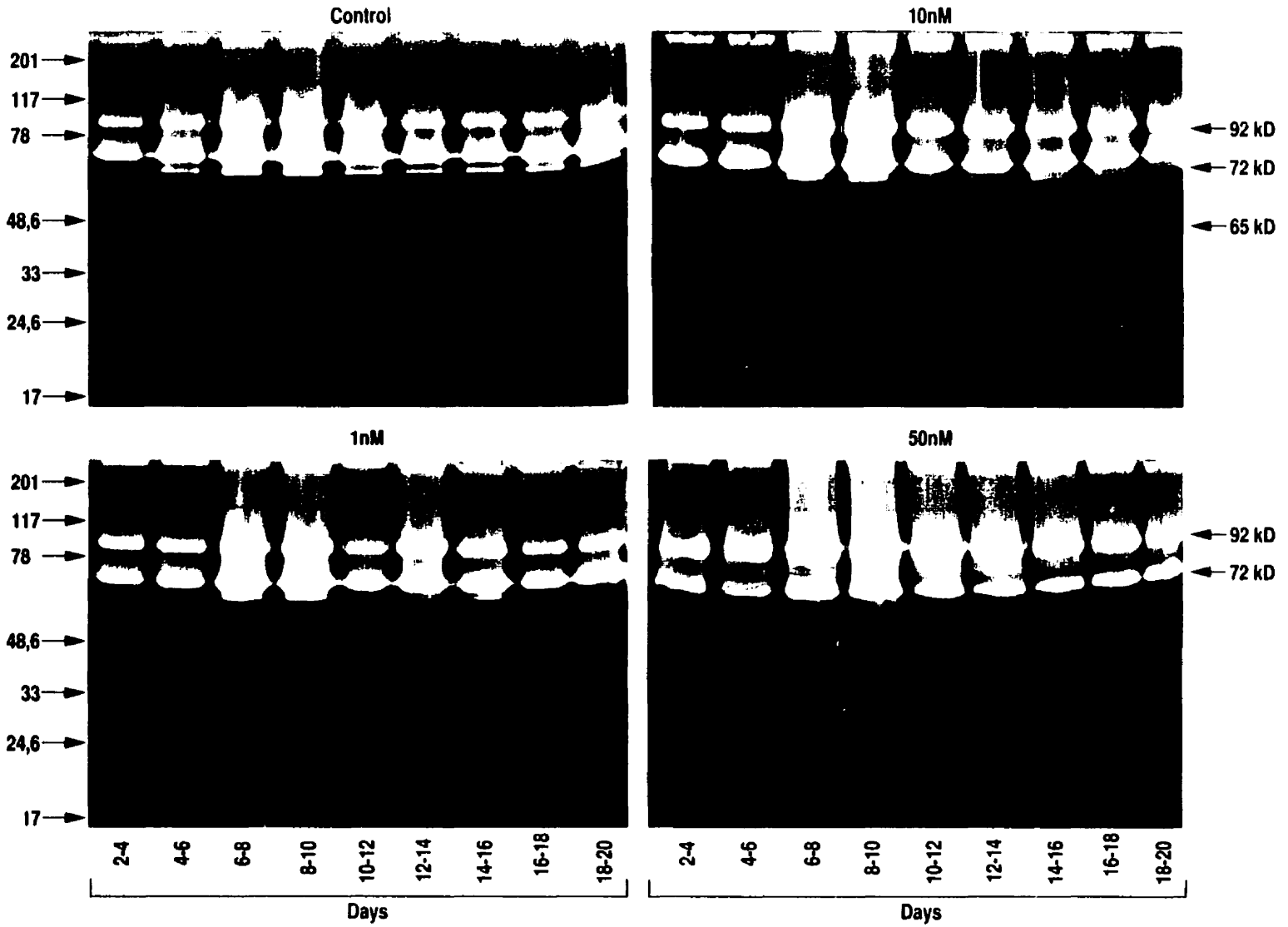

Control
1nM
10nM
50nM
201
117
78
48,6
33
24,6
17
2-4
4-6
6-8
8-10
10-12
12-14
14-16
16-18
18-20
Days

Fig. 3.31 The effect of RS 102,481, a synthetic preferential inhibitor of MMP-13, on gelatinase activity in culture medium from bovine fetal growth plate chondrocyte C subpopulation during maturation.

Conditioned media (20 µl) collected every 2 days were examined for gelatinolytic activity. All cells produced 92 kD and 72 kD gelatinases during culture in the absence or presence of RS 102. 481 inhibitor. There is however more active 72 kD gelatinase in control cultures vs. 1. 10 and 50 nM. A band at 65 kD is present in control cultures at days 18 and 20 which are absent in the others. There is a high amount of gelatinolytic activity in 50 nM cultures as compared with total protein content (Fig. 3.30). (Fetal age = 221 days).


Control
10nM
1nM
50nM
201
117
78
48,6
33
24,6
17
92 kD
72 kD
65 kD
2-4
4-6
6-8
8-10
10-12
12-14
14-16
16-18
18-20
Days

or 50 nM RS 102,481. However, due to decrease in total and newly synthesized proteins in cultures at 50 nM, one would expect a decrease in gelatinase activity. This is not so, and therefore gelatinase activity is higher in the 50 nM cultures. The active form of 72 kD gelatinase can be seen throughout the culture period in control cultures. There is a decrease of this form in the cultures with 1, 10 and 50 nM RS 102,481. There is also another 65kD band present only in control cultures at days 18- 20. This band is probably MMP-13 in view of its size and the time at which it is first detected.

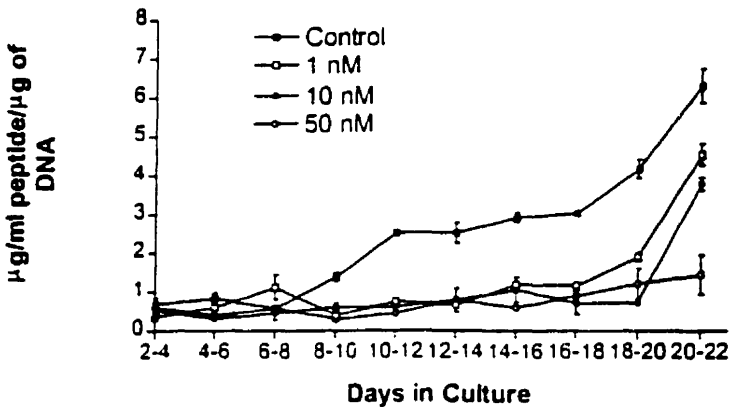
3.9.5 Effects of inhibitor on collagenase cleavage and total type II collagen content

Type II collagen denaturation was measured in α–chymotrypsin digests of bovine fetal growth plate C subpopulation chondrocyte cell layers (fetal age = 221 days) by the CB11B immunoassay (Fig 3.32a). The denatured type II collagen epitope increased in content when chondrocytes started to synthesize type X collagen in control cultures (days 8-10). The generation of this epitope was inhibited with 1, 10 and 50 nM RS 102, 481. The amount of denatured type II epitope was inhibited in a dose dependent manner with the inhibitor. An increase in this epitope can be observed at days 20-22 in cultures grown in the presence of 1 and 10 nM RS 102,481. The inhibitor may have caused a delay in hypertrophy and therefore degradation was seen later. There was essentially no release from cultures grown in the presence of 50 nM 102,481. This is probably due to toxicity of the inhibitor on the cells at this concentration.

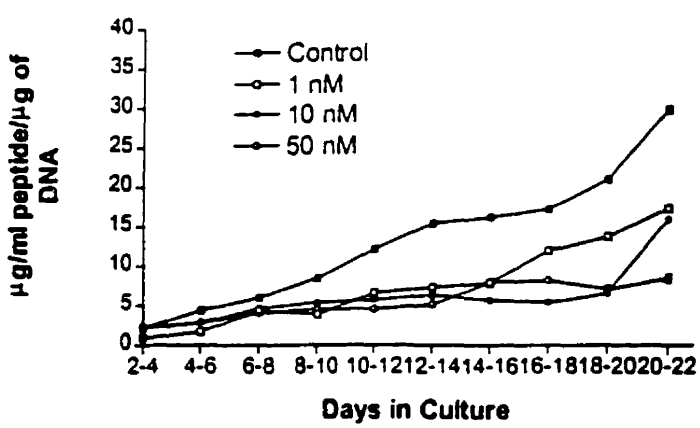
Fig. 3.32 The effect of RS 102,481, a synthetic preferential inhibitor of MMP-13, on the generation (cell layer) of the COL2-3/4m epitope during the maturation of bovine fetal growth plate chondrocyte C subpopulation.

Cell layers were assayed for denatured collagen (COL2-3/4 m epitope) using the CB11B assay (A). Epitope is first detected in the control cultures at days 8-10. No increase in epitope was detected in 1, 10 or 50 nM cultures until day 20-22. Inhibition of this epitope followed a dose-dependent inhibition. Total type II collagen content was also assayed using the CB11B assay (B). Total type II collagen content in cell layers increased with time in culture. Total type II collagen content was less in the inhibitor containing cultures. RS 102, 481 had a dose dependent inhibition on total type II collagen content in the cell layers. Each data point represents the mean ± standard deviation of triplicate wells. (Fetal age = 221 days)

A


Cell Layer (α-chymotrypsin)
μg/ml peptide/μg of DNA
Control
1 nM
10 nM
50 nM
0
1
2
3
4
5
6
7
8
2-4
4-6
6-8
8-10
10-12
12-14
14-16
16-18
18-20
20-22
Days in Culture

B


Cell layer
(α-chymotrypsin + proteinase K)
μg/ml peptide/μg of DNA
Control
1 nM
10 nM
50 nM
0
5
10
15
20
25
30
35
40
2-4
4-6
6-8
8-10
10-12
12-14
14-16
16-18
18-20
20-22
Days in Culture

Total type II collagen content was determined by addition of the CB11B epitope measured in the α-chymotrypsin and proteinase K digests of bovine fetal growth plate C subpopulation chondrocyte cell layers (fetal age = 221 days) (Fig. 3.32b). Total type II collagen content increased with time in cell layers of control cultures. However, there was a time dependent delay in the increase in total type II collagen content in cell layers that were cultured in the presence of 1, 10 and 50 nM RS 102,481.

3.9.6 Effects of inhibitor on proteoglycan release

Proteoglycan release into culture medium from bovine fetal growth plate C subpopulation chondrocytes (fetal age = 221 days) was measured with the DMMB assay (Fig. 3.33). An increase in proteoglycan release was first observed in control medium when chondrocytes first expressed type X collagen (days 8-10). Proteoglycan release remained constant during maturation (days 20-22) . The RS 102,481 inhibitor reduced proteoglycan release at 1 and 10 nM in a dose dependent manner. There was much reduced proteoglycan release at 50 nM of inhibitor, probably due to toxicity of the inhibitor on the cells.

3.9.7 Effect of inhibitor on matrix calcification

Matrix calcification was measured in bovine fetal growth plate C subpopulation cell layers (fetal age = 221 days) utilizing $^{45}Ca^{2+}$ incorporation (Fig.3.34). $^{45}Ca^{2+}$ accumulation occurred only in the control cell layers. $^{45}Ca^{2+}$ incorporation started at days 14-16 and persisted till days 18-20 in the control cell layers. This incorporation is maximal when cells are synthesizing type X collagen.

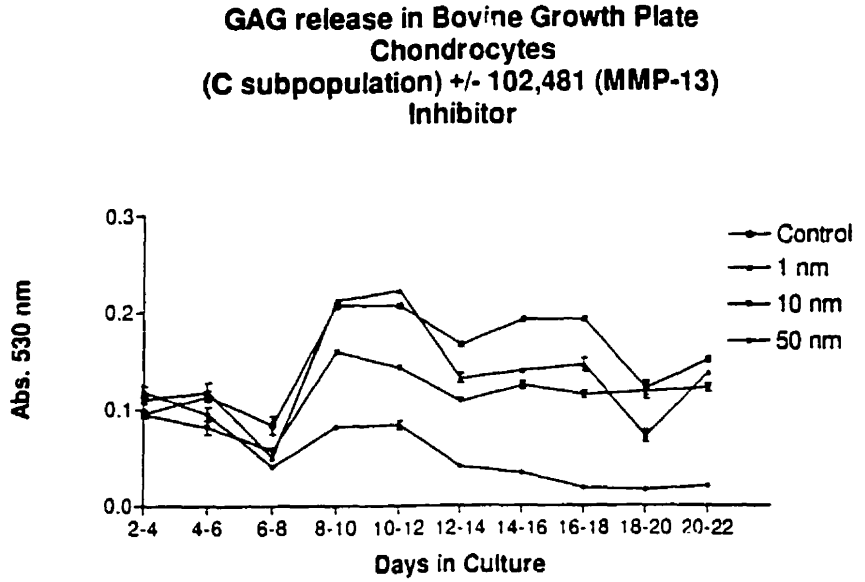


Fig. 3.33 The effect of RS 102,481, a synthetic preferential inhibitor of MMP-13, on the release (media) of proteoglycans during the maturation of bovine fetal growth plate chondrocyte C subpopulation.

GAG release was measured by the DMMB assay. 10 µl of conditioned medium were assayed. GAG release was highest in the control cultures vs the inhibitor cultures. GAG release began when cells started to synthesize type X collagen (days 8-10). GAG release remained constant throughout the rest of the culturing period (days 10-22). GAG release was inhibited in a dose-dependent manner in cultures with 1, 10 or 50 nM of inhibitor RS 102,481. Each data point represents the mean ± standard deviation of triplicate wells. (Fetal age = 221 days)

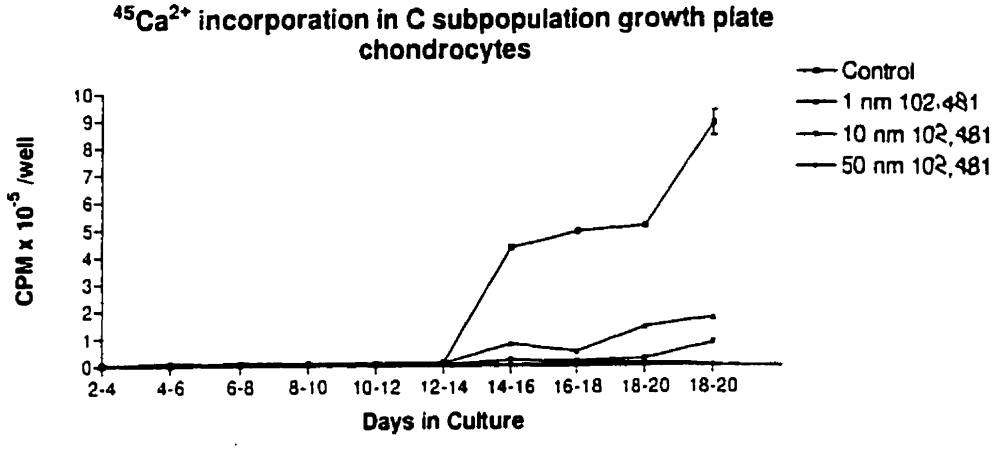


Fig. 3.34 The effect of RS 102,481, a synthetic preferential inhibitor of MMP-13, on $^{45}Ca^{2+}$ incorporation during the maturation of bovine fetal growth plate chondrocyte C subpopulation.

Mineralization was followed by $^{45}Ca^{2+}$ incorporation into cell layers. Cells cultured in the presence of RS 102, 481 exhibited a dose-dependent reduction in $^{45}Ca^{2+}$ incorporation. Mineralization was initiated in control cultures at days 14-16. This is after the initiation of type X collagen synthesis and type II collagen and proteoglycan degradation. Each data point represents the mean ± standard deviation of triplicate wells. (Fetal age = 221 days)

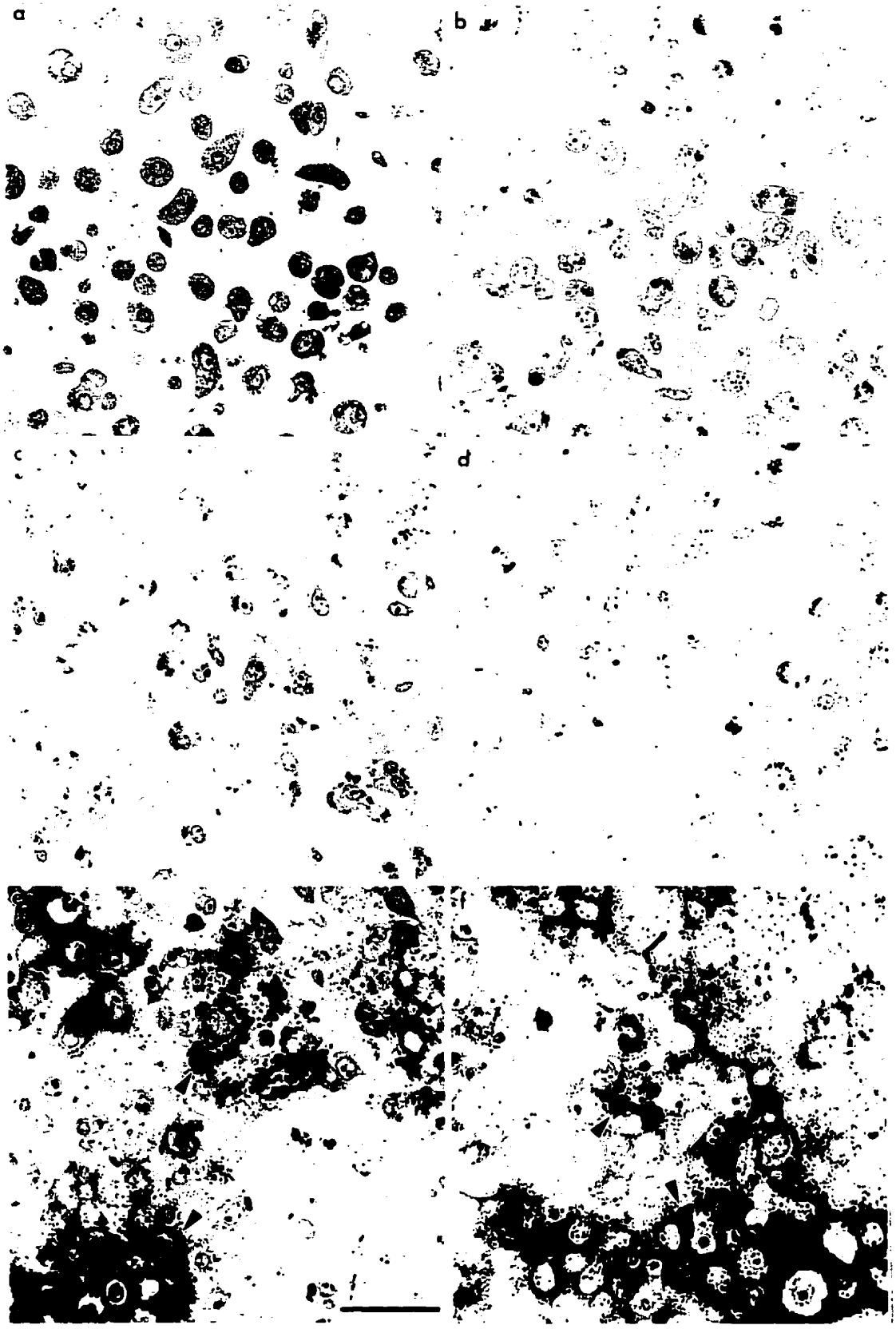
Cell layers grown in the presence of 1 and 10 nM of MMP-13 inhibitor RS 102,481 had little or no accumulation of $^{45}Ca^{2+}$. At 50 nM there was essentially no incorporation. Mineralization was initiated in control cells after they became hypertrophic and after proteoglycan and type II collagen loss had started.

3.9.8 Histological analysis of chondrocyte cell layers

Bovine fetal growth plate C subpopulation chondrocyte cell layers (fetal age = 221 days) at the end of the culturing period (22 days) were examined by histological analysis (Fig. 3.35). There was no pronounced differences in cell size between the control cells and cells grown in the presence of 1 or 10 nM RS 102,481. Calcification was detected in the control cells by staining with Von Kossa's reagent, whereas cells grown in the presence of inhibitor were negative for mineral. Cells grown in the presence of 50 nM RS 102,481, were irregular in shape and had large intracellular vacuoles (data not shown). This morphology is typical for a cell under toxic stress.

Fig. 3.35 Histological analyses of chondrocyte C subpopulation cell layers cultured with or without RS 102,481.

Chondrocyte morphology (toluidine blue: a, c and e) and mineral deposited (Von Kossa's: b, d and f) in the extracellular matrix of cell layers grown in the presence (a and b, 1 nM; c and d, 10 nM) or absence (e and f) of RS 102,481 inhibitor. C subpopulation cells have enlarged after 20 days in culture. There is no significant difference in cell size in cells grown in the presence (a, b, c, and d) or absence (e and f) of inhibitor. There is however, no matrix mineralization in cells grown in the presence of 1 nM (b) or 10 nM (d) of RS 102,481 as compared to control (f). Arrows indicate areas of mineral in control cultures (e and f). Fetal age=221 days. Bar=50μm.


a
b
c
d

4.DISCUSSION

4.1 Optimization of total RNA extraction from bovine cell layers and cartilage

An efficient method of RNA isolation from cell layers and cartilage was required to study gene expression. This can be hindered due to low cellularity. high amounts of RNAases and the extensive extracellular matrix produced by chondrocytes. A common method of choice for total RNA extraction is the direct guanidine isothiocyanate/phenol/chloroform method of Chomczynski and Sacchi, (1987). This is, however, ineffective with cartilaginous tissue or chondrocyte cell layers grown in long term cultures due to the synthesis of large proteoglycans such as aggrecan which contain many highly negative charged GAGs. I have produced a simple method that involves some important modifications to the direct guanidine isothiocynate/phenol/chloroform method (Chomczynski and Sacchi, 1987) that allows us to extract increased amounts of contaminant-free total RNA. In this method, proteins and nucleic acids are precipitated with 100% isopropanol. The protein and RNases are digested with proteinase K. This allows an efficient separation of nucleic acids into the aqueous phase during the phenol-chloroform extraction. Subsequently, all contaminating GAGs are removed with 4 M LiCl.

The effect of GAG contamination was measured with the addition of shark CS into purified bovine fetal skin fibroblast total cellular RNA. Northern blot analysis was performed on these RNA preperations. Signal intensity of GAPDH by Northern blot analysis was inhibited with increasing concentrations of shark

CS. However, MMP-1 signal intensity was not as greatly affected. This interference with certain mRNA species and not others, may be due to the molecular weight and migration of the GAGs in the agarose gel. The fluorescence of the 18S ribosomal RNA is more inhibited than that of the 28S ribosomal RNA. This observation suggests that mRNAs migrating in the region of 18S would be affected more than the higher molecular weight mRNAs. Methylene blue staining of the nylon membrane which contains the transferred total cellular RNA is also affected by GAG contamination. This could lead to misinterpretation of the mRNA levels in these samples. If GAPDH was used as a housekeeping gene in these gels, it could lead to a false interpretation of MMP-1 mRNA levels. Therefore it is essential that these GAGs be removed from the total cellular RNA preparations. These simple modifications result in large amounts of intact RNA which can be used for analysis of gene expression in cartilage.

4.2 MMP-1 expression in bovine fetal growth plate chondrocytes

The presence of a " collagenase" has been documented in the rat (Blair *et al.*, 1989, Dean *et al.*, 1989, 1990), mouse (Mattot *et al.*, 1994; Gack, *et al.*, 1995), and rabbit (Brown *et al.*, 1989, Ballock *et al.*, 1993) growth plates. With the cloning of MMP-13 and the recognition that rodents possess only one collagenase (Shapiro, 1997), it was unclear which collagenases are present in non-rodent growth plates. The antisera previously used to investigate collagenases were made using whole recombinant or purified MMP-1. These reagents could cross react with the different collagenases. Therefore the results of these earlier studies,

concerning the presence of collagenases are inconclusive. Furthermore, earlier studies in rat (Dean *et al.*, 1989,1990), revealed only the presence of collagenase activity, and not the identity of the collagenase(s). Also, in the rabbit studies, the identity and function of collagenase(s) during endochondral ossification were never identified (Brown *et al.*., 1989).

When these studies were initiated MMP-1 was the only enzyme thought to present in cartilage (Matrisian, 1992; Birkedal-Hansen, *et al.*, 1993). To study MMP-1 expression during the progression of cellular maturation in the bovine growth plate, we used an *in vitro* cell culture system that had been developed in the laboratory (Alini *et al.*, 1994, 1996). It was shown that isolated subpopulations would undergo the same maturation to the hypertrophic phenotype as *in vivo* (Alini *et al.*, 1994) and could be cultured in serum-free conditions (Alini *et al.*, 1996). There was also evidence that in this system that there was type II collagen denaturation (Alini *et al.*, 1992, 1994). This was also observed *in situ* (Alini *et al.*, 1992) suggesting the presence of a collagenase when cells became hypertrophic and calcification was initiated.

We showed that MMP-1 mRNA expression was not detectable by Northern blot analysis. MMP-1 mRNA was undetectable in growth plate chondrocyte subpopulation at isolation and during culture. Type II collagen mRNA was shown to be expressed at all times in these cultures and type X collagen mRNA was expressed when the chondrocytes became hypertrophic in culture. MMP-1 protein was also not detectable in conditioned media of cultures analyzed by Western blot.

To rule out the possibility of low expression of MMP-1 in these chondrocytes, IL-1α was used in these cultures. IL-1 has been shown to be a potent stimulator of MMP-1 in chondrocytes (Schnyder *et al.*, 1987; Smith *et al.*, 1989) and in both skin and synovial fibroblasts (Birkedal-Hansen *et al.*, 1993; Benbow and Brinckerhoff, 1997). However, expression of MMP-1 mRNA and protein synthesis could still not be detectable in bovine fetal growth plate or epiphyseal chondrocytes when cultured with 1 or 10 ng/ml of IL-1α. However, bovine fetal skin fibroblasts that were stimulated with IL-1α were shown to express MMP-1 mRNA and synthesize protein. Although MMP-1 mRNA and protein was undetectable in these chondrocyte cultures, a collagenase activity could be detected in the conditioned media using ^{14}C-labeled type II collagen as the substrate in a fibril assay. It was apparent therefore that another collagenase was likely produced by these chondrocytes. I then went on to identify this as MMP-13 as the collagenase (see below). Recently, this finding has been confirmed in human fetal growth plate *in vitro* (Johansson *et al.*, 1997). These authors also could not detect MMP-1 mRNA in cultured primary human fetal chondrocytes by Northern analysis.

4.3 Cloning and expression of bovine collagenase-3 (MMP-13) in bovine fetal growth plate chondrocytes

A novel collagenase, cloned from a human breast carcinoma, was reported at the end of 1994: it was called collagenase-3 or MMP-13 (Freije *et al.*, 1994) It was shown to be the homologue of rat and mouse interstitial collagenases. This

collagenase was also detected in human adult normal and osteoarthritic chondrocytes (Borden *et al.*, 1996; Mitchell *et al.*, 1996; Reboul *et al.*, 1996). MMP-13 was also shown to degrade type II collagen about 6-10 fold more effectively than type I and III collagen (Knäuper *et al.*, 1996a; Mitchell *et al.*, 1996). It also displayed a 50-fold stronger gelatinolytic activity than MMP-1 and MMP-8 (Knäuper *et al.*, 1996a; Mitchell *et al.*, 1996). This collagenase was therefore a good candidate for the bovine fetal growth plate collagenase. Hence I decided to determine whether the bovine homologue of this collagenase was present in the bovine fetal growth plate. By using PCR with conserved sequences found in human, rat, and mouse MMP-13, I was able to clone the entire coding region of the bovine MMP-13 using mRNA isolated from bovine hypertrophic chondrocytes. The cDNA sequence showed a 92%, 82% and 82% homology to human, mouse and rat cDNA, respectively. The deduced amino acid sequence showed a 90%, 85%, and 85% homology to human, mouse and rat protein respectively. A comparison of the MMP-13 amino acid sequences of all the characterized MMP-13s permitted the identification of highly homologous domains. The sequence PRCGVPD located between residues 95-101, which is thought to be characteristic of the propeptide domain of MMPs, is highly conserved and may play a role of enzyme activation mechanism (van Wart and Birkedal-Hansen, 1990). The zinc binding region located between residues 220 and 230 identifies the 11 residue consensus sequence VAAHE L/F GH S/A L/M G which is considered to be a part of the active site of these enzymes (van Wart

and Birkedal-Hansen, 1990). The three putative N-glycosylation sites at amino acids; 117, 152, and 409 are also conserved. On Northern analysis, there are two mRNA of 2.0 and 2.8 kb in size. The human MMP-13 mRNA has three different size transcripts of 3.0, 2.5 and 2.0 kb. (Freije *et al.*, 1994). These different transcripts are due to different polyadenylation sites in the 3' untranslated region. This may be also be the reason for the two transcript sizes in bovine MMP-13 mRNA.

Expression of MMP-13 mRNA was shown to be restricted to cultured bovine fetal hypertrophic growth plate chondrocytes. Only cells that were synthesizing type X collagen expressed MMP-13 mRNA. This is in agreement with *in situ* hybridization studies in mouse (Mattot *et al.*, 1994; Gack *et al.*, 1994) and human (Johansson *et al.*, 1997; Ståhle-Bäckdahl *et al.*, 1997) growth plates where MMP-13 mRNA expression was also restricted to hypertrophic chondrocytes.

The presence of MMP-1 and MMP-13 protein in bovine fetal growth plate chondrocyte cultures were analyzed using specific anti-peptide antibodies that I prepared. These antibodies were collagenase specific. Anti-MMP-1 antiserum recognized only bovine and human forms of pro-MMP-1 and the activated molecules in the presence or absence of bound TIMP-1. Anti-MMP-13 antiserum only recognized mouse, bovine and human forms of pro-MMP-13 and the active molecules in the presence or absence of bound TIMP-1. Western blot and immunoprecipitation revealed the presence of the pro-MMP-13 (65 kD) but not

the active form (55kD) in these cultures. This is surprising since collagenase activity was measured from conditioned medium when cells expressed MMP-13 at hypertrophy and this activity increased with time. The activation mechanism of MMP-13 in these cultures is unknown but. conditioned medium from these cultures contained active 72 kD gelatinase (MMP-2). revealed by gelatin zymography, which has been shown to be able to activate MMP-13 *in vitro* (Knäuper *et al.*, 1996b). However. there were proteolytic fragments that also showed reactivity with the anti-MMP-13 antibody (30kD). These may be autolytic fragments of the kind described for MMP-1 (Clark and Cawston, 1989).They may have arisen from active enzyme that had undergone autolysis. That MMP-13 is the sole collagenase synthesized by hypertrophic chondrocytes of the growth plate makes good sense since this is the most potent collagenase and gelatinase with respect to cleavage of type II collagen. Recently. MMP-13 mRNA and protein has been shown by *in situ* hybridization and immunohistochemistry to be present in human hypertrophic chondrocytes (Ståhle-Bäckdahl *et al.*, 1997) during fetal development. The collagenase provides the chondrocytes in the growth plate with the opportunity to rapidly remodel its extracellular matrix by quick effective removal of type II collagen fibrils.

4.4 Inhibition of collagenase-3 (MMP-13) activity and the expression of hypertrophy in bovine fetal growth plate chondrocyte cultures with a preferential inhibitor of MMP-13, RS 102,481.

Since, MMP-13 protein is synthesized by hypertrophic chondrocytes and is

active, we wanted to see if inhibition of the enzyme would have any affect on chondrocyte hypertrophy. At low concentrations (1 and 10 nM), the synthetic inhibitor RS 102, 481 is very selective against MMP-13. It belongs to the class of small peptide inhibitors that can bind and chelate the zinc moiety in the active site of MMP-13. It's active component is a carboxylic acid which exploits the deep S_1' pocket of MMP-13. In these studies, we did not have to be concerned with MMP-1 because of its demonstrated absence in this system. There were pronounce effects on chondrocyte maturation at the concentrations tested: 1, 10 and 50 nM. The chondrocytes that were grown in the presence of 50 nM of inhibitor exhibited evidence of cellular toxicity. These chondrocytes contained large intracellular vacuoles and protein synthesis was inhibited. This inhibitor may have also inhibited other classes of metalloproteases at this concentration and affected other cellular functions. This toxic effect was not observed at 1 and 10 nM. The results obtained at 50 nM will therefore not be discussed further.

The amount of type II collagen denaturation in the cell layers as measured by the COL2-3/4m assay (Hollander *et al.*, 1994) was decreased in a dose dependent manner with RS 102, 481. In the control culture denatured type II collagen was detected at days 8-10 and increases until the end of the culturing period at 20-22 days. In the cell layers grown in the presence of 1 and 10 nM of 102,481 there was an absence of denatured collagen until days 20-22. The inhibitor thus prevented the generation of this epitope in these cell layers. This denaturation likely reflects collagenase activity since only this can knowingly

produce denaturation by enzymatic means. This shows that this inhibition decreases type II collagen degradation, reflected as denaturation, as well as retarding synthesis. Only when sufficient degradation has occurred, can the degradation cascade and maturation continue. This denaturation measured by the COL2-3/4m assay, has been shown to be a directly correlated with type II collagen cleavage by collagenases in human normal and osteoarthritic cartilage (Billinghurst *et al.*, 1997).

Although type II collagen synthesis was not detectably affected, total type II collagen content was reduced in the cell layers cultured in the presence of inhibitor. These observations raise the question of whether the degradation products from the remodeling of the extracellular matrix may play a role in chondrocyte maturation and matrix turnover. Fibronectin fragments have been shown to induce collagenase and stromelysin gene expression in fibroblasts through engagement of integrin receptors (Werb *et al.*, 1989; Huhtala *et al.*, 1995). Type I collagen synthesis is also modulated by integrins on fibroblasts (Langholz *et al.*, 1995). Although, the integrin expression pattern is not known in these fetal bovine growth plate chondrocytes, it is possible that type II collagen fragments which are generated in the initiation of chondrocyte hypertrophy signal back to the chondrocyte to produce MMP-13 and stimulate type II and initiated X collagen synthesis. MMP-13 mRNA and protein has been shown to be synthesized when hypertrophic chondrocytes express type X collagen in the absence of inhibitor. MMP-13 mRNA expression and protein synthesis were not measured in cultures

grown in the presence of inhibitor due to limited number of cells recovered for the analysis. These experiments would be very important in answering if MMP-13 mRNA expression and protein synthesis were affected by this inhibitor. If the production of these fragments is inhibited either by the lack of MMP-13 protein or activity, this may explain the inhibition of type II and X synthesis. Recently, concanvalin A (con A) was shown to promote chondrocyte hypertrophy and matrix calcification in rabbit growth plate chondrocytes *in vitro* (Yan *et al.*, 1997). Con A has been shown to be a potent stimulator of MMPs in fibroblasts (Overall and Sodek, 1990). Con A may ligate integrins or other receptors that stimulate MMP-13 production and thus create a cascade of degradative events that leads to chondrocyte hypertrophy and mineralization.

The most dramatic effect of the inhibitor was on type X collagen synthesis. At both 1 and 10 nM, type X collagen synthesis was completely inhibited, although the synthesis of type II collagen and other proteins measured by ^{3}H proline and ^{35}S methionine incorporation into secreted proteins were generally unaffected. Although, measurement of total type II collagen content with the COL2-3/4 m immunoassay revealed a decrease in total type II collagen in the cell layers of chondrocytes grown in the presence of the inhibitor at all concentrations, this difference could be due to the difference in the sensitivity of the two assays. The difference in type II collagen synthesis could not be detected by autoradiography at the exposures time used. Perhaps different exposure times would allow differentiation between the different cultures. However, it is

possible that inhibition of hypertrophy leads not only a decrease in type II collagen cleavage but a reduction of synthesis as well. Therefore this may lead to a drop in total type II content because synthesis could determine content more than degradation. This inhibition of an MMP leading to the suppression of the hypertrophic phenotype is reminiscent of the recent study where doxycycline was shown to inhibit type X collagen synthesis in chick hypertrophic chondrocytes (Davies *et al.*, 1996). Doxycycline has been shown to inhibit collagenase and gelatinase activity (Yu *et al.*, 1992) and inhibit resorption of chick embryonic tibias *in vitro* (Cole *et al.*, 1994). The mechanism of its inhibition of MMP activity or production is not clearly understood, but it is conceivable that it may have inhibited synthesis and/or activity of MMP-13 in chick hypertrophic chondrocyte cultures. Although the exact function of type X collagen is still unknown, its location in hypertrophic chondrocytes suggests that it may be important during endochondral ossification. The only other tissue in which the presence of the type X collagen has been reported is in human osteoarthritic (OA) cartilage (Hoyland *et al.*, 1991; von der Mark *et al.*, 1992; Walker *et al.*, 1995). MMP-13 mRNA and protein have also been shown to be highly expressed in human OA cartilage (Borden *et al.*, 1996; Mitchell *et al.*, 1996; Reboul *et al.*, 1996). It would seem that the expression of these two molecules are related to one another and therefore excessive degradation of type II collagen in osteoarthritis may cause a feedback of type II collagen fragments to the chondrocyte that leads to upregulation of MMP-13 and type X collagen. Type X collagen expression could be linked to a

chondrocyte that is highly collagenolytic. It would be interesting to see if RS 102, 481 could inhibit type X collagen synthesis in human osteoarthritic cartilage.

Matrix mineralization was completly inhibited in the cell layers cultured in the presence of RS 102, 481. Mineralization in control cell layers was observed only after type II collagen and proteoglycan degradation. In all cultures, proteoglycan release began at days 8-10 and continued for the duration of the culture period. Proteoglycan release occurs slightly ahead of type II collagen denaturation. This supports the theory that the molecular status of proteoglycans must be changed for matrix mineralization to occur (Poole, 1991; Howell and Dean, 1992). MMP-13 has been shown to be able to degrade aggrecan *in vitro* (Fosang *et al.*, 1996) Cell layers grown in the presence of MMP-13 inhibitor, RS 102, 481 had lower proteoglycan release into the conditioned media which may have resulted in an inhibition of matrix mineralization. Histological analysis of the chondrocytes cultured in the presence or absence of RS 102, 481 showed that matrix mineralization occurred only in the cell layers cultured without the inhibitor. Interestingly, chondrocyte cell shape was similar in the cell layers grown in the presence or absence of inhibitor. This is surprising since this suggests normal cellular enlargement yet the chondrocytes did not produce type X collagen and thus were not "hypertrophic". This observation may indicate that there was enough degradation in the presence of the inhibitor to allow for cellular hypertrophy, but not enough for complete progression to the full hypertrophic phenotype.

Gelatinolytic activity, measured by gelatin zymography was not affected in the conditioned medium from growth plate chondrocytes grown in the presence of MMP-13 inhibitor 102,481. Cultures contained both 92 and 72 kD gelatinase activity irrespective of the inhibitor being present. There was also active 72 kD gelatinase activity in the control and inhibitor containing cultures. Recently it was been shown *in vitro* that TIMP-free 72 kD gelatinase can degrade type I collagen (Aimes and Quiqley, 1995). It is not known that if this gelatinase possesses the ability to degrade type II collagen. Although active 72 kD gelatinase activity appears early on in these cultures, we are unable to detect denatured type II collagen using the COL2-3/4m assay at the same time period in these cultures. Therefore, it is probably unable to cleave type II collagen in the growth plate cell layers and not a collagenase in this system.

The inhibition of MMP-13 activity in bovine fetal growth plate chondrocytes leads to an inhibition of the hypertrophic phenotype. These results suggest that MMP-13 is essential for normal chondrocyte maturation and endochondral ossification. The lack of its degradative properties during normal turnover in the hypertrophic zone, could lead to disruption of bone formation. Interestingly, tibial dyschondroplasia (TD) in poultry (Orth, and Cook, 1994) is a disorder of growth plate cartilage that fails to resorb. It has been shown that TD results from a decrease of collagenolytic and gelatinolytic activity (Rath *et al.*, 1996). Although the exact MMP responsible for this disorder has yet to be identified, evidence demonstrated in our studies in the bovine fetal growth plate

suggest it may be MMP-13. It is also conceivable that some human chondrodysplasias could arise from a defect in MMP-13. In summary, a diagrammatic representation of the proposed involvement of MMP-13 in the maturation of growth plate chondrocytes is shown in fig. 4.1.

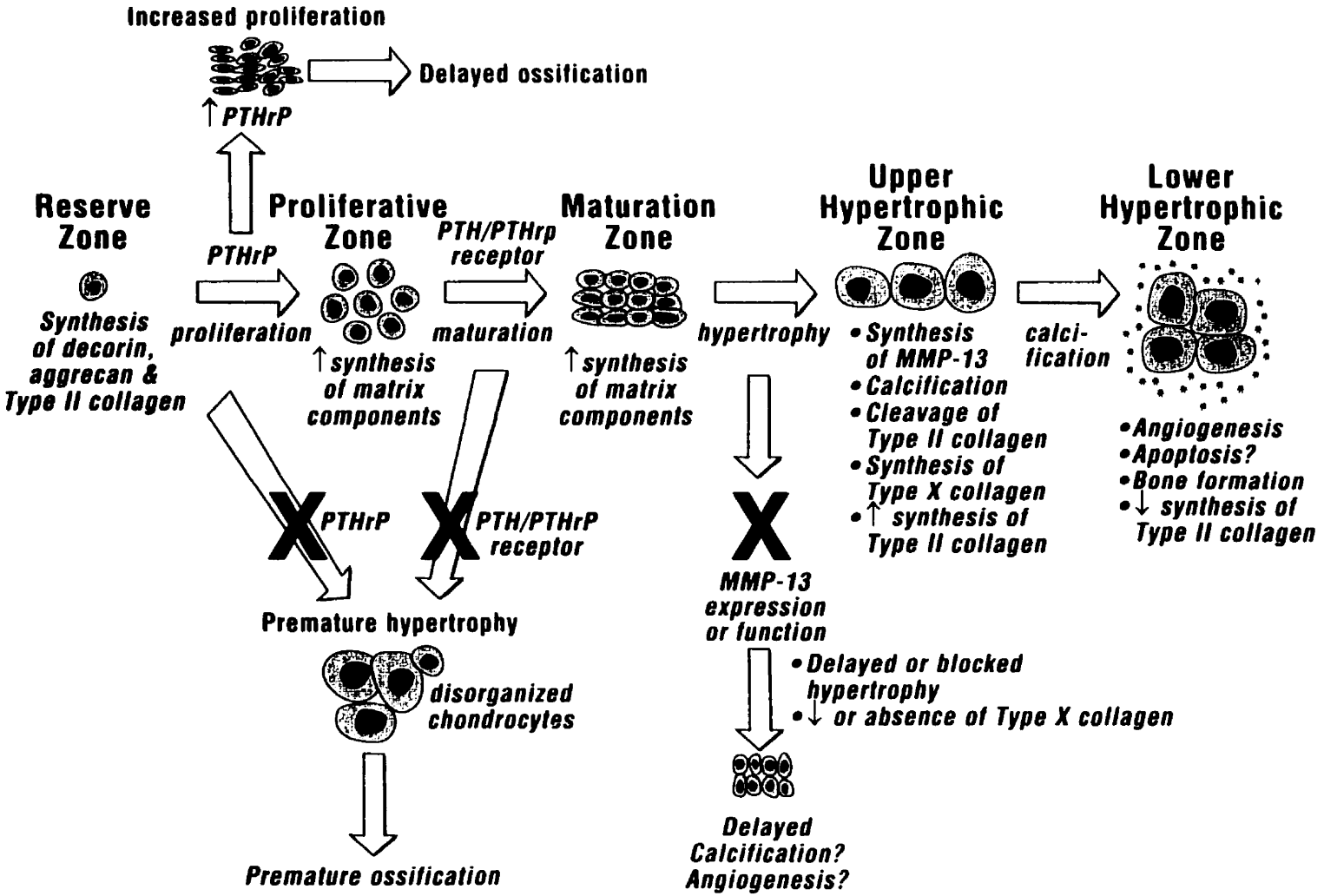


Fig. 4.1 Diagrammatic representation of possible pathways during the maturation of fetal growth plate chondrocytes during endochondral ossification.

5.CONCLUSIONS

In this study, the identity of the collagenase that is involved in the degradation of the extracellular matrix during hypertrophy of bovine fetal growth plate chondrocytes was established. A novel collagenase was cloned from fetal bovine growth plate chondrocytes and was shown to be the bovine homologue of human collagenase-3 (MMP-13). This study also provides molecular and biochemical data to indicate that MMP-13 is the collagenase that is required by bovine fetal growth plate chondrocytes to mature from the prehypertrophic to the hypertrophic phenotype. As a result of this study the following conclusions can be made:

- The interstitial collagenase (MMP-1) gene is not expressed nor is MMP-1 synthesized by bovine fetal growth plate chondrocytes *in vitro*.
- IL-1α does not stimulate MMP-1 mRNA expression or protein synthesis in bovine fetal chondrocytes *in vitro*.
- MMP-13 mRNA is expressed and MMP-13 protein is synthesized in bovine fetal growth plate chondrocytes when cells become hypertrophic as defined by synthesis of type X collagen.
- Type II collagen denaturation and proteoglycan release occurs at the time when MMP-13 is expressed and synthesized.
- MMP-13 may be required by bovine fetal growth plate chondrocytes to progress through hypertrophy and endochondral ossification. Inhibition of MMP-13 activity results in suppression of expression of the hypertrophic

phenotype characterized by type X collagen synthesis, mineralization, type II collagen degradation, and proteoglycan release.

6. STATEMENTS OF ORIGINAL CONTRIBUTIONS

1. Optimization of a simple and rapid method for efficient extraction of total RNA from bovine chondrocyte cell layers and cartilage.
2. Interstitial collagenase (MMP-1) mRNA and protein are absent from bovine fetal growth plate chondrocytes.
3. Cloning of a novel bovine collagenase that is the homologue of human, rat, and mouse MMP-13.
4. Preparation of specific cDNA probes and specific antisera to MMP-1 and MMP-13 that will allow for more detailed studies on these collagenases.
5. MMP-13 is expressed in bovine fetal growth plate chondrocytes *in vitro* during hypertrophy and is probably responsible for type II collagen degradation.
6. Analysis of mRNA, protein and activity of MMP-13 in bovine fetal growth plate chondrocytes *in vitro*.
7. Inhibition of MMP-13 activity in bovine fetal growth plate chondrocytes *in vitro* results in inhibition of expression of the hypertrophic phenotype characterized by type X collagen synthesis, matrix mineralization, type II collagen degradation and proteoglycan release.

7. PUBLICATIONS

The following manuscripts and abstracts were derived from the work presented in this thesis:

Abstracts:

- Wu, C.W. and Poole, A.R. MMP-1 is absent in fetal bovine cartilages. Canadian Connective Tissue Conference. Montreal. QC. June, 1995.
- Wu, C.W., Billinghurst, R.C., Pidoux, I., Tanzer, M., Brooks, E. and Poole, A.R. .Immunolocalization of collagenases MMP-1 and 13 in human normal and arthritic articular cartilages in relationship to cleavage of type II collagen. Canadian Orthopeadic Research Society Meeting. Quebec City, QC. May, 1996.
- Wu, C.W., Billinghurst, R.C., Pidoux, I., Tanzer, M., Brooks, E. and Poole, A.R. Immunolocalization of collagenases MMP-1 and 13 in human normal and arthritic articular cartilages in relationship to cleavage of type II collagen., Canadian Connective Tissue Conference. Toronto. ON. June, 1996.
- Wu, C.W., Hasty, K.A. and Poole, A.R. MMP-13 but not MMP-1 is synthesized in fetal bovine growth plate chondrocytes., 43rd Orthopedic Research Society Annual Meeting, Vol. 22 pp.618, San-Francisco, CA, February, 1997.
- Wu, C.W., Hasty, K.A. and Poole, A.R. MMP-13 but not MMP-1 is synthesized in fetal bovine growth plate chondrocytes, Matrix Metalloproteinases, Gordon Research Conference. New London, NH, July,

1997.

Manuscripts:

1. Alini, M., Kofsky, Y., Wu, W., Pidoux, I., and Poole, A .R.. 1996 In Serum-Free Culture Thyroid Hormones Can Induce Full Expression of Chondrocyte Hypertrophy Leading to Matrix Calcification.. J. Bone and Mineral Res. 11(1) 105-113.
2. Wu, C.W., Billinghurst, R.C., Pidoux, I., Tanzer, M., Brooks, E., and Poole, A.R.. Immunolocalization of Collagenase MMP-1 and 13 in Human Normal and Arthritic Articular Cartilages in Relationship to Cleavage of Type II Collagen.. manuscript in preparation.
3. Wu, C.W., Hasty, K.A. and Poole, A.R. MMP-13 but not MMP-1 is synthesized in fetal bovine growth plate chondrocytes: Role and Regulation. manuscript in preparation.

8. LIST OF REFERENCES

Aigner, T., Dietz, U., Stöss, H. and von der Mark, K. 1995. Differential expression of collagen types I, II, III and X in human osteophytes. Lab. Invest. 73:236-243.

Alexander, C.M., and Werb, Z. 1991. Extracellular matrix degradation. **In:** Cell biology of extracellular matrix. 2nd ed. E.D. Hay eds. Plenum Press, New York, New York. pp. 255-294.

Alini, M., Matsui, Y., Dodge, G.R., and Poole, A.R. 1992. The extracellular matrix of cartilage in the growth plate before and during calcification: Changes in composition and degradation of type II collagen. Calcif. Tissue Int. 50:327-335.

Alini, M., Carey, D., Hirata, S., Grynpas, M.D., Pidoux, I., and Poole, A.R. 1994. Cellular and matrix changes before and after the time of calcification in the growth plate studied in vitro: arrest of type X collagen synthesis and net loss of collagen when calcification is initiated. J. Bone and Mineral Res. 9:1077-1087.

Alini, M., Kofsky., Wu, W., Pidoux, I., and Poole, A.R. 1996. In serum free culture thyroid hormones can induce full expression of chondrocyte hypertrophy leading to matrix calcification J. Bone and Mineral Res. 11:105-13.

Ames, R.T., and Quigley, J.P. 1995. Matrix metalloproteinase-2 is an interstitial collagenase. J. Biol. Chem. 270:5872-5876.

Amizuka, N., Warshawsky, H., Henderson, J.E., Goltzman, D., and Karaplis, A.C. 1994. Parathyroid hormone-related peptide-depleted mice show abnormal epiphyseal cartilage development and altered endochondral bone formation. J. Cell Biol. 126:1611-1623

Amizuka, N., Henderson, J.E., Hoshi, K., Warshawsky, H., Ozawa, H., Goltzman, D., and Karaplis, A.C. 1996. Programmed cell death of chondrocytes and aberrant chondrogenesis in mice homozygous for parathyroid hormone-related peptide gene deletion. Endocrinology. 137:5055-5067.

Anderson, H.C. 1969. Vesicles associated with calcification in the matrix of epiphyseal cartilage. J. Cell. Biol. 41:59-72.

Anderson, H.C. 1976. Matrix vesicles calcification. Fed. Proc. 35:105-108

Angel, P., Baumann, I., Stein, B., Delius, H., Rahmsdorf, H.J., and Herrlich, P. 1987. 12-O-tetradecanoyl-phorbol-13-acetate induction of the human collagenase gene is mediated by an inducible enhancer element located in the 5'- flanking region. Mol. Cell. Biol. 7:2256-2266.

Antonsson, P., Heinegård, D., and Oldberg, A. 1989. The keratan sulfate-enriched region of bovine cartilage proteoglycan consists of a consecutively repeated hexapeptide motif. J. Biol. Chem. 264:16170-16173.

Apte, S.S., Mattei, M-G., and Olsen, B.R. 1994. Cloning of the cDNA encoding human tissue inhibitor of metalloproteinases-3 (TIMP-3) and mapping of the TIMP3 gene to chromosome 22. Genomics. 19:86-90.

Apte, S.S., Olsen, B.R., and Murphy, G. 1995. The gene structure of tissue inhibitor of matrix metalloproteinases (TIMP)-3 and its inhibitory activities define a distinct TIMP gene family. J. Biol. Chem. 270:14313-14318.

Arner, E.C., Decicco, C.P., Cherny R., Tortorella, M.D. 1997. Cleavage of native cartilage aggrecan by neutrophil collagenase (MMP-8) is distinct from endogenous cleavage by aggrecanase. J. Biol. Chem. 272:9294-9299.

Arsenault, A.L. 1987. Microvascular organization at the epiphyseal-metaphyseal junction of growing rats. J. Bone Miner. Res. 2:143-149

Arsenault, A.L., and Hunziker, E.B. 1988. Electron microscopic analysis of mineral deposits in the calcifying epiphyseal growth plate. Calcif. Tissue Int. 42:119-126

Atkin, I., Pita, J.L., Ornoy, A., Agundez, A., Castiglione, G., and Howell, D.S. 1985. Effects of vitamin D metabolites on healing of low phosphate, vitamin D-deficient induced rickets in rats. Bone. 6:113-120.

Ballock, R.T., Hydemann., A., Wakefield, L.M., Flanders, K.C., Roberts, A.B., and Sporn, M.B. 1993. TGF-beta 1 prevents hypertrophy of epiphyseal chondrocytes: regulation of gene expression for cartilage matrix proteins and metalloproteases. Dev. Biol. 158:414-429.

Ballock, R.T., and Reddi, H.R. 1994. Thyroxine is the serum factor that regulates morphogenesis of columnar cartilage from isolated chondrocytes in chemically defined medium. J. Cell. Biol. 126:1311-1318.

Balmain, N., Leguellec, D., Elkak, A., Nars, G., Toury, R., and Schoevaert D. 1995. Zonal variations of types II, IX, XI collagen mRNAs in rat epiphyseal cartilage chondrocytes:quantitative evaluation of in situ hybridization by image analysis of autoradiography. Cellular and Molecular Biology. 41:197-212.

Balwin, C.T., Reginato, A.M., and Prockop, D.J. 1989. A new epidermal growth factor-like repeat in the human core protein for the large cartilage-specific proteoglycan. J. Biol. Chem. 264:15747-15750.

Banda, M.J., Werb, Z., and McKerrow, J.H. 1987. Elastin Degradation. Methods in Enzymology. 144:288-305.

Becker, J.W., Mary, A.I., Rokosz, L.L., Axel, M.G., Burbaum, J.J., Fitzgerald, P.M.D., Cameron, P.M., Esser, C.K., Hagmann, W.K., Hermes, J.D., and Springer, J.P. 1995. Stromelysin-1: Three-dimensonal structure of the inhibited catalytic domain and the C-truncated proenzyme. Protein Sci. 4:166-1976

Benbow, U., and Brinckerhoff, C.E. 1997. The AP-1 site and MMP gene regulation: What is all the fuss about? Matrix Biol. 15:519-526.

Bernard, M., Yoshioka, H., Rodriguez, E., van der Rest, M., Kimura, T., Ninomiya, Y., Olsen, B.R., and Ramirez, F. 1988. Cloning and sequencing of pro-α1(XI) collagen cDNA demonstrates that type XI belongs to the fibrillar class of collagens and reveals that the expression of the gene is not restricted to cartilagineous tissue. J. Biol. Chem. 263:17159-17166.

Bigg, H.F., Shi, Y.E., Liu, Y.E., Steffenson, B., and Overall, C.M. 1997. Specific, high affinity binding of tissue inhibitor of metalloproteinase-4 (TIMP-4) to the COOH-terminal hemopexin-like domain of human gelatinase A. J. Biol. Chem. 272:15496-15500.

Billinghurst, R.C., Dahlberg, L., Ionescu, M., Reiner, A., Bourne, C., Rorabeck, P., Mitchell, P., Hambor, J., Dikemann, O., Tschesche, H., Chen, J., Van Wart, H., and Poole, A.R. 1997. Enhanced cleavage of type II collagen by collagenases in osteoarthritic articular cartilage. J. Clin. Invest. 99:1534-1545.

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.

Blair, H.C., Dean, D.D., Howel, D.S., Teitelbaum, S.L., and Jeffrey, J.J. 1989. Hypertrophic chondrocytes produce immunoreactive collagenase in vivo. Conn. Tissue. Res. 23:65-73.

Blundell, T.L., 1994. Metalloproteinase superfamilies and drug design. Strut. Biol. 1:73-75.

Böhme, K., Coscience-Egli, M., Tschan, T., Winterhalter, K.H., and Bruckner, P. 1989. Induction of proliferation or hypertrophy of chondrocytes in serum-free culture: the role of insulin-like growth factor-I, insulin or thyroxine. J. Cell. Biol. 116:1035-1042.

Bonnet, F., Pèrin, J., Lorenzo, F., Jollès, J., and Jollès, P. 1986. An unexpected sequence homology between link proteins of the proteoglycan complex and the immunoglobulin-like proteins. Biochim. Biophys. Acta. 873:152-155.

Borden, P., Solymar, D., Sucharczuk, A., Lindman, B., Cannon, P., and Heller, R.A. 1996. Cytokine control of interstitial collagenase and collagenase-3 gene expression in human chondrocytes. J. Biol. Chem. 271:23577-23581.

Bottomley, K.M., Borkakoti, N., Bradshaw, D., Brown, P.A., Broadhurst, M.J., Budd, J.M., Elliott, L., Eyers, P., Hallam, T.J., Handa, B.K., Hill, C.H., James, M., Lahm, H-W., Lawton, G., Merritt, J.E., Nixon, J.S., Röthlisberger, U., Whittle, A., and Johnson, W.H. 1997. Inhibition of bovine nasal cartilage degradation by selective matrix metalloproteinase inhibitors. Biochem. J. 323:483-488.

Bourdon, M., Oldberg, A., Piersbacher, M., and Rouslahti, E. 1985. Molecular cloning and sequence analysis of a chondroitin sulfate proteoglycan cDNA. Proc. Natl. Acad. Sci. USA. 82:1321-1325.

Bourdon, M., Krusius, T., Campbell, S., Schwartz, N., and Ruoslahti, E. 1987. Identification and synthesis of a recognition signal for the attachment of glycosaminoglycans to proteins. Proc. Natl. Acad. Sci. USA. 84:3194-3198.

Bowness, J and Lee, K. 1967. Effects of chondroitin sulphates on mineralization in vitro. Biochem. J. 103:382-390.

Boyan, B., Schwartz, Z., Carnes, D., and Ramirez, V. 1988. The effects of vitamin D metabolites on the plasma and matrix vesicle membranes of growth and resting cartilage cells in vitro. Endocrinology. 122:2851-2860.

Boyde, A., and Shapiro, M. 1980. Energy dispersive X-ray elemental analysis of isolated epiphyseal growth plate chondrocyte fragments. Histochemistry. 69:85-94.

Brown, R.A., Hembry, R.M., and Reynolds, J.J. 1989. Immonulocalization of metalloproteinases and their inhibitor in the rabbit growth plate. J. Bone Jt. Surg. American. Vol. 71:580-593.

Brown, R.A., Kayser, M., Mclaughlin, B., and Weiss, J.B. 1993. Collagenase and gelatinase production by calcifying growth plate chondrocytes. Exp. Cell Res. 208:1-9.

Buckwalter, J., Rosenberg, L., and Tang, L. 1984. The effect of link protein on proteoglycan aggregate structure. An electron microscopic study of the molecular architecture and dimensions of proteoglycan aggregates reassembled from the proteoglycan monomers and link proteins of bovine fetal ephiphyseal cartilage. J. Biol. Chem. 259:5361-5363.

Cao, J., Sato, H., Takino, T., and Seiki, M. 1995. The C-terminal region of membrane type matrix metalloproteinase is a functional transmembrane domain required for pro-gelatinase A activation. J. Biol. Chem. 270:801-805.

Cancedda, F.D., Gentili, C., Manduca, P., and Cancedda, R. 1992. Hypertrophic chondrocytes undergo further differentiation in culture. J. Cell. Biol. 117:427-435.

Cawston, T.E., Ellis, A.J., Humm, G., Lean, E., Ward, D., Curry, V. 1995. Interleukin-1 and oncostatin M in combination promote the release of collagen fragments from bovine nasal cartilage in culture. Biochem. Biophys. Res. Commun. 215:377-85.

Chan, D., Cole, W.G., Rogers, J.G., and Bateman, J.F. 1995. Type X collagen mutimer assembly in vitro is prevented by a gly618 to val mutation in the a1(X) NC1 domain resulting in Schmid metaphyseal chondrodysplasia. J. Biol. Chem. 270:4558-4562.

Carey, D.E., Alini, M., Matsui, Y., and Poole, A.R., 1993. Density gradient separation of growth plate chondrocytes. In vitro. Cell Dev. Biol. 29A:117-119.

Chichester, C.O., Barrach, H-J. Srinivas, G.R., and Mitchell, P. 1996. Immunological detection of type II collagen degradation: use in the evaluation of anti-arthritic therapies. J. Pharm. Pharmacol. 48:694-98.

Chomczynski, P., and Sacchi, N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162:156-159.

Chopra, I.J. 1977. A study of extrathyroidal conversion of thyroxine (T_4) to 3,3', 5-triiodothyronine (T_3) in vitro. Endocrinology. 101:453-463.

Chubinskaya, S., Huch, K., Mikecz K., Cs-Szabo, G., Hasty, K.A., Kuettner, K.E., and Cole, A.A. 1996. Chondrocyte matrix metalloproteinase-8: up-regulation of neutrophil collagenase by interleukin-1β in human cartilage from knee and ankle joints. Lab. Invest. 74:232-40.

Clark, I.M., and Cawston, T.E. 1989. Fragments of human fibroblast collagenase. Purification and characterization. Biochem. J. 263:201-206.

Clarke, N.J., O'Hare, M.C., Cawston, T.E., and Harper, G.P. 1990. Nucleotide sequence for porcine type I collagenase, obtained by PCR. Nucleic Acids Res. 18:6703.

Cole, A.A., Chubinskaya, S., Luchene, L.J., Chlebek, K., Orth, M.W., Grenwald, R.A., Kuettner, K.E., and Schmid, T.M. 1994. Doxycycline disrupts chondrocyte differentiation and inhibits cartilage matrix degradation. Arthritis Rheum. 37:1727-1734.

Cole, A.A., Chubinskaya, S., Schumacher, B., Huch, K., Cs-Szabo, G, Yao, J., Mikecz, K., Hasty, K.A., and Kuettner, K.E. 1996. Chondrocyte matrix metalloproteinase-8: human articular chondrocytes express neutrophil collagenase. J. Biol. Chem. 271:11023-11026.

Collier, I.E., Wilhelm, S.M., Eisen, A.Z., Marmer, B.L., Grant, G.A., Seltzer, J.L., Hunziker, E.B., Schenk, R.K., and Cruz-Orive, L-M. 1987. Quantitation of chondrocyte performance in growth plate cartilage during longitudinal bone growth. J. Bone Jt. Surg. American vol. 69:162-172.

Corvol, M., Ulmann, A., and Garabedian, M. 1980. Specific nuclear uptake of 24, 25 dihydroxycholefcalciferol: a vitamin D_3 metabolite biologically active in cartilage. FEBS Lett. 116:273-278.

Davies, S., Cole, A., and Schmid, T. 1996. Doxycycline inhibits type X collagen synthesis in avian hypertrophic chondrocyte cultures. J. Biol. Chem. 271:25966-25970.

Dean, D.D., Muniz, O.E., Howell, D.S. 1989. Association of collagenase and tissue inhibitor of metalloproteinases (TIMP) with hypertrophic cell enlargement in the growth plate. Matrix. 9:366-375.

Dean, D.D., and Woessner, Jr. J.F., 1985. A sensitive, specific assay for tissue collagenase using telopeptide-free [^{3}H] acetylated collagen. Anal. Biochem. 148:174-81.

Dean, D.D., Muniz, O.E., Woessner, Jr., J.F., and Howell, D.S., 1990. Production of collagenase and tissue inhibitor of metalloproteinases (TIMP) by rat growth plates in culture. Matrix. 10:320-330.

de Bernard, B., Bianco, P., Bonucci, E., Constantini, M., Lunazzi, G.C., Martinuzzi, P., Modricky, C., Moro, L., Panfili, E., Polleslo, P., Stagni, N., and Vittur, F. 1986. Biochemical and immunohistochemical evidence that in cartilage an alkaline phosphatase is a Ca^{2+} -binding glycoprotein. J. Cell. Biol. 103:1615-1623.

DeClerck, Y.A., Yean, T.D., Lu, H.S., Ting, J., and Langley, K.E. 1991. Inhibition of autoproteolytic activation of interstitial procollagenase by recombinant metalloproteinase inhibitor MI/TIMP-2. J. Biol. Chem. 266:3893-3899.

DeClerck, Y., Szpirer, C., Aly, M.S., Cassiman, J.-J., Eeckhout, Y., and Rousseau, G. 1992. The gene for tissue inhibitor of metalloproteinases-2 is localized on human chromosome arm 17q24. Genomics. 14:782-784.

Devarajan, P., Johnston, J.J. Ginsber, S.S., VanWart, H.E., and Berliner, N. 1992. Structure and expression of neutrophil gelatinase cDNA. Identity with type IV collagenase from HT1080 cells. J. Biol. Chem. 267:25228-25232.

Dhanaraj, V., Ye, Q-Z., Johnson, L.L., Hupe, D.J., Ortwine, D.F., Dunbar, Jr. J.B., Rubin, J.R., Pavlovsky, A., Humblet, C., and Blundell, T.L. 1996. X-ray structure of a hydroxamate inhibitor complex of stromelysin catalytic domain and its comparison with members of the zinc metalloproteinase superfamily. Structure. 4:375-386.

DiMartino, M.J., Wolff, C.E., High, W., Crimmin, M.J., and Galloway, W.A. 1991. Anti-inflammatory and chondroprotective activities of a potent metalloproteinase inhibitor. J. Cell. Biochem. Suppl. 19E:179-182

DiPasquale, G., Caccese, R., Pasternak, R., Conaty, J., Hubbs, S., and Perry, K. 1986. Proteoglycan- and collagen-degrading enzymes from human interleukin 1-stimulated chondrocytes from several species: proteoglycanase and collagenase inhibitors as potentially new disease-modifying antiarthritic agents. Proc. Soc. Exp. med. 183:262-67.

Docherty, A.S..D., O'Connell, J., Crabbe, T., Angal, S., and Murphy, G. 1992. The matrix metalloproteinases and their natural inhibitors: prospects for treating degenerative tissue diseases. TIBS. 10:200-20.

Doege, K.J., Sasaki, M., Kimura, T., and Yamada, Y. 1991. Complete coding sequence and deduced primary structure of the human cartilage large aggregating proteoglycan, Aggrecan. J. Biol. Chem. 266:894-902.

Dott, N.M. 1923 Investigation Into the function of the pituitary and thyroid glands. J. Exp. Physiol. 13:241-282.

Dziewiatkowski, D.D. 1987. Binding of calcium by proteoglycans in vitro. Calcif. Tissue Res. 16:111-120.

Ewenson, A., Laufer, R., Frey, J., Chorev, M., Selinger, Z., and Gilon, C. 1992. Dehydro keto methylene and keto methylene analogues of substance P. Synthesis and biological activity. Eur. J. Med. Chem. 27:179-186.

Eyre, D.R., Apone, S., Wu, J.J., Ericsson, L.H., and Walsh, K.A. 1987. Collagen type IX: evidence for covalent linkages to type II collagen in cartilage. FEBS Lett. 220, 337-341.

Farndale, R.W., Buttle, D.J., and Barrett, A.J. 1986. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. Biochem. Biophys. Acta. 883:173-177.

Farnum C.E., Wilsman N.J. 1987. Morphologic stages of the terminal hypertrophic chondrocyte of growth plate cartilage. Anat. Rec. 219:221-232.

Fassler, R., Schnegelsberg, P.N.J., Dausman, J., Shinya, T., Maragaki, Y., McCarthy, M.T., Olsen, B.R., and Jaenisch, R. 1994. Mice lacking a1(IX) collagen develop noninflammatory degenerative joint disease. Proc. Natl. Acad. Sci. USA 91:5070-5074.

Felbor, U., Stöhr, H., Amann, T., Schönherr, U., and Weber, B.H.F. 1995. A novel Ser156Cys mutation in the tissue inhibitor of metalloproteinases-3 (TIMP3) in Sorsby's fundus dystrophy with unusual clinical features. Hum. Mol. Genet. 4:2415-1416.

Fine, N., Binderman, I., Somjen, D., Earon, Y., Edlestein, S., and Weisman, Y. 1985. Autoradiographic localization of 24,25-dihydroxyvitamin D_3 in epiphyseal cartilage. Bone 6:99-106.

Fini, M.E., Pluciniska, I.M., Mayer, A.S., Gross, R.H., and Brinckerhoff, C.E. 1987. A gene for rabbit synovial cell collagenase: Member of a family of metalloproteinases that degrade the connective tissue matrix. Biochemistry 26:6156-6165.

Flannery, C.R., Lark, M.W., and Sandy, J.D., 1992. Identification of a stromelysin cleavage site within the interglobular domain of human aggrecan. Evidence for proteolysis at this site in vivo in human cartilage. J. Biol. Chem. 267:1008-1014.

Fosang, A. J., and Hardingham, T.E., 1988. Isolation of the N-terminal globular protein domains from cartilage proteoglycans. Biochem. J. 261:801-9.

Fosang, A.J., and Hardingham, T.E., 1991 I-C-6 epitope in cartilage proteoglycan G2 domain is masked by keratan sulfate. Biochem. J. 273:369-373.

Fosang, A.J., Neame, P.J., Last, K., Hardhingham, T.E., Murphy, G., and Hamilton, J.A. 1992. The interglobular domain of cartilage aggrecan is cleaved by PUMP, gelatinases, and cathepsin B. J. Biol. Chem. 267:19470-19474.

Fosang, A.J., Last, K., Knäuper, V., Neame, P.J., Murphy, G., Hardingham, T.E., Tschesche, H., and Hamilton, J.A. 1993. Fibroblast and neutrophil collagenases cleave at two sites in the cartilage aggrecan interglobular domain. Biochem. J. 295:273-276.

Fosang, A.J., Last, K., Neame, P.J., Murphy, G., Knäuper, V., Tschesche, H., Hughes, C.E., Caterson, B., and Hardingham, T.E., 1994. Neutrophil collagenase (MMP-8) cleaves at the aggrecanase site E373-A374 in the interglobular domain of cartilage aggrecan. Biochem. J. 305:347-351.

Fosang, A.J., Last, K., Knäuper, V., Murphy, G., and Neame, P.J., 1996. Degradation of cartilage aggrecan by collagenase-3 (MMP-13). FEBS Lett. 380:17-20.

Franzen, A., Heinegård D., Reiland, S., Olsson, S-E. 1982. Proteoglycans and calcification of cartilage in the femoral head epiphysis of the immature rat. J. Bone. Jt. Surg. American vol. 64:558-566.

Freije, J.M., Diez-Itza, I., Balbin, M., Sanchez, L.M., Blasco, R., Tolivia, J., and Lopez-Otin, C. 1994. Molecular cloning and expression of collagenase-3, a novel human matrix metalloproteinase produced by breast carcinomas. J. Biol. Chem. 269:16766-16773.

Freshney, R.I. 1987. Culture of animal cells. A manual of basic technique. 2nd ed. Alan R. Liss, Inc., New York, New York. pp. 257-288

Furoto, D.K., and Miller, E.J. 1983 Different levels of glycosylation contribute to the heterogeneity of α1(II) collagen chains derived from a transplantable rat chondrosarcoma. Arch. Biochem. Biophys. 226:604-611.

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.

Gerstenfeld, L.C., Kelly, C.M., von Deck, M., Lian, J.B. 1990a. Effect of 1,25 dihydroxyvitamin D_3 on induction of chondrocyte maturation in culture: extracellular matrix gene expression and morphology. Endocrinology. 126:1599-1609.

Gerstenfeld, L., Kelly, C.M., von Deck, M., Lian, J.B., 1990b. Comparative morphological and biochemical analysis of hypertrophic, nonhypertrophic and 1,25 $(OH)_2D_3$ treated non-hypertrophic chondrocytes. Connect. Tissue. Res. 24:29-39.

Gibson, G.J. and Flint, M.H. 1985. Type X collagen synthesis by chick sternal cartilage and its relationship to endochondral development. 1985 J. Cell. Biol. 101:277-284.

Gibson, G.J., Bearman, C.H., and Flint, H.M. 1986. The immunoperoxidase localization of type X collagen in chick cartilage. Coll. Rel. Res. 6:163-184

Goldberg, G.I., Marmer, B.L., Grant, G.A., Eisen, A.Z., Wilhelm, S., and He, C. 1989. Human 72-kilodalton type IV collagenase forms a complex with a tissue inhibitor of matrix metalloproteinases designated TIMP-2. Proc. Natl. Acad. Sci. USA. 86:8207-8211

Goldstein, L.A., Zhou, D.F.H., Picker, L.J., Minty, C.N., Bargatze, R.F., Ding, J.F., and Butcher, E.C. 1989. A human lymphocyte homing receptor, the Hermes antigen, is related to cartilage proteoglycan core and link proteins. Cell. 56:1063-72.

Goltzman, D., Peytremann, A., Callahan E., Tregear, G.W., and Potts, Jr., J.T. 1975. Analysis of the requirements for parathyroid hormone action in renal membranes with the use of inhibiting analogues. J. Biol. Chem. 250:3199-3203.

Gordon, M.K., and Olsen, B.R. 1990. The contribution of collagenous proteins to tissue-specific matrix assemblies. Curr. Opin. Cell Biol. 2:833-838.

Gray, R.D., Miller, R.B., and Spatola, A.F. 1987. MMP inhibitors **In**: Proteinases in Biological Control and Biotechnology. D.D. Cunningham, and G.L. Long, eds. Vol.57, pp. 37-44 Alan R. Liss, New York, New York.

Greene, J., Wang, M., Liu, Y.E., Raymond, L.A., Rosen, C., and Shi, Y.E. 1996. Molecular cloning and characterization of human tissue inhibitor of metalloproteinase 4. J. Biol. Chem. 271:30375-30380.

Gross, J. and Lapiere, C.M. 1962. Collagenolytic activity in amphibian tissues: a tissue culture assay. Proc. Natl. Acad. Sci. USA. 54:1197-1204.

Hanglow, A.C., Lugo, A., Walsky, R., Finch-Arietta, M., Lusch, L., Visnick, M., and Fotouhi, N. 1993. Peptides based on the conserved prodomain sequence of matrix metalloproteinases inhibit human stromelysin and collagenase. Agents Actions 39:C148-150.

Hardingham, T.E., and Fosang, A.J. 1992. Proteoglycans-Many forms and many functions. FASEB J. 6:861-870.

Hargest, T.E., Gay, C.V., Schraer, H., and Wasserman, A.J. 1985. Vertical distribution of elements in cells and matrix of epiphyseal growth plate cartilage determined by quantitative electron probe analysis. J. Histochem. Cytochem. 33:275-286.

Hascall, V., and Sajdera, S. 1969. Protein-polysaccharide complex from bovine nasal cartilage: the function of glycoprotein in the formation of aggregates. J. Biol. Chem. 244:2384-96.

Hascall, V.C., and Heinegård, D. 1974. Aggregation of cartilage proteoglycans: The role of hyaluronic acid. J. Biol. Chem. 249:4332-4441.

Hasty, K.A., Pourmotabbled, T.F., Goldberg, G.I., Thompson, J.P., Spinella, D.G., Stevens, R.M., and Mainardi, C.L. 1990. Human neutrophil collagenase: a distinct gene product with homology to other matrix metalloproteinases. J. Biol. Chem. 265:11421-24.

Henriet, P., Rousseau, G.G., and Eeckhout, Y. 1992. Cloning and sequencing of mouse collagenase cDNA. Divergence of mouse and rat collagenases from the other mammalian collagenases. FEBS. Lett. 310:175-178.

Hinek, A., and Poole, A.R. 1988. The influence of vitamin D metabolites on the calcification of cartilage matrix and the C-propeptide of type II collagen (chondrocalcin). J. Bone Miner. Res. 3:421-429.

Hirschman, A., and Dziewiatkowski, D.D. 1966 Protein polysaccharide loss during endochondral ossification: immunochemical evidence. Science. 54:393-395.

Hollander, A.P., Heathfield, T.F., Webber, C., Iwata, Y., Bourne, R., Rorabeck, C., and Poole, A.R. 1994. Increased damage to type II collagen in osteoarthritic articular cartilage detected by a new immunoassay. J. Clin. Invest. 93:1722-1732.

Howell, D.A., and Carlson, L. 1968. Alterations in the composition of growth cartilage septa during calcification studied by microscopic X-ray elemental analysis. Exp. Cell. Res. 51:185-196.

Howell, D.S., and Dean, D.D. 1992. The biology, chemistry, and biochemistry of the mamamalian growth plate. **In:** Disorders of Bone and Mineral Metabolism. F.L. Coe and H.L. Fauys, eds. Raven Press Ltd. pp. 313-353.

Hoyland, J.A., Thomas, J.T., Donn, R., Marriott, A., Ayad, S., Boot-Handford, R.P., Grant, M.E., and Freemont, A.J. 1991. Distribution of type X collagen mRNA in normal and osteoarthritic cartilage. Bone and Mineral, 15:151-164.

Hunter, G.K. 1987. An ion-exchange mechanism of cartilage calcification. Connet. Tissue. Res. 16:111-120.

Huhtala, P., Humphries, M.J., McCarthy, J.B., Tremble, P.M., Werb, Z., and Damsky, C.H. 1995. Cooperative signaling by α5β1 and α4β1 integrins regualates metalloproteinase gene expression in fibroblasts adhering to fibronectin. J. Cell. Biol. 129:867-879.

Hunter, G.K., Wong, K.S., and Kim, J.J. 1988. Binding of calcium to glycosaminoglycans: an equilibrium dialysis study. Arch. Biochem. Biophys. 260:161-167.

Hunziker, E.B., Schenk, R.K., and Cruz Orive, L.M. 1987. Quantitation of chondrocyte performance in growth-plate cartilage during longitudinal bone growth. J. Bone Jt. Surg. American Vol. 69:162-173.

Hunziker, E.B., Herrmann, W., Cruz Orive, L.M., and Arsenault, A.L. 1989. Image analysis of electron micrographs relating to mineralization in calcifying cartilage: theoretical considerations. J. Electron Microsc. Tech. 11:9-15.

Iwamoto, M., Sato, K., Nakashima, K., Shimazu, A., and Kato, Y. 1989. Hypertrophy and calcification of rabbit permanent chondrocytes in pelleted cultures: synthesis of alkaline phosphatase and 1.25-dihydroxycholecalciferol receptor. Dev. Biol. 136:500-507.

Iyama, K.I., Kitaoka. M., Monda, M., Ninomiya, Y., and Hayashi, M. 1994. Co-expression of collagen types II and X mRNAs in newly formed hypertrophic chondrocytes of the embryonic chick vertebral body demonstrated by double-flourescence in situ hybridization. Histochemical J. 26: 844-849.

Johansson, N., Saarialho-Kere, U., Airola, K., Herva, R., Nissinen, L., Westermarck, J., Vuorio, E., Heino, J., and Kähäri, V-M. 1997. Collagenase-3 (MMP-13) is expressed by hypertrophic chondrocytes, periosteal cells, and osteoblasts during human fetal bone development. Dev. Dynamics. 208:387-397.

Jüppner. H. 1994. Molecular cloning and characterization of a parathyroid hormone (PTH)/PTH-related peptide (PTHrP) receptor: A member of an ancient family of G protein-coupled receptors. Curr. Opin. Nephrol. Hypertens. 3:371-378.

Jüppner. H. 1995. Functional Properties of the PTH/PTHrP receptor. Bone 17: Suppl. 2 : 39S-42S.

Karaplis, A.C., Luz, A., Glowacki, J., Bronson, R.T., Tybulewicz, V.L.J., Kronenberg, H.M., and Mulligan, R.C. 1994. Lethal skeletal dysplasia from targeted disruption of the parathyroid hormone-related peptide gene. Genes and Dev. 8:277-289.

Keene, D.R., Oxford, J.T., and Morris, N.P. 1995. Utrastructural localization of collagen types II, IX and XI in the growth plate of human rib and fetal bovine epiphyseal cartilage: type XI is restricted to thin fibrils. J. Histochem. Cytochem. 43:967-79.

Kerr, L.D., Miller, D.B., Matrisian, L.M. 1990. TGF-beta 1 inhibition of transin/stomelysin gene expression is mediated through a FOS binding site. Cell. 57:267-278.

Kember, N.F. 1960. Cell division in endochondral ossification: a study of cell proliferation in rat bones by the method of tritiated thymidine autoradiography. J. Bone Jt. Surg. Br. Vol. 42:824-839.

Kessler, E., Takahara, K., Biniaminov, L., Brusel, M., and Greenspan, D.S. 1996. Bone morphogenic protein-1: The type I collagen C-proteinase. Science. 271:360-362.

Kimura, T., Cheah, K.S.E., Chan, S.D.H., Lui, V.C.H., Mattei, M-G., van der Rest, M., Ono, K., Solomon, E., Ninomiya, Y., and Olsen, B.R. 1989. The human α2(XI) chain. Molecular cloning of cDNA and genomic DNA reveals characteristics of a fibrillar collagen with differences in genomic organization. J. Biol. Chem. 264: 13910-13916.

Kirsch, T., and von der Mark, K. 1991. Isolation of human type X collagen and immunolocalization in fetal human cartilage. Eur. J. Biochem. 196:575-580.

Kirsch, T., and Wuthier, R.E. 1994. Stimulation of calcification of growth plate cartilage matrix vesicles by binding to type II and X collagen. Biol. Chem. 269:11462-11469.

Kirsch, T., Ishikawa, Y., Mwale, F., and Wuthier, R.E. 1994. Roles of the nucleational core complex and collagens (types II and X) in calcification of growth plate cartilage matrix vesicles. J. Biol. Chem. 269:20103-20109.

Knäuper, V., Osthues, A., DeClerck, Y.A., Langley, K.E., Bläser, J., and Tschesche, H. 1993. Fragmentation of human polymorphonuclear-leucocyte collagenase. Biochem. J. 291:847-854.

Knäuper, V.C., Lopez-Otin, C., Smith, B., Knight, G., and Murphy, G. 1996a. Biochemical characterization of human collagenase-3. J. Biol. Chem. 271:1544-1540.

Knäuper, V.C., Will, H., Lopez-Otin, C., Smith, B., Atkinson, S.J., Stanton, H., Hembry, R.M., and Murphy, G. 1996b. Cellular mechanisms for human procollagenase-3(MMP-13) activation-evidence that MT1-MMP (MMP-14) and gelatinase A (MMP-2) are able to generate active enzyme. J. Biol. Chem. 271:17124-17131.

Knäuper, V.C., Cowell, S., Smith, B., Lopez-Otin, C., O' Shea, M., Morris, H., Zardi, L., and Murphy, G. 1997. The role of the C-terminal domain of human collagenase-3 (MMP-13) in the activation of procollagenase-3, substrate specificity and tissue inhibitor of metalloproteinase interaction. J. Biol. Chem. 272:7608-7616.

Kozaci, L.D., Buttle, D.J., and Hollander, A.P., 1997. Degradation of type II collagen, but not proteoglycan, correlates with matrix metalloproteinase activity in cartilage explant cultures. Arthritis. Rheum. 40: 164-74.

Krane, S.M., Byrne, M.H., Lemaitre, V., Henriet, P., Jeffrey, J.J., Witter, J.P., Liu, X., Wu, H., Jaenisch, R., and Eeckhout, Y. 1996. Different collagenases gene products have different roles in degradation of type I collagen. J. Biol. Chem. 271:28509-28515.

Kremer, R., Sebag, M, Champigny, C., Meerovitch, K., Hendy, G.N., White, J., and Goltzman, D. 1996. Identification and characterization of 1,25-dihydroxyvitamin D_3-responsive repressor sequences in the rat parathyroid hormone-related peptide gene. J. Biol. Chem. 271:16310-16316.

Kronberger, A., He. C., Bauer, E.A., and Goldberg, G.I. 1988. H-ras oncogene transformed bronchial epithelial cells (TBE-1) secrete a single metalloprotease capable of degrading basement membrane collagen. J. Biol. Chem 263:6579-6597.

Kwan, A.P.L., Cummings, C.E., Chapman, J.A., and Grant, M.E. 1991. Macromolecular organization of chicken type X collagen in vitro. J. Cell Biol. 114:597-604.

Kwan, K.M., Pang, M.K.M., Zhou, S., Cowan, S.K., Kong, R.Y.C., Pfordte, T.P., Olsen, B.R., Sillence, D.O., Tam, P.P.L., and Cheah, K.S.E. 1997. Abnormal compartmentalization of cartilage matrix components in mice lacking collagen X: implications for function. J. Cell Biol. 136:459-471.

Labarca, C., Paigen, K. 1980. A single, rapid and sensitive DNA assay procedure. Anal. Biochem. 102:344-352.

Lachman, R.S., Rimoin, D.L., and Spranger, J. 1988. Metaphyseal chondrodysplasia, Schmid type clinical and radiographic deliniation with a review of literature. Pediatr. Radiol. 18:93-102.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Landis, W.J., and Glimcher, M.J. 1982. Electron optical and analytical observations of rat growth plate cartilage prepared by ultramicrotomy: the failure to detect a mineral phase in matrix vesicles and identification of heterodispersed particles as the initial solid phase of calcium phosphate deposited in the extracellular matrix. J. Utrastruct. Res. 78:227-268.

Langholz, O, Röckel, D., Mauch, C., Kozowska, E., Bank, I., Krieg, T., and Eckes, B. 1995. Collagen and collagenase gene expression in three-dimensional collagen lattices are differentially regulated by α1β1 and α2β1 integrins. J. Cell. Biol. 131:1903-1915.

Lanske, B., Karaplis, A.C., Lee, K., Luz, A., Vortkamp, A., Pirro, A., Karperien, M., Defize, L.H.K., Ho. C., Mulligan, R.C., Abou-Samra, A-B., Jüppner, H., Segre, G.V., and Kronenberg, H.M. 1996. PTH/PTHrp receptor in early development and indian hedgehog-regulated bone growth. Science. 273:663-666.

Lark, M.W., Gordy, J.T., Weidner, J.R., Ayala, J., Kimura, J.H., Williams, H.R., Mumford, R.A., Flannery, C.R., Carlson, S.S., Iwata, M., and Sandy, J.D. 1995. Cell mediated catabolism of aggrecan. Evidence that cleavage at the "aggrecanase" site (Glu373-Ala374) is a primary event in proteolysis of the interglobular domain. J. Biol. Chem. 270:2550-2556.

Larson, P.R., Silva, J.E., and Kaplan, N.M., 1981. Relationship between circulating and intracellular thyroid hormones: physiological and clinical implications. Endocr. Rev. 2:87-102.

Lasky, R.A., and Hills, A.D. 1975. Quantitative film detection of ^{3}H and ^{14}C in polyacrylamide gels by fluorography. Eur. J. Biochem. 56:335-341.

Lee, E.R., Matsui, Y., and Poole, A.R. 1990 Immunochemical and immunocytochemical studies of the c-propeptide of type II procollagen in chondrocytes of the growth plate. J. Histochem. Cytochem. 38:659-673.

Lee, K.C., Lanske, B., Karaplis, A.C., Deeds, J.D., Kohno, H., Nissenson, R.A., Kronenberg, H.M., and Segre, G.V. 1996. Parathyroid hormone-related peptide delays terminal differentiation of chondrocytes during endochondral bone development. Endocrinology. 137:5109-5118.

Lelievre, Y., Bouboutou, R., Boiziau, J., Faucher, D., Achard, D., and Cartright, T. 1990. Low molecular weight, sequence based, collagenase inhibitors selectively block the interaction between collagenase and TIMP (tissue inhibitor of matrix metalloproteinases). Matrix, 10:292-299.

Lemaitre, V., Jungbluth, A., and Eeckhout, Y. 1997. The recombinant catalytic domain of mouse collagenase-3 depolymerizes type I collagen by cleaving its aminotelopeptides. Biochem. Biophys. Res. Comm. 230:202-205.

Leonard, J.L., and Visser, T.J. 1986 Biochemistry of deiodination. **In**: Hennemann G (ed). Thyroid Hormone Metabolism. Marcel Dekker. New York, pp. 186-230.

Lewinson, D., Harel, Z., Shenzer, P., Sibermann, M., and Hochberg, Z. 1989. Effect of thyroid hormone and growth hormone on recovery from hypothyroidism of epiphyseal growth plate cartilage and its adjacent bone. Endocrinology. 124:937-945.

Lewinson, D., and Sibermann, M. 1992. Chondroclasts and endothelial cells collaborate in the process of cartilage resorption. Anat. Res. 233:504-514.

Lewinson, D., Bialik, G.M., and Hochberg, Z. 1994. Differential effects of hypothroidism on the cartilage and the osteogenic process in the mandibular condyle: recovery by growth hormone and thyroxine. Endocrinology. 135: 1504-1510.

Li, S.W., Sieron, A., Fertala, A., Yoshio, H., Arnold, W.V., and Prockop, D.J. 1996. The C-proteinase that processes procollagens to fibrillar collagens is identical to the protein previously identified as bone morphogenic protein-1. Proc. Natl. Acad. Sci. USA. 93:5127-5130.

Li, Y., Lacerda, A., Warman, M.L., Beier, D.R., Yoshioka, H., Ninomiya, Y., Oxford, J.T., Morris, N.P., Andrikopoulos, K., Ramirez, F., Wardell, B.B., Lifferth, G.D., Teuscher, C., Woodward, S.R., Taylor, B.A., Seegmiller, R.E., and Olsen, B.R. 1995. A fibrillar collagen gene, Col11a1, is essential for skeletal morphogenesis. Cell. 80:423-430.

Liu, X., Wu., H., Byrne, M., Jeffrey, J. Krane, S., and Jaenisch, R. 1995. A targeted mutation at the know collagenase cleavage site in mouse type I collagen impairs tissue remodeling. J. Cell. Biol. 130:227-237.

Lohmander, S., and Hjerpe, A. 1975. Proteoglycans of mineralizing rib and epiphyseal cartilage. Biochem. Biophys. Acta. 404:93-109.

LuValle, P., Daniels, K., Hay, H.D., and Olsen, B.R. 1992. Type X collagen is transcriptionally activated and specifically localized during sternal cartilage maturation. Matrix. 12:404-413.

LuValle, P., Iwamoto, M., Pacifici, M. and Olsen, B.R. 1993. Multiple, promotor-specific negative elements restrict type X collagen gene expression to hypertrophic chondrocytes. J. Cell. Biol. 101:277-284.

Mallya, S.K., Mookhtiar, K.A., Goa, Y., Brew, K., Dioszegi, M., Birkedal-Hansen, H., and Van Wart, H.E. 1990. Characterization of 58-kilodalton human neutrophil collagenase: Comparison with human fibroblast collagenase. Biochemistry. 29:10628-10634.

Mallein-Gerin, F and Olsen, B.R. 1993. Expression of simian virus 40 large T (tumor) oncogene in mouse chondrocytes induces cell proliferation without loss of the differentiated phenotype. Proc. Natl. Acad. Sci. USA. 90:3289-3293.

Mangin, M., Ikeda, K., Broadus, A.E. 1990. Structure of the mouse gene encoding parathyroid hormone-related peptide. Gene. 95:195-202.

Marriott, A., Ayad, S., and Grant, M.E. 1991. The synthesis of type X collagen by bovine and human growth-plate chondrocytes. J. Cell Sci. 99: 641-649.

Martin, T.J., Moseley, J.M., and Gillespie, M.T. 1991. Parathyroid hormone-like protein: Biochemistry and molecular biology. Crit. Rev. Biochem. Mol. Biol. 26:377-395.

Matrisian, L.M., 1992. The matrix-degrading metalloproteinases. BioEssays. 14:455-463.

Matsui, Y., Alini, M., Webber, C., and Poole, A.R. 1992. Characterization of aggregating proteoglycans from the proliferative, maturing, hypertrophic, and calcifying zones of the cartilaginous physis. J. Bone. Jt. Surg. American Vol. 73:1064-1074.

Mattei, M-G., Roeckel, N., Olsen, B.R., and Apte, S.S. 1997. Genes of the membrane-type matrix metalloproteinase (MT-MMP) gene family, MMP14, MMP15 and MMP16, localize to human chromosomes 14, 16 and 8 respectively. Genomics. 40:168-169.

Mattot, V., Raes, M.B., Henriet, P., Eeckhout, Y., Stehelin, D., Vanderbunder, B., and Desbiens, X. 1995. Expression of interstitial collagenase is restricted to skeletal tissue during mouse embryogenesis. J. Cell Sci. 108:529-535.

Mauviel, A., Chung, K-Y., Agarwal, A., Tamai, K., and Uitto, J. 1996. Cell-specific induction of distinct oncogenes of the Jun family is responsible for differential regulation of collagenase gene expression by transforming growth factor-β in fibroblasts and keratinocytes. J. Biol. Chem. 271:10917-10923.

Mayne, R., and M.H., Irwin. 1986. Collagen types in cartilage. In Articular Cartilage Biochemistry. K.E. Kuetnner, R. Schleyerbach, and V.C. Hascall, editors. Raven Press, New York, New York. pp. 23-35.

Mayne, R. 1989. Cartilage collagens. Arthritis Rheum. 32:241-246.

Melchiori, A., Albini, A., Ray, J.M., and Stetler-Stevenson, W.G. 1992. Inhibition of tumor cell invasion by a highly conserved peptide sequence from the matrix metalloproteinase enzyme prosegment. Cancer Res. 52:2353-2356.

Mendler, M., Eich-Bender, S.G., Vaughan, L., Winterhalter, K.H., and Bruckner, P., 1989. Cartilage contains mixed fibrils of collagen types II, IX, and XI. J. Cell. Biol. 108:191-197.

Miller, E.J., and Matukas, V.J. 1969. Chick cartilage collagen: a new type of α1 chain not present in bone or skin of the species. Proc. Natl. Acad. Sci. USA. 64:1264-1268.

Miller, E.J. 1971. Isolation and characterization of a collagen from chick cartilage containing three identical α chains. Biochemistry. 10:1652-1659.

Mitchell, N., Shepard, N., and Harrod, J. 1982. The measurement of proteoglycan in the mineralizing region of the growth plate. An electron microscopic and x-ray microanalytical study. J. Bone Jt. Surg. American Vol. 64:32-38.

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. 7:761-68.

Morris N.P., and Bachinger, H.P. 1987. Type XI collagen is a heterodimer with the composition (1α,2α,3α) retaining non-triple-helical domains. J. Biol. Chem. 262:11345-50.

Mort, J.S., Dodge, G.R., Roughley, P.J., Liu, J., Finch, S.J., DiPasquale, G., and Poole, A.R. 1993. Direct evidence for the active metalloproteinases mediating matrix degradation in interleukin 1-stimulated articular cartilage. Matrix 13:95-102.

Muragaki, Y., Nishimura, I., Henney, A., Ninomiya, Y., and Olsen, B.R. 1990. The α1(IX) collagen gene give rise to two different transcripts in both mouse embryonic and human fetal RNA. Proc. Nat. Acad. Sci. USA 87:2400-2404.

Murphy G., Allan, J.A., Willenbrock, F., Cockett, M.I., O'Connell, J.P., and Docherty, A.J.P. 1992. The role of the C-terminal domain in collagenase and stomelysin specificity J. Biol. Chem. 267:9612-9618.

Murphy, G., and Knäuper, V. 1997. Relating matrix metalloproteinase structure to function: why the "hemopexin" domain? Matrix Biology 15:511-518.

Mundlos, S., Engel, H., Michel-Behuke, I., and Zabel, B. 1990. Distribution of type II collagen gene expression during the development of human long bones. Bone 11:275-279.

Nagase, H., Enghild, J.J., Suzuki,K., and Salvesen G., 1990. Stepwise activation mechanisms of the precusor of matrix metalloproteinase-3 (stromelysin) by proteinases and (4-aminophenyl) mercuric acetate. Biochemistry 29:5783-5789.

Neame, P.J. 1993. Extracellular Matrix of Cartilage: Proteoglycans. **In** Joint Cartilage Degradation: Basic and Clinical Aspects. eds. J.F. Woessner Jr. D.S. Howell., Marcel Dekker, New York, New York, pp. 255-294.

Nishimura, I., Muragaki, Y. and Olsen, B.R. 1989. Tissue-specific forms of type IX collagen-proteoglycan arise from the use of two widely separated promoters. J. Biol. Chem. 264:20033-20041.

Nixon, J.S., Bottomley, K.M., Broadhurst, M.J., Brown, P.A., Johnson, W.H., Lawton, G., Marley, J., Sedgwick, A.D., and Wilkinson, S.E., 1991. Potent collagenase inhibitors prevent interleukin-1 induced cartilage degradation in vitro. Int. J. Tissue. React. 13:237-243.

Ohlsson, C., Nilsson, A., Saksson, O., Benthan, J., and Lindahl, A. 1992. Effect of tri-iodothyronine and insulin-like growth factor-I (IGF-I) on alkaline phosphatase activity, [^{3}H] thymidine incorporation and IGF-I receptor mRNA in cultured rat epiphyseal chondrocytes. J. Endocrinol. 135:114-123.

Ohuchi, E., Imai K., Fujii, Y., Sato, H., Seiki, M., and Okada, Y. 1997. Membrane type 1 matrix metalloproteinase digests interstitial collagens and other extracellular matrix macromolecules. J. Biol. Chem. 272:2446-2451.

Orth, M.W., and Cook, M.E. 1994. Avian tibial dyschondroplasia: a morphological and biochemical review of the growth plate lesion and its causes. Vet. Pathol. 31:403-414.

Overall, C.M., and Sodek, J. 1990. Concanavalin A produces a matrix-degradative phenotype in human fibroblasts. Induction and endogenous activation of collagenase, 72- kDa gelatinase, and Pump-1 is accompanied by the suppression of the tissue inhibitor of matrix metalloproteinases. J. Biol. Chem. 265:21141-21151.

Pal, S., Tang, L-H., Choi, H., Haberman, E., Rosenberg, L.C., Reiner, A., and Poole, A.R., 1983. Isolation and characterization of a 35,000 molecular weight subunit fetal cartilage matrix protein. J. Biol. Chem. 258:655-661.

Pei, D., Weiss, S.J. 1995. Furin-dependent intracellular activation of the human stromelysin-3 zymogen. Nature 375:244-247.

Pei, D., Weiss, S.J. 1996. Transmembrane-deletion mutants of the membrane-type metalloproteinases-1 process progelatinase A and express intrinsic matrix-degrading activity. J. Biol. Chem. 271:9135-9140.

Pendas, A.M., Matilla, T., Estiuill, X. and Lopez-Otin, C. 1995. The human collagenase-3 (CL3) gene is located on chromosome 11q22.3. clustered to other members of the matrix metalloproteinase gene family. Genomics 26:615-618.

Pendas, A.M., Santamaria, I., Alvarez, M. V., Pritchard, M., and Lopez-Otin, C. 1996. Fine physical mapping of the human matrix metalloproteinase genes clustered on chomosome 11q22.3. Genomics. 37:266-268.

Poole, A.R., Pidoux, I., and Rosenberg, L.C., 1982. Role of proteoglycans in endochondral ossification: immunofluorescent localization of link protein and proteoglycan monomer in bovine fetal epiphyseal growth plate. J. Cell. Biol. 92:249-260.

Poole, A.R., Pidoux, I., Reiner, A., Choi, H., and Rosenberg, L.C. 1984. Association of an extracellular protein (chondracalcin) with the calcification of cartilage in endochondral bone formation. J. Cell Biol., 98:54-60.

Poole, A.R., and Pidoux, I. 1989. Immunoelectron microscopic studies of type X collagen in endochondral ossification. J. Cell. Biol. 109:2547-2554.

Poole, A.R. 1991. The growth plate: cellular physiology, cartilage assembly and mineralization. **In**: Cartilage: Molecular Aspects. B.K., Hall and S.A., Newman, eds. CRC Press Inc., Boca Raton, Florida. pp. 179-211.

Potts, J.T. Jr., Kronenberg, H.M., and Rosenblatt, M. 1982. Parathyroid hormone: chemistry, biosynthesis, and mode of action. Adv. Protein Chem. 35:323-396.

Puissant C., and Hondebine, L-M. 1990. An improvement of the single-step method of RNA isolation by acid guanidinium thio-cyanate-phenol-chloroform extraction. Biotechniques 8:148-149.

Quarto, R., Campanile, G., Cancedda, R., and Dozin, B. 1992. Thyroid hormone, insulin and glucocorticoids are sufficient to support chondrocyte differentiation to hypertrophy: a serum-free analysis. J. Cell. Biol. 119:989-995.

Quinn, C.O., Scott, D.K., Brinckerhoff, C.E., Matrisian, L.M., Jeffery, J.J., and Partridge, N.C. 1990. Rat collagenase. Cloning, amino acid sequence comparison, and parthyroid hormone regulation in osteoblastic cells. J. Biol. Chem. 265:22343-22347.

Raisz, LG and Kream B.E. 1983a. Regulation of bone formation (first of two parts). N. Engl. J. Med. 309:29-35.

Raisz, LG and Kream B.E. 1983b. Regulation of bone formation (second of two parts). N. Engl. J. Med. 309:83-89.

Rath, N.C., Huff, W.E., Balog, J.M., Bayyari, G.R., and Reddy, R.P. 1997. Matrix metalloproteinase activities in avian tibial dyschondroplasia. Poultry Sci. 76:501-505.

Ray, R.D., Asling, C.W., Walker, D.G., Simpson, M.E., Li, C.H., and Evans, H.M. 1954 Growth and differntiation of the skeleton in thyroidectomized hypophysectomized rats treated with thyroxin, growth hormone and the combination. J. Bone Joint Surg. American. 36:94-103.

Reginato, A.M., and Jimenez, S.A. 1991. Biochemical characterization of the native tissue form of type X collagen from embryonic chick sternal cartilage and identification of a chymotrypsin-sensitive site within its triple-helical domain. Biochem. J. 273:333-338.

Reboul, P., Pelletier, J-P., Tardiff, G., Clouter, J-M., and Martel Pelletier, J. 1996. The new collagenase, collagenase-3, is expressed and synthesized by human chondrocytes but not by synoviocytes. J. Clin. Invest. 97:2011-2019.

Reinemer, P., Grams, F., Huber, R., Kleine, T., Schnierer, S., Piper, M., Tschesche, H., and Bode, W. 1994. Structural implications for the role of the N-terminus in the "superactivation" of collagenases. FEBS Lett. 338:227-233.

Roach, HI., Erenpreisa, J., and Aigner, T. 1995. Osteogenic Differentiation of hypertrophic chondrocytes involves asymmetric cell divisions and apoptosis. J. Cell Biol. 131:483-494.

Roberts, W.H., Borkakoti, N.A., and Johnson, N. 1987. Collagenase inhibitors: Their design and therapeutic use. J. Enzyme Inhibition 2:1-22.

Rosati, R., Horan, G.S.B., Pinero, G.J., Garofalo, S., Keene, D.R., Horton, W.A., De Crombrugghe, B., and Behringer, R.R. 1994. Normal long bone growth and development in type X collagen-null mice. Nature Genet. 8:129-135.

Sambrook, J., Fritsch, E.F., and Maniatis, T. 1986. Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, New York.

Sanchez-Lopez, R., Alexander, C.M., Behrendtsen, O., Breathnach, R., and Werb, Z. 1993. Role of zinc-binding- and hemopexin domain- encoded sequences in the substrate specificity of collagenase and stromelysin-2 as revealed as chimeric proteins. J. Biol. Chem. 268:7238-7247.

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.

Sandell, L.J., Sugai, J.V., and Tripple, S.B. 1994. Expression of collagens I, II, X, and XI and aggrecan mRNAs by bovine growth plate chondrocytes in situ. J. Ortho. Res. 12: 1-14.

Sandy, J.D., Neame, P.J., Boynton, R.E., and Flannery, C.R., 1991. Catabolism of aggrecan in cartilage explants. Identification of a major cleavage site within the interglobular domain. J. Biol. Chem. 266:8683-8685.

Sanger, F., and Coulson, A.R. 1975. A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. J. Mol. Biol. 94:441.

Sarras Jr., M.P. 1996. BMP-1 and the astacin family of metalloproteinases: a potential link between the extracellular matrix, growth factors and pattern formation. Bioessays. 18:439-442.

Schnyder, J., Payne, T., and Dinarello, C. 1987. Human monocyte or recombinant interleukin-1s are specific for the secretion of a metalloproteinase from chondrocytes. J. Immunol. 138:496-503.

Schenk, R.K., Spiro, D., and Wiener, J. 1967. Cartilage resorption in the tibial epiphyseal growth plate of growing rats. J. Cell Biol. 34:275-291.

Schenk, R.K., Wiener, J. and Spiro, D. 1968. Fine structural aspects of vascular invasion of the tibial epiphyseal plate of growing rats. Acta. Anat. 68:1-17.

Scherf, J.P., and Moskalweski, S. 1984. The amount of proteoglycan in cartilage matrix and the onset of mineralization. Metab. Bone Dis. Rel. Res. 5:195-203.

Schmid, T.M., and Lisenmayer, T.F. 1985. Immunohistochemical localization of short chain cartilage collagen (type X) in avian tissues. J. Cell. Biol. 100:598-605.

Schmid, T.M., Mayne, R., Jeffrey, J.J., and Linsenmayer, T.F. 1986. Type X collagen contains two cleavage sites for a vertebrate collagenase. J. Biol. Chem. 261:4184-4189.

Schmid T.M., and Linsenmayer. T.F. 1987. Type X collagen. In Structure and function of collagen types. Mayne, R., and Burgeson, R.E. eds. Academic Press, Orlando, Florida. pp. 223-259.

Schmid, T.M., and Linsenmayer, T.F. 1990. Immunoelectron microscopy of type X collagen: supramolecular forms within embryonic chick cartilage. Dev. Biol. 138:53-62.

Ståhle-Bäckdahl, M., Sandstedt, B., Bruce, K., Lindahl, A., Jiménez, M.G., Vega, J.A., and Lopéz-Otin, C. 1997. Collagenase-3 (MMP-13) is expressed during human fetal ossification and re-expressed in postnatal bone remodeling and in rheumatoid arthritis. Lab. Invest. 76:717-728.

Stoolman, L.M., 1989 Adhesion molecules controlling lymphocyte migration. Cell. 56:907-10.

Schonthal, A., Herrlich, P., Rahmsdorf, H.J., and Ponta, H. 1988. Requirement for fos gene expression in the transcriptional activation of collagenase by other oncogenes and phorbol esters. Cell. 54:32-334.

Seo, E.G., Schwartz, Z., Dean, D.D., Norman, A.W., and Boyan, B.D. 1996. Preferential accumulation in vivo of 24R, 25-dihydroxyvitamin D-3 in growth plate cartilage of rats. Endocrine. 5:147-155.

Shaw, L.M., and Olsen, B.R. 1991. FACIT collagens: diverse molecular bridges in extracellular matrices. Trends Biochem. Sci. 16:191-194.

Schipani, E., Kruse, K., and H. Jüppner. 1995. A constitutively active mutant PTH-PTHrP receptor in Jansen-type metaphyseal chondrodysplasia. Science 268:98-100.

Shapiro, S.D. 1997. Might mice: transgenic technology "knocks out" questions of matrix metalloproteinase function. Matrix Biol. 15:527-533.

Shepard, N., and Mitchell, N. 1985. Ultrastructural modifications of proteoglycans coincident with mineralization in local regions of rat growth plate. J. Bone Jt. Surg. American Vol. 67:455-464.

Sibermann, M. 1983 Hormones and cartilage. **In**: Cartilage Hall B.K. ed. Academic Press, New York, New York. pp.32.

Simpson, M.E., Ashing, C.W., and Evans, H.M. 1950. Some endocrine infleunces on skeletal growth and differentiation. Yale J. Biol. Med. 23:1-27.

Sires, U.I., Schmid, T.M., Fliszar, C.J., Wang, Z.Q., Gluck, S.L. and Welgus, H.G. 1995. Complete degradation of type X collagen requires the combined action of interstitial collagenases and osteoclast-derived cathepsin-B. J. Clin. Invest. 95:2089-2095.

Smith, R.J., Rohloff, N.A., Sam, L.M., Justen, J.M., Diebel, M.R., and Cornette, J.C. 1989. Recombinant interleukin-α and recombinant human interleukin-β stimulate cartilage matrix degradation and inhibit glycosaminoglycan synthesis. Inflammation. 13:367-382.

Springman, E.B., Angleton, E.L., Birkedal-Hansen, H., and van Wart, H.E. 1990. Multiple modes of activation of latent fibroblast collagenase: evidence for the role of a Cys73 active-site zinc complex in latency and a "cysteine switch" mechanism for activation. Proc. Natl. Acad. Sci. USA. 87:364-368.

Stamenkovic, I., Amiot, M., Pesando, J.M., and Seed, B. 1989. A lymphocyte molecule implicated in lymph node homing is a member of the cartilage link protein family. Cell 56:1057-1062.

Stricklin, G.P. 1986. Human fibroblast tissue inhibitor of metalloproteinases: glycoslyation and function. Collagen Rel. Res. 6:219-228.

Suzuki, K., Enghid, J.J., Morodomi, T., Salvesen,G., and Nagase, H. 1990. Mechanisms of activation of tissue procollagenase by matrix metalloproteinase-3 (stromelysin) Biochemistry. 29:10261-10270.

Tamura, M., Shimokawa, H., and Sasaki, S. 1994. Primary structure of bovine interstitial collagenase deduced from cDNA sequence DNA Seq. 5:63-66.

Thiede, M.A. and Rodan, G.A. 1988. Expression of a calcium-mobilizing parathyroid hormone-like peptide in lactating mammary tissue. Science. 242:278-280.

Thomas, J.T., Kwan, A.P.L., Grant, M.E., and Boot-Handford, R.P. 1991. Isolation of cDNA encoding the complete sequence of bovine type X collagen gene. Evidence for the condensed nature of mammalian type X collagen genes. Biochem. J. 273:141-148.

Thorngren, K.G., and Hansson, L.I. 1981. Cell production of different growth plates in the rabbit. Acta. Anat., 110:121-127.

van der Rest, M., Rosenberg, L.C., Olsen, B.R., and Poole, A.R. 1986. Chondrocalcin is identical to the C-propeptide of type II procollagen. Biochem. J., 297:923-930.

van der Rest, M., and Mayne, R. 1988. Type IX collagen from cartilage is covalently crosslinked to type II collagen. J. Biol. Chem. 263, 1615-1618.

van der Rest, M., and Garrone, R. 1991. Collagen family of proteins. FASEB 5:2814-2823.

Vasios, G., Nishimura, I., Konomi, H., van der Rest, M., Ninomiya, Y., and Olsen, B.R. 1988. Type IX collagen-proteoglycan contains a large amino-terminal globular domain encoded by multiple exons. J. Biol. Chem. 263, 2324-2329.

von der Mark, K., Kirsch, T., Nierlich, A., Kuss, A., Wesohol, G., Gluckert, K., and Stoss, H., 1992. Type X collagen synthesis in human osteoarthritic cartilage . Indication of chondrocyte hypertrophy. Arthritis Rheum. 35:806-811.

Vornehm, S.I., Dudhia, J., Von der Mark, K., and Aigner, T. 1996. Expression of collagen IX and XI and other major cartilage matrix components by human fetal chondrocytes in vivo. Matrix Biol. 15:91-98.

Walker, G.D., Fischer, M., Thompson, R.C., Jr., and Oegema, T.R., Jr. 1995. Expression of type-X collagen in osteoarthritis. J. Orthop. Res. 13:4-12.

Warman, M.L., Abbot, M., Apte, S.S., Hefferon, T., McIntosh, I., Cohn, D.H., Hecht, J.T., Olsen, B.R., and Francomano, C.A. 1993. A type X collagen mutation causes Schmid metaphyseal chondrodysplasia. Nature Genet. 5: 79-82.

Weber, B.H.F., Vogt, G., Pruett, R.C., Stöhr, H., and Felbor, U., 1994. Mutations in the tissue inhibitor of metalloproteinases-3 (TIMP-3) in patients with Sorsby's fundus dystrophy. Nature Genet. 8:352-356.

Weir, E.C., Philbrick, W.M., Amling, M., Neff, L.A., Baron, R., and Broadus, A.E. 1996. Targeted overexpression of parathyroid hormone-related peptide in chondrocytes causes chondrodysplasia and delayed endochondral bone formation. Proc. Natl. Acad. Sci. USA. 93:10240-10245.

Welch, A.R., Holman, C.M., Huber, M., Brenner, M.C., Browner, M.F., and van Wart, H.E., 1996. Understanding the P_1' specificity of the matrix metalloproteinases: effect of S_1' pocket mutations in matrilysin and stomelysin-1. Biochemistry 35:10103-10109.

Welgus, H.G., Kobayashi, D.K., and Jeffrey, J.J. 1983. The collagen substrate specificity of rat uterus collagenase. J. Biol. Chem. 258:14162-14165.

Werb, Z., Tremble, P.M., Behrendtsen, O., Crowley, E., and Damsky, C.H. 1989. Signal transduction through the fibronectin receptor induces collagenase and stromelysin gene expression. J. Cell. Biol. 109:877-889.

Whitham, S.E., Murphy, G., Angel, P., Rahmsdorf, H.J., Smith, B.J., Lyons, A., Harris, T.J., Reynolds, J.J., Herrlich, P., and Docherty, A.J. 1986. Comparision of human stromelysin and collagenase by cloning and sequence analysis. Biochem. J. 240:913-916.

Wick, M., Härönen, R., Mumberg, D., Bürger, C., Olsen, B.R., Budarf, M.L., Apte, S.S., and Müller, R. 1995. Structure of the human TIMP-3 gene and its cell cycle-regulated promoter. Biochem. J. 311:549-554.

Wilkins, L., 1965 The diagnosis and treatment of endocrine disorders in childhood and adolescence, 3rd Ed. Charles C. Thomas, Springfield, Illinois. pp. 114.

Williard, H.F., Durfy, S.J., Mohtani, M..M., Dorkins, H., and Williams, B.R.G. 1989. Regional localization of the TIMP gene on the human X chromosomes: extension of observed synteny and linkage group on proximal Xp. Hum. Genet. 81:234-238.

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

Wu, H., Byrne, M.H., Stacey, A., Goldring, M.B., Birkhead, J.R., Jaenaisch, K., and Krane, S.M. 1990. Generation of collagenase-resistant collagen by site-directed mutagenesis of murine pro alpha 1(I) collagen gene. Proc. Natl. Acad. Sci. USA 87:5888-5892.

Wu, J.J., Lark, M.W., Chun, L.E., and Eyre, D.R. 1991. Sites of stromelysin cleavage in collagen types II, IX, X and XI of cartilage. J. Biol. Chem. 266:5625-5628.

Yan, W., Pan, H., Ishida, H., Nakashima, K., Suzuki, F., Nishimura, M., Jikko, A., Oda, R., and Kato, Y. 1997. Effects of concanvalin A on chondrocyte hypertrophy and matrix calcification. J. Biol. Chem. 272:7833-7840.

Yasuda, T., Banville, D., Rabbani, S.A., Hendy, G.N., and Goltzman, D. 1989. Rat parathyroid hormone-like peptide: Comparison with the human homologue and expression in malignant and normal tissue. Mol. Endocrinol. 3:518-525.

Yu, L.P., Jr., Smith, G.N., Jr., Brandt, K.D., Meyers, S.L., O'Connor, B.L., and Brandt, D.A. 1992. Reduction of the severity of canine osteoarthritis by prophylactic treatment with oral doxycycline. Arthritis Rheum. 35:1150-1159.

Zenymo, M., Komiya, S., Rikimaru, K., Sasaguri, Y., Inoue, A., and Morimatasu, M. 1996. Morphological and biochemical evidence for apoptosis in the terminal hypertrophic chondrocytes of the growth plate. J. of Pathology 180:430-433.