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Expression and regulation of tissue inhibitor of metallaproteinases-4 in joints

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Mémoire accepté le: .....

To My Family

SUMMARY

Arthritis is a prevalent disease that affects joints of patients. The matrix metalloproteinases (MMPs) and aggrecanases are expressed at increased levels in joint tissues of patients with rheumatoid arthritis (RA) and osteoarthritis (OA). Four members of the tissue inhibitor of metalloproteinases (TIMP) family, TIMP-1, TIMP-2, TIMP-3 and TIMP-4, have been characterized so far, which are capable of specifically inhibiting the activities of all known MMPs. Physiological and pathological degradation of cartilage extracellular matrix (ECM) is regulated by the balance between MMPs and their inhibitors TIMPs. TIMP-4 is the newest member of the TIMP family which is selectively elevated in cardiac tissue and is expressed at lower level in liver, kidney and some other organs.

1) In this study, we examined for the first time whether TIMP-4 RNA and protein is expressed in joints. By utilizing semi-quantitative RT-PCR, immunohistochemistry and Western blot assay, we demonstrated that TIMP-4 gene is expressed in human arthritic and non-arthritic cartilage. TIMP-4 gene expression is also found in human synovial membrane, human and bovine chondrocyte primary cultures and bovine cartilage.

2) In comparison with non-arthritic patients, TIMP-4 gene expression is increased in the cartilage of OA and RA patients, but not in the synovial membranes. Expression in OA cartilage was mainly in the superficial zone of articular cartilage. This suggests that it may play a role in remodeling and pathogenesis of arthritic cartilage but may not efficiently defend against cartilage destruction by excessive multiple MMPs and aggrecanases.

3) The expression of MMPs and TIMP-1, TIMP-3 are regulated by inflammatory cytokines and growth factors, such as interlukin-1 (IL-1), tumor necrosis factor-α (TNF-α), oncostain M (OSM) and transforming growth factor-β (TGF-β). Unlike TIMP-1 and TIMP-3, TIMP-4 expression is not up-regulated by these factors. This suggests that TIMP-4 may play a distinct role in the arthritic disease process whose regulation remains to be investigated.

RÉSUMÉ

L'arthrite est une maladie qui affecte les articulations. Les métalloprotéases matricielles (MMPs) et les aggrécanases sont hautement exprimées chez les patients souffrant de l'arthrite rhumatoïde (RA) et de l'arthrose (OA). Quatre inhibiteurs tissulaires specifiques des métalloprotéinases (TIMPs): TIMP-1, TIMP-2, TIMP-3 et TIMP-4, identifiées à date, sont capables d'abolir spécifiquement les activités des MMPs. La dégradation physiologique et pathologique de la matrice extracellulaire du cartilage est réglée par un équilibre entre MMPs et leurs inhibiteurs TIMPs, TIMP-4 étant le plus récent membre de la famille TIMP. La distribution tissulaire de TIMP-4 nous montre qu'elle est abondamment présente dans le tissu cardiaque, par contre elle est moins exprimée dans le foie, le rein, et les autres organes.

1) Dans ce travail nous avons démontré pour la première fois que l'ARN et la protéine correspondante TIMP-4, sont exprimés dans le cartilage grâce aux techniques d'Immunohistochimie, RT-PCR, et Western blot. Également nous avons mis en évidence que le gène TIMP-4 est exprimé dans le cartilage arthritique et non-arthritique. Le gène TIMP-4 est aussi exprimé dans les membranes synoviales, les chondrocytes primaires humaines et les chondrocytes bovines ainsi que dans le cartilage bovin.

2) Chez les patients la comparaison de l'expression du gène TIMP-4 indique bien la surexpression de ce dernier dans le cartilage des patients ostéoarthritiques et arthritiques. Nous avons remarqué que l'expression de TIMP-4 dans le cartilage chez les patients ostéoarthritiques, était principalement dans la zone superficielle du cartilage articulaire. Ceci suggère qu'il pourrait avoir un rôle dans le remodelage et dans le processus pathologique du cartilage arthritique. La présence et l'augmentation des TIMPs ne protégent pas contre la destruction du cartilage par une surabondance des MMPs et des aggrécanases.

3) La régulation de l'expression des MMPs, TIMP-1, TIMP-3 se fait par les cytokines et des facteurs de croissance inflammatoires : IL-1, TNF-α, l'oncostatin M (OSM) et TGF-β. Alors que celle de TIMP-4 n'est pas réglée par ces facteurs. Ceci

suggère que TIMP-4 pourrait jouer un rôle distinct dans le processus de la maladie arthritique dont les mécanismes restent à étudier.

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ADAM	a disintegrin and metalloproteinase
ADAMTS	a disintegrin and metalloproteinase with thrombospondin motifs
COX-2	cyclooxygenase-2
ECM	extracellular matrix
IL-1	interleukin-1
IPF	idiopathic pulmonary fibrosis
LIF	leukemia inhibitory factor
MMPs	matrix metalloproteinases
MTx-MMP	membrane-type x-MMP
NSAID	non-steroidal anti-inflammatory drug
OA	osteoarthritis
OSM	oncostatin M
PCR	polymerase chain reaction
PMN	polymorphonuclear leukocyte
RA	rheumatoid arthritis
RT-PCR	reverse transcription-PCR
SF	synovial fluid
TGF-β	transforming growth factor-beta
TIMPs	tissue inhibitors of metalloproteinases
TNF-α	tumor necrosis factor alpha

I. INTRODUCTION

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I.1. Articular cartilage and chondrocyte

Cartilage is a tough white flexible tissue attached with the bones, which is made up of small numbers of cells called chondrocytes within an extensive extracellular matrix and provides permanent flexible components for the skeleton. The extracellular matrix of cartilage is a fibre-reinforced gel which is resilient and pliant. It is an elaborate framework of macromolecules—including collagen, protecoglycans and non-collagenous proteins (glycoproteins). It is beautifully structured in molecular terms for its purpose and is produced by surprisingly few cells. For example, in adult human femoral head cartilage, there are only 10,000 cell/mm3 (Venn and Maroudas, 1997). In adult articular cartilage, cell volume attains approximately 2% of the total cartilage volume (Poole et al., 2001). For normal articular cartilage, the structure and composition varies from its surface to its connection with subchondral bone. From the free surface to the subchondral bone, Articular cartilage can be divided into four zones, referred to as the superficial zone (or tangential), the middle zone (or transition), the deep (or radial) zone, and the zone of calcified cartilage.

The chondrocyte is a distinctive cell different from other types of mesenchymal cell. It is round, highly differentiated cell which is the sole architect of cartilage. Chondrocytes rely on diffusion of nutrients and metabolites through the matrix to produce the matrix of cartilage and differentiate during development. In healthy adult articular cartilage, chondrocyte cells stop dividing after growth has ceased. Thus, articular chondrocytes normarlly survives for long time. The chondrocytes in the free surface bathed by synovial fluid are flattened and aligned parallel to the surface. The rest of chondrocytes in the cartilage are arranged differently.

From the superficial zone to the deep zone, the cell density becomes lower (Poole et al., 2001).

The function of chondrocytes is to synthesize and maintain the matrix of cartilage and a resilient ECM. In articular cartilage, collagen contributes about 50% of the tissue's dry weight and forms the fibrillar meshwork that gives cartilage its shape and tensile strength (reviewed in Mayne, 1989). The proteoglycans and the glycoproteins complete the macromolecular framework by binding to the collagenous meshwork and are physically trapped within it. Collagens are extended extracellular proteins composed of three polypeptide chains. Three α-chains are twisted tightly into a right-handed helix to form the rope-like collagen fibrils. Collagen fibrils are further stabilized by cross-links with lysine residues. the diameter of collagen fibrils becomes larger from cartilage surface to deep zone. Among many different collagens, fibrillar type II collagen is specific to cartilage and is a marker of chondrocyte differentiation, and collagen type VI, IX, XII and XIV, as a quantitatively minor components, also have important structural and functional properties. Type II collagen forms a fibrillar network with the minor collagens type IX and type XI (reviewed in Muir, 1995). Type X collagen is a shorter-chain collagen synthesized by hypertrophic chondrocytes of calcified ossifying zone. Its expression in osteoarthritic cartilage was altered in several locations (Walker et al., 1995). In OA and RA, the chondrocytes may exhibit an altered phenotype and express a different pattern of collagens. Type X and type III collagens are not expressed in healthy cartilage, but they have been detected in OA cartilage (reviewed in Poole, 1999; and Goldring, 2000). The low level type VI collagen in normal cartilage was also increased in OA cartilage (Hambach et al., 1998). Such alterations in gene expression could change matrix structure and cause the cartilage erosion and loss of tissue repair in OA and RA.

Proteoglycan is another major component in articular cartilage that contributes 30-35% of the tissue's dry weight (reviewed in Greenwald,

1978). The large cartilage-specific proteoglycan is named aggrecan. Aggrecan belongs to the family of hyaluronan-binding protein (reviewed in Hardingham and Fosang, 1992). Aggrecan molecule is a bottlebrush structure with a core protein that contains three globular domains, including two N-terminal globular domain G1 and G2 following with a long C-terminal domain G3. Between G1 and G2 there is a region known as the interglobular domain (IGD). Along the length between G2 and G3 there are approximately 100 chondroitin sulfate chains and 30 karatan sulphate chains attached to this structure. Aggrecan binds to hyaluronan through its amino terminal G1 globular domain. Many aggrecans and link proteins can bind each other with high affinity to form huge multimolecular aggregates. Aggregates are tertiary complexes consisting of about 100 aggrecan molecules. They are too large to be immobilized in the collagen network. Aggrecans mostly concentrate in the deep zone of cartilage where collagen is at its lowest content (Poole et al., 2001).

There are several other matrix components that may play important roles in cartilage structure and function, such as decorin, biglycan, fibromodulin and lumican. They are small leucine-rich proteoglycans that help to maintain cartilage structure by interacting with the collagen network and binding growth factors such as TGF-β.

I.2. Osteoarthritis and rheumatoid arthritis

Osteoarthritis (OA) is a common disease of joints. It is a slowly progressing musculoskeletal disorder characterized by gradual loss of articular cartilage. The breakdown of the cartilage matrix leads to the development of fibrillation, appearance of gross ulcerations and ultimately the disappearance of the full thickness of articular cartilage. This is accompanied by bone changes such as osteophyte formation. OA causes changes not only in cartilage but also in synovial membrane, where a variable degree of inflammatory reaction is often observed (reviewed in Martel-Pelletier, 1998

and 2001). OA is caused possibly by multiple factors such as joint injuries and trauma, obesity, joint dysplasia, aging, estrogen loss, gender, life style and gene mutations (reviewed in Goldring, 2000).

Rheumatoid arthritis (RA) is a more aggressive disease affecting many joints, which has more immunological etiology and much pronounced synovial inflammation and hyperplasia. The proliferating synovial fibroblasts invade cartilage leading to its loss. The cause of RA is not clearly known, but it is thought that small molecular mediators of inflammation, cytokines, growth factors, chemokine, adhesion molecules, p53 tumor suppressor gene mutations and metalloproteinases play important roles in this disease.

The destruction of articular cartilage is a complex process in which proinflammatory cytokines (IL-1, TNF-α) alter connective tissue metabolism, including inhibition of collagen and aggrecan synthesis (reviewed in Westacott and Sharif, 1996; and Poole 1994). These cytokines also stimulate various cell-types (chondrocyte, synovial fibroblast) to express matrix-digesting proteases. Current knowledge indicates a major involvement of matrix metalloproteinases (MMPs), aggrecanases, and the tissue inhibitors of metalloproteinases (TIMPs) in the cartilage and other damage in joints (reviewed in Cawston, 1998). Current evidence suggests that proinflammatory cytokines are responsible for initiating the catabolic process occurring in the arthritic tissues. The disease is currently treated with non-steroidal anti-inflammatory drugs (NSAIDs), cyclooxygenase-2 (COX-2) inhibitors or steroid injections. Ultimately joint replacement surgery (e.g. hip, knee) is performed where synthetic joints are constructed to improve the quality of life for patients. The development of therapeutic agents, such as proteinase inhibitors, cytokine antagonists, and cytokine receptor blocking antibodies could be used to prevent or retard the progression of the OA and RA articular lesion in the future (reviewed in Goldring, 2000).

I.3. Properties of matrix metalloproteinases (MMPs) and aggrecanases

The matrix metalloproteinases (MMPs) are a unique family of metalloenzymes, which includes at least 20 human zinc-dependent endopeptidedases, collectively capable of degrading essentially all ECM components (reviewed in Nagase and Woessner, 1999; Nelson et al., 2000). Most MMPs are secreted as latent precursors that are proteolytically activated in the extracellular environment (reviewed in Murphy and Knauper, 1997). In general, all the MMPs contain a signal peptide, a propeptide, and a catalytic domain with the highly conserved zinc binding site and a hemopexin-like domain (except MMP-7) linked to the catalytic domain by a hinge region. According to their substrate specificity and structure, members of the MMP gene family can be classified into 5 groups (reviewed in Westermarck and Kahari, 1999). Group I, collagenase subgroup, includes collagenase-1 (MMP-1), collagenase-2 (MMP-8), collagenase-3 (MMP-13); Group II, stromelysin subgroup, includes stromelysin-1 (MMP-3), stromelysin-2 (MMP-10), metalloelastase (MMP-12), matrilysin (MMP-7); Group III, includes gelatinase-A (MMP-2), gelatinase-B (MMP-9); Group IV, the membrane-type MMP, includes MT1-MMP (MMP-14), MT2-MMP (MMP-15), MT3-MMP (MMP-16), MT4-MMP (MMP-17); Group V, includes all the other MMPs, such as stromelysin-3 (MMP-11), MMP-19 and enamelysin (MMP-20) (Table. A).

In group I, Collagenase-1, -2, -3 are the principal secreted neutral proteinases capable of initiating degradation of native fibrillar collagens of type I, II, III, and V, and apparently play a crucial role in degradation of collagenous ECM in various physiological and pathological situations (reviewed in Kahari and Saarilho-Kere, 1997; and Shapiro, 1998). They all cleave fibrillar collagens at a specific site, resulting in generation of amino-terminal ¾ and carboxylterminal ¼ fragments, which then rapidly denature at body temperature and are further degraded by other MMPs. In group II, MMP-3 and MMP-10 are expressed by fibroblastic cells and by normal and

transformed squamous epithelial cells (Airola et al., 1997; Johansson et al, 1999). MMP-3 and MMP-10 degrade basement membrane components, type IV collagen, nidogen, and fibronectin; MMP-7 and MMP-12 have the ability to degrade elastin (reviewed in Kahari and Saarilho-Kere, 1997; and Shapiro, 1998). In group III, MMP-2 is expressed by a variety of normal and transformed cells. MMP-9 is produced by keratinocytes, monocytes, alveolar macrophages, PMN leukocytes, and a large variety of malignant cells. Both of them degrade gelatin, laminin, nidogen, and MMP-2 also have the ability to degrade native type I collagen and proteolytiallly activate MMP-9 and MMP-13 (reviewed in Basset et al., 1997; and Murphy and Knauper, 1997). Group IV is the membrane-type MMP. MT1-MMP and MT2-MMP degrade type I, II and III collagens, gelatin, fibronectin, laminin, vitronectin, and aggrecan. Active MT1-MMP serves as a cell membrane receptor for the complex formed of latent MMP-2 (proMMP-2) and TIMP-2, MT1-MMP and MT2-MMP are also reported to proteolytically activate proMMP-2 at the cell surface (Johansson et al, 1999; Gilles et al., 1997). Group V includes all the other MMPs. MMP-11 degrades serine proteinase inhibitors, α1-proteinase inhibitor and α1-antitrypsin (Kahari and Saarilho-Kere, 1997; Shapiro, 1998). MMP-20 is observed only in dental tissues, where it degrades dental amelogenin (Llano et al., 1997). The ability of MMP-19 to degrade native ECM components is still unknown (Johansson et al, 1999, Pendas et al., 1997). Many MMPs, such as MMP-3, MMP-13 and MMP-8 can also degrade aggrecan molecules at a specific cleavage site. In vitro, many of the potential substrates of MMPs have been tested (Table 1).

A large number of studies indicate that the MMPs are involved in the pathological destruction of joint tissue (Brinkerhoff, 1991; Vincenti et al., 1994). The significant up-regulation of MMP gene expression early in the OA process is seen in animal models of OA (Pelletier et al., 1988; Bonassar et al., 1995). Significantly increased MMP activity has also been measured in human cartilage sampled from patients with debilitating OA (Fosang et al.,

1996; Lark et al., 1997; Billinghurst et al., 1997). Recent studies on aggrecan release suggest that MMPs are not responsible for the turnover of this proteoglycan (Sandy and Verscharen, 2001). The activity of MMPs in extracellular environment is specifically inhibited by tissue inhibitors of metalloproteinases (TIMPs), which bind to the highly conserved zinc binding site of active MMPs at molar equivalence (reviewed in Gomez et al., 1997). MMPs are also controlled by various cytokines, growth factors and other agents, which can stimulate the synthesis and secretion of pro-MMPs. It was demonstrated that IL-1 or TNF-α could induce MT1-MMP (Rajavashisth et al., 1999), MMP-7 (Ohta et al., 1998), MMP-13 (Mitchell et al., 1996; Reboul et al., 1996), aggrecanase-1 and aggrecanase-2 (Tortorella et al., 2001); IL-1 can induce MMP-1 (reviewed in Mauviel, 1993), MMP-8 (Cole et al., 1996) and MMP-13 (Reboul et al., 1996). On the other hand, certain anti-inflammatory cytokines such as IL-4, IL-10 and TGF-β, can down-regulate some MMPs (van Roon et al., 1996).

ADAM (a disintegrin and metalloproteinase) is a novel gene family, which contains two groups according to the domain structure: membrane type ADAM and ADAM with thrombospondin motifs (ADAMTS). ADAMTS now is considered as a family of extracellular proteinases. Among this family, ADAMTS-4 and ADAMTS-5 are identified as aggrecanase, so called aggrecanase-1 and aggrecanase-2. These two enzymes play a key role in the destruction of articular cartilage through aggrecan degradation in human OA and RA (Fosang et al., 2000). Aggrecanases have a somewhat similar structure as MMPs, including a signal peptide, propeptide and a zinc-binding metalloproteinase active site sequence (reviewed in Tang, 2001) (Table. A). Aggrecanases can recognize and cleave aggrecan molecule at the site Glu^{373}-Ala^{374}, but not the cleavage site Asn341- Phe342 bond in the aggrecan molecule which is targeted by MMPs (Amanda J et al., 1996; Caterson B, 2000). Aggrecanases activity is induced by IL-1 or retinoic acid (Clare E et al., 1998; Sandy et al., 1998) and inhibited by TIMPs such as TIMP-3

(Hashimoto et al., 2001) and glucosamine (Sandy et al., 1998). Aggrecanases and MMPs are independent and uncoupled in the process of aggrecan degradation.

I.4. Tissue inhibitors of metalloproteinases (TIMPs)

Tissue inhibitors of metalloproteinases (TIMPs) are endogenous proteins capable of inhibiting the activity of all known MMPs (reviewed in Matrisian, 1992). TIMPs are expressed in many tissue and body fluids. At present, the TIMP family consists of four structurally related members, TIMP-1, TIMP-2, TIMP-3 and TIMP-4 (Table. B). Mature TIMPs are comprised of two domains containing 12 cysteine residues, and forming six S-S bonds. One domain, the amino-terminal domain is necessary for the inhibitory activity; the other domain, the carboxyl-terminal regulatory domain can bind to the progelatinases and also has growth factor properties for some cells (reviewed in Murphy et al., 1991; Gomez et al, 1997). TIMPs bind noncovalently to active MMPs in a 1:1 molar ratio. Inhibition is accomplished by their ability to interact with the zinc-binding site within the catalytic domain of active MMPs.

There is a certain degree of specificity in the activity of different TIMPs toward distinct members of the MMP family, whereas TIMP-1 potently inhibits the activity of most MMPs, with the exception of MMP-2, MT1-MMP. TIMP-2 is a potent inhibitor of most MMPs, except MMP-9. TIMP-3, which is insoluble and binds to the ECM, has been shown to bind MMP-1, -2, -3, -9 and –13. TIMP-4 inhibits MMP-1, -3, -7 and –9 and shows a high level of expression in adult human cardiac tissue (Liu et al., 1997; Greene et al., 1996).

TIMP-1 is an extensively glycosylated protein, the unglycosylated TIMP-1 has a molecular mass of approximately 20 kDa and the glycosylated form

reaches to 28.5 kDa (Gomez et al., 1997), although it can range from 30 to 34 kDa, depending on the degree of glycosylation (Tolley et al., 1993; Williamson et al., 1990). A site-directed mutagenesis study identified an important sequence between cysteine-3 and cysteine-13 in the N-terminal domain for the MMP inhibitory activity (Bodden et al., 1994). TIMP-1 is also a potent inhibitor of aggrecanase-1 (Arner et al., 1999). TIMP-2 is a 21 kDa unglycosylated protein, which shares 40% amino acid identity with TIMP-1 (Stetler-Stevenson et al., 1998). A complex of proMMP-2/TIMP-2/MT1-MMP is important in the cell-surface activation of pro-MMP-2 (Strongin et al., 1995). TIMP-3 is the only member of TIMP family that is found exclusively in the ECM (Leco et al., 1994; Greene et al., 1996). TIMP-3 is a 21 kDa protein as unglycosylated form and 29 kDa as glycosylated form which shares 30% homology with TIMP-1 and 38% homology with TIMP-2 (Pavloff et al., 1992; Apte et al., 1995). Overexpression of TIMP-3 induces the apoptotic cell death of a number of cancer cell line (Baker et al., 1999) and rat vascular smooth muscle cells (Baker et al., 1998). TIMP-3 is a strong inhibitor of aggrencanase, the major enzyme responsible for degradation of aggrecan. (Kashiwagi et al., 2001). TIMP-4 shows 37% sequence homology with TIMP-1 and 51% homology with TIMP-2 and TIMP-3. TIMP-4 was shown to have high expression level in heart and low or undetectable expression level in other tissues. It is a somewhat tissue-specific protein (Liu et al., 1997; Dollery et al., 1999).

TIMP protein, of molecular weight 20 to 30 kDa, is heavily cystein-crosslinked with two N-linked glycosylation sites (Williamson et al., 1990). Glycoproteins have diverse biological roles, and the carbohydrate moiety can be the active component or may have an auxiliary role to the protein function (Varki, 1993). Normally, protein glycosylation comprises conjugation of oligosaccharide(s) to one or more N-linked (Asn-Xxx-Thr/Ser) and/or O-linked (Ser/Thr) sites. It is confirmed that the 2 glycosylation sites Asn30 and Asn78 of TIMP exhibited extensive heterogeoeity comprising mainly fucosylated complex oligosaccharides, but in different proportions (Sutton et

al., 1994). Glycoproteins and oligosaccharides on glycolipids have important functions, but for the most part these functions are not known. N-linked glycosylation is prevalent in all eucaryotes including yeasts, but is absent from prokaryotes. Because one or more N-linked oligosaccharides are present on most proteins transported through ER and Golgi apparatus—a pathway that is unique to eucaryotic cells. With the study on TIMP-1 mutant protein, it was reported that double glycosylation mutant protein was expressed at a level that was 2-4 fold lower than that of the wild-type or the single glycosylation mutant. In osteoarthritic and normal cartilage, collagen, aggrecan and link proteins are also crosslinked by nonenzymed glycation (Pokharna and Pottenger, 1997). This suggests that glycosylation of protein occurs very often in cartilage.

TIMPs are important participants in various physiological processes that involve tissue remodeling. A major role of TIMPs is their inhibitor activity for MMPs and aggrecanases (Table. B). Recent studies show that TIMPs inhibited tumor growth, invasion and metastases. Also TIMPs may affect the angiogenic process in several ways, i.e. by inhibiting endothelial cell migration, preventing MMP-mediated endothelial cell detachment, blocking the release of matrix-bound angiogenic factors, and preventing degradation of the ECM (Murphy et al., 1993; Schnaper et al., 1993).

I.5. Metalloproteinases and their inhibitors in arthritis

Normally, the activity of MMPs is quite low in articular cartilage with regulatory control over their synthesis and activation responsible for the low turnover of ECM proteins (Malemud and Goldberg, 1999) (Figure. A). But MMPs are up-regulated during physiological and pathological remodeling process such as embryonic development, tissue repair and inflammation. Once the MMPs are fully activated, they have an imbalance against TIMPs, which could contribute to the cartilage destruction in the diseases such as OA

and RA. The significant up-regulation of MMP gene expression early in the OA process is seen in animal models of OA (Pelletier et al., 1990; Bonassar et al., 1995; Pelletier et al., 1998).

Among the MMPs which have been implicated in this process, MMP-1 (collagenase-1), MMP-3, MMP-2, MMP-9, MMP-8, MMP-13, MMP-7 and aggrecanases have received the most attention. Levels of MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, MMP-7, and MMP-13 were significantly higher in RA synovial fluid than in OA synovial fluid. MMP-3 level was extremely high compared with those of other MMPs (Yoshihara et al., 2000; Smith, 1999). MMP-3 was considered as the most important proteinase in the cartilage degradation of OA and RA before (Ishiguro et al., 2001), but now it is believed that MMP-13 plays a pivotal role in the pathogenesis of OA. With a transgenic mouse model, a study confirmed that excessive MMP-13 activity alone can result in articular cartilage degradation and induced-OA in mice joints (Neuhold et al., 2001). Both MMPs and aggrecanases can digest aggrecan into different fragments, while MMPs and aggrecanases have different cleavage site in the aggrecan molecules.

The enzymic activities of these MMPs are strictly controlled by inhibition with specific inhibitors—TIMPs (TIMP-1, TIMP-2, TIMP-3, TIMP-4) (Figure. A). The level of latent MMP synthesis in OA cartilage greatly exceeds the up-regulation of TIMP gene expression that also occurs during OA process (Malemud and Goldberg, 1999). The role of TIMPs in controlling cartilage ECM protein turnover is to inhibit MMPs once they are activated. The amount of TIMP isoform produced by OA or RA cartilage is insufficient to inhibit the level of MMPs produced by chondrocytes (Morgunova et al., 1999). It was reported that the calculated values for molar ratios of MMPs to TIMPs in RA synvial fluid were ~44 fold on average, however, the molar ratios of MMPs to TIMPs were ~9 fold on average in OA synovial fluid (Yoshihara et al., 2000). Different TIMPs may play different role in OA and RA disease.

Studies showed that TIMP-1 RNA expression by synovial membrane derived from OA patients was elevated compared to synovial membrane derived from non-OA patients (Zafarullah et al., 1993), while TIMP-2 RNA expression was comparable in both tissues (Zafarullah et al, 1996). TIMP-1 and TIMP-2 have also been detected in the synovial fluid (SF) of patients with erosive joint disease, OA and RA. The levels of TIMP-1 was expressed significantly higher in RA SF than in OA SF but TIMP-2 expression level did not differ between OA and RA SFs (Yoshihara et al., 2000; Osthues et al., 1992). The presence of TIMP-1, TIMP-2, TIMP-3 mRNA in OA synovia may be related to growth factor activity of TIMPs (Zafarullah et al., 1993; Hayakawa et al., 1994; Su et al., 1999), that may have a mitogenic effect and so play an important role in the repairing process of OA and RA disease. TIMP-4 expression has not been reported in joints. We detected it for the first time in human cartilage, synovial membrane tissue and chondrocytes. The level of TIMP-4 in OA and RA cartilage was elevated compared to that in non-OA cartilage. This suggested that like other TIMPs, TIMP-4 also played an important role in the erosive joint disease.

I.6. Role of cytokines and growth factors in arthritis

Cytokines are hormone-like proteins that enable immune cells to communicate. Cytokines include interleukin family (interleukin-1, -2, -3, -4, -6, -10, -17, -18 etc.), gamma interferon, tumor necrosis factor (TNF-α, $-\beta$), chemokines, transforming growth factor, fibroblast growth factor and several other recently discovered factors. Cartilage matrix synthesis and degradation is controlled by endogenous and exogenous cytokines and growth factors, such as IL-1 and TNF-α (Figure. A).

Interleukin-1 (IL-1) is a ubiquitous family of polypeptides with a wide range of biologic activity. Its action makes it a candidate for the major amplification factor and translator of the inflammatory response of RA into a

proliferative one. The potential for a major role for IL-1 in these responses is great. It was inferred that IL-1 was inducing chondrocytes or synovial cells, or both, to generate enzymes that degrade proteoglycans. (Robak and Gladalska, 1997).

Tumor necrosis factor- α was named by its activity to cause necrosis in certain tumors in mice. There are at least two forms, TNF-α and TNF-β, that have similar cytotoxic effects on neoplastic cell lines. TNF-α has been detected in rheumatoid synovial fluid and serum (Miossec et al., 1990). IL-1 and TNF-α have similar activities, including the ability to enhance cytokine production, adhesion molecule production by cultured synoviocytes. Many studies showed that the levels of IL-1 and TNF-α are increased in arthritic tissues. IL-1 and TNF-α are accepted as the pivotal mediators in cartilage degradation in OA and RA joints (reviewed in Feldmann et al., 1996; Martel-Pelletier et al., 1999; Goldring, 2000). IL-1 and TNF-α stimulate numerous cells to produce other proinflammatory cytokines such as IL-6, IL-8 and leukemia inhibitory factor (LIF), as well as induce cartilage-degrading enzymes such as MMPs (Feldmann et al., 1996; Martel-Pelletier et al., 1999). With animal models of articular cartilage, injection of combination of IL-1 and TNF-α elicited more severe cartilage damage than the injection of either IL-1 or TNF-α alone (Goldring, 1999). Other studies showed that both IL-1 and TNF-α can cause cartilage damage in vitro. IL-1 and TNF-α stimulate the degradation of the matrix and the release of proteoglycan fragments within 12-24h when they are added to the cartilage (Arner et al., 1998). At the same time, the synthesis of matrix components, like type II and IX collagens and proteoglycan, are also down-regulated (van de Loo et al., 1995). In addition to their ability to promote cartilage and bone destruction by enhanced MMP production, IL-1 and TNF-α can induce joint cells, such as chondrocytes and synovial fibroblasts, to produce other cytokines like IL-6, IL-8, LIF and their own products (Feldmann et al., 1996; Henrotin et al., 1996). In the process of human RA, TNF-α induced most of the IL-1

production (van den Berg, 1998). Large amount of IL-1 was synthesized in rheumatoid synovial tissue (Robak and Gladalska, 1997). Blocking IL-1 activity reduced the production of IL-6 and IL-8 but not TNF-α (Feldmann et al., 1996). TNF-α is a pivotal cytokine in joint swelling; yet IL-1 is the dominant cartilage destructive cytokine (Kuiper et al., 1998).

Transforming growth factor (TGF) is a family of proteins that stimulate cells to lose contact inhibition. Two major forms have been isolated and characterized, TGF-α and TGF-β. Although TGF-β alone has a modest effect on the expression of genes for collagenase and collagenase inhibitor, in the presence of other growth factors (such as EGF) it not only represses the production of collagenase (in contrast to IL-1 or TNF-α, which stimulate it) but also can superinduce expression of TIMPs (Henderson and Pettipher, 1989). Large amount of TGF-β are present in synovial fluid and the mRNA can be detected in OA and RA synovial tissue (Taketazu et al., 1994; Fahlgren et al., 2001). The role of TGF-β in OA and RA is quite complex as demonstrated by its conflicting results in various animal models (Allen et al., 1990; Fahlgren et al., 2001). Although earlier studies identified TGF-β as an inducer of TIMP expression (Gunther et al., 1994; Su et al., 1996), more recent work has also shown it to be a stimulant of MMP-13 expression in some patients (Moldovan et al., 1997). Excessive TGF-β is also implicated in the osteophyte formation in OA as well as synovial hyperplasia and inflammation in RA (van Beuningen et al., 2000). These studies suggest that TGF-β is an important mediator of tissue repair and it can alter the balance between extracellular matrix production and destruction in arthritis.

Oncostatin M (OSM), belonging to the IL-6 family of cytokine, is a 28 kDa glycoprotein first identified from activated monocytes and T lymphocytes (Brown et al., 1987). Subsequently, OSM was characterized as a growth regulator for different cell types, being able to either inhibit or stimulate the cell proliferation (Horn et al., 1990). OSM was present in the synovial fluid

of RA patients and its mRNA and protein increased in the RA synovial cells (Hui et al., 1997), however OSM was undetectable in the synovial fluid of OA patients (Okamoto et al., 1997). OSM has pro-inflammatory and cartilage destruction properties. Injection of human recombinant OSM into goat joints promoted cartilage resorption and proteoglycans synthesis depression (Bell et al., 1999). It can up-regulate TIMP-3 gene expression in bovine articular cartilage (Li et al., 1998), and studies showed that OSM inhibits inflammatory cytokines such as TNF-α and IL-8 production. These studies suggested its complicated implication in the pathogenesis of rheumatoid joint erosion.

In summary, cytokines and growth factors control cartilage matrix homeostasis. An individual cytokine can negate or promote the effect of another. They play an important role in joint lesion and reparation mechanism. In the context of current study, the impact of these cytokine/growth factors on TIMP-4 gene expression is not known.

I.7. Characteristics of TIMP-4

TIMP-4 is the newest member of the TIMP family. It was first cloned from a human heart cDNA library (Greene et al., 1996). The full-length cDNA sequence contains 1,189 bp with a 672 bp open reading frame. The open reading frame encodes a 224-amino acid precursor including a 29-residue secretion signal peptide. Removal of the signal sequence may result in a mature protein of 195 amino-acids. The expected TIMP-4 mature protein was calculated to a molecular mass of 22 kDa.

TIMP-4 protein has an isoelectric point of 7.34, this is the most neutral human TIMP protein at the physiological condition (pH7.4) compared with values of 8.00, 6.45, and 9.04 for human TIMP-1, TIMP-2 and TIMP-3 proteins respectively (Wilde et al., 1994). Similar to other TIMP proteins, the

structure of TIMP-4 also contains 12 completely conserved cystein residues in the corresponding positions that form intrachain disulfide bonds that fold the protein into a two domain structure (Greene et al., 1996; Williamson et al., 1990). TIMP-4 has 37% DNA sequence identity and 57% protein similarity to TIMP-1 and 51% DNA identity and 70% protein similarity to TIMP-2 and –3. TIMP-4 also has a highly conserved amino-terminal domain similar to other TIMPs, which has been shown to be adequate for the inhibition of MMPs (Murphy et al., 1991; Willenbrock et al., 1993; DeClerk et al., 1993). In this region, the first 22 amino-acids of the mature proteins is the most conserved among the TIMPs; 16 of the first 22 amino acids (73%) are identical among human TIMP-1, TIMP-2 and TIMP-3 (Apte et al., 1995), however TIMP-4 has a less conserved sequence in this region with only 12 of 22 amino acids identical in all four TIMPs (Greene et al., 1996).

It was initially believed that TIMP-4 transcription is somewhat tissue-specific. TIMP-4 RNA transcripts of 4.1, 2.1, 1.4, 1.2, and 0.97 kb were detected in heart, with the 1.4 kb band representing at least 90% of the hybridization signal (Greene et al., 1996). Similar bands were obtained in the kidney, pancreas, colon, and testes with much less accumulation in their relative intensity, and none of them was present in the liver, brain, lung, small intestine, thymus and spleen (Greene et al., 1996). Recently, TIMP-4 expression was detected in idiopathic pulmonary fibrosis (IPF) lungs, but not in normal lung as examined before by RT-PCR (Selman et al., 2000). The roles of TIMP-1 to TIMP-4 were evaluated in idiopathic pulmonary fibrosis (IPF) lungs. TIMP-1 was found in interstitial macrophages and TIMP-2 in fibroblast foci, TIMP-3 revealed an intense staining mainly decorating the elastic lamina in vessels. TIMP-4 was highly expressed in IPF lungs by epithelial and plasma cells, while in normal lungs, no transcripts of TIMP-4 was detected (Selman et al., 2000). Another study showed that in normal and aberrant wound healing, TIMP-1 to TIMP-4 could be detected, where TIMP-4 protein was found in stromal cells of chronic ulcers near blood vessles (Vaalamo et al., 1999). In human fetal membrane, TIMP-4 expression was

examined by RT-PCR while the expression of other TIMPs was demonstrated also in tissue from laboring and nonlaboring women as well as in cultured membranes (Fortunato et al., 1998).

The C-terminal domain of TIMP-4 is a regulatory domain, it is divergent and may enhance the selectivity to the target enzyme (Fujimoto et al., 1996; Douglas et al., 1997). A more detailed structure comparison indicated that TIMP-4 shares a relatively high identity with TIMP-2 particularly in the loop 4 and 5 with the C-terminal domain (Douglas et al., 1997). It was determined that this is a high affinity interaction between TIMP-4 and the C-terminal domain of MMP-2. It was shown that TIMP-4 bound both full-length MMP-2 and the C-terminal domain of MMP-2 in a manner similar to TIMP-2 (Bigg et al., 1997).

Human recombinant TIMP-4 protein can effectively inhibit human MMP-1, MMP-2, MMP-3, MMP-7 and MMP-9; this protein can also inhibit the growth of human breast cancer cells (Liu et al., 1997; Bigg et al., 2001). It was demonstrated that TIMP-4 mediated anti-tumor and anti-metastasis activities of TIMP-4 overexpressing breast cancer cells in the animal model (Wang et al., 1997). One study showed that TIMP-4 could bind to progelatinase A on the hemopexin C domain (Bigg et al., 1997) and inhibit gelatinase A (MMP-2) activation by its inhibition of MTI-MMP activity (Bigg et al., 2001). Analysis of TIMP-4 expression in human mammary specimens indicated that TIMP-4 protein is increased in mammary carcinoma cells compared with normal mammary epithelial cells. The growth factor-like activity of TIMP-4 can up-regulate the anti-apoptotic proteins Bcl-2 and Bcl-X_L. TIMP-4 also inhibits apoptosis in human breast cancer cells in vitro and mammary tumor in vivo (Jiang et al., 2001).

I.8. Research hypothesis and experimental approach

The overall goal of this research was to elucidate the expression and regulation of tissue inhibitor of metalloproteinases (TIMPs) genes in normal joints and those with osteoarthritis (OA) and rheumatoid arthritis (RA). So far, nothing is known about the expression of the novel inhibitor, TIMP-4, in joint tissue and its significance in arthritis. This work focused on the investigation of the expression and regulation of TIMP-4 gene. Each TIMP family member could have a distinct mode of regulation and function. Alternatively the regulation and function of TIMP-4 may be similar to one of the TIMP family members although TIMP-4 showed a relatively tissue-specific character. Based on our previous work with other TIMPs, we hypothesized that TIMP-4 may be expressed in bovine and human joint tissues, and its expression may be regulated by cytokines such as TGF-β, TNF-α, IL-1 and OSM, which induced TIMP-3 and certain MMPs in bovine and human chondrocytes.

We tested these hypotheses using in vitro model systems consisting of primary cultures of bovine and human chondrocytes and with human femoral head cartilage obtained from hip fracture or arthritis-related surgery.

The specific aims of our study were 1) To examine the expression of TIMP-4 in human cartilage. Using non-arthritic and arthritic cartilage from femoral head and knee, we investigated the TIMP-4 RNA expression by RT-PCR in different patients. TIMP-4 protein was also analyzed by using Western blotting to further confirm the TIMP-4 gene expression. 2) To determine the location of TIMP-4 protein in human cartilage by using immunohistochemistry staining method with specific TIMP-4 antibody. 3) To investigate the mechanism of TIMP-4 regulation by different cytokines such as TGF-β, TNF-α, IL-1 and OSM by using Western blotting to measure TIMP-4 protein expression in the primary, cultured chondrocytes. 4) To

examine whether TIMP-4 is expressed in human knee synovial membrane from non-arthritic and arthritic patients.

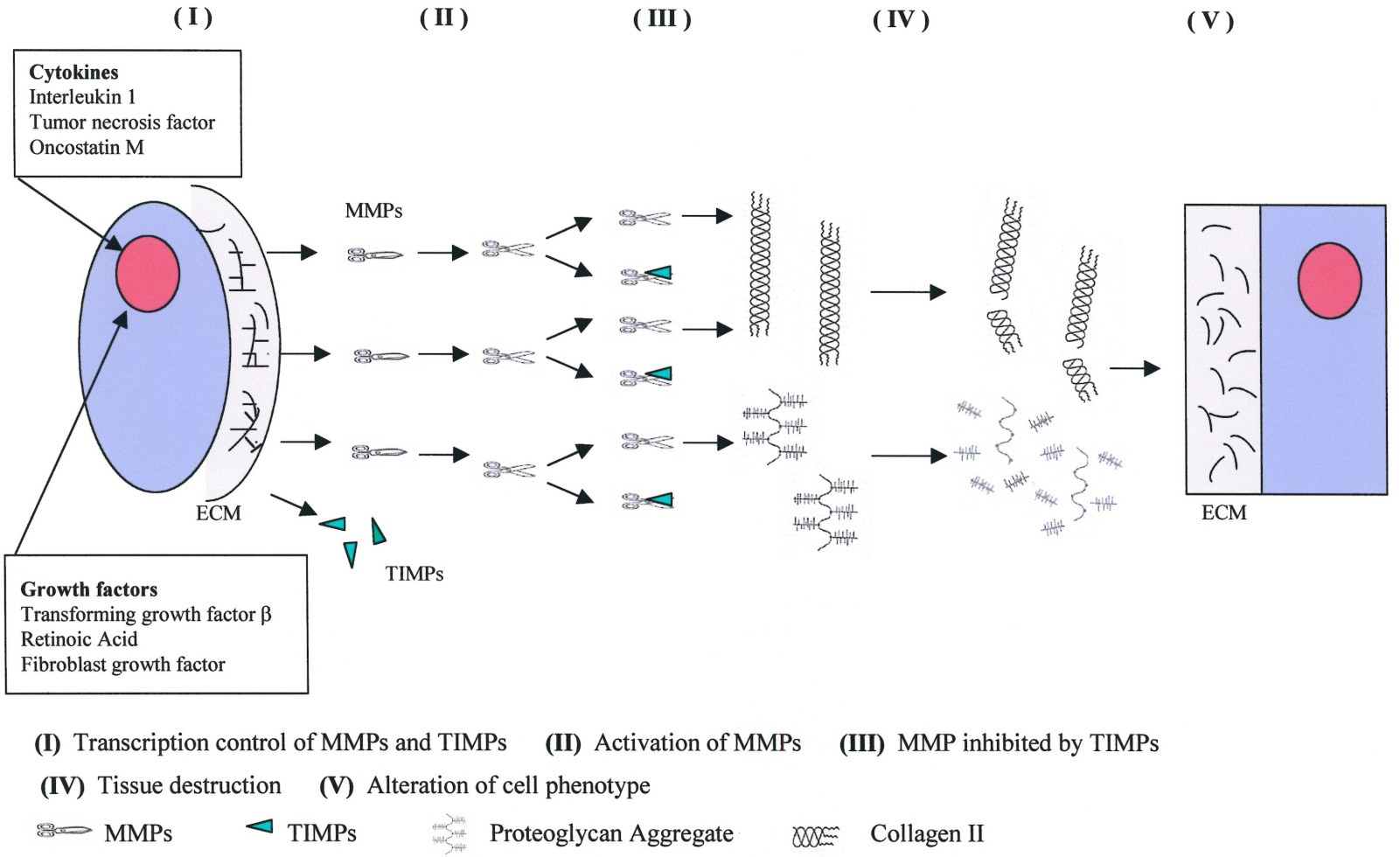

Figure A. Matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs) activities (Based on Ref: Cawston, 1998; Sternlicht and Werb, 2001)

Table A. The matrix metalloproteinase family (MMPs)

Number/name		Substrate	Structure
Collagenase	MMP-1/Collagenase-1	Aggrecan; Collagen type I, II, III, VII, VIII, X, XI; Entactin/Nidogen; Fibronectin; Gelatin; Laminin; Link protein; tenascin,	
	MMP-8/Collagenase-2	Aggrecan; Collagen type I, II, III, VII, X, XI	
	MMP-13/Collagenase-3	Aggrecan; Collagen type I, II, III, VI, VII, IX, X; Gelatin	
Stromelysin	MMP-3/Stromelysin-1	Aggrecan; Collagen type III, IV, V, IX, X, XI; Decorin; Elastin; Entactin/Nidogen; Fibronectin; Gelatin;Laminin; Link protein; Tenasin; Vitronectin	
	MMP-10/Stromelysin-2	Aggrecan; Collagen type III, IV, V, IX; Elastin: Fibronectin; Gelatin; Link protein	
	MMP-12/Metalloelastase	Aggrecan; Collagen type I, IV; Elastin; Entactin/Nidogen; Fibronectin; Fibrillin; Gelatin; Laminin; Vitronectin	
	MMP-7/Matrilysin	Aggrecan; Collagen type I, IV; Docorin; Elastin; Entactin/Nidogen; Fibronectin; Gelatin;Laminin; Link protein; Osteonectin; Tenascin;	
Gelatinase	MMP-2/Gelatinase-A	Aggrecan; Collagen type I, III, IV, V, VII, X, XI; Decorin; Elastin; Entactin/Nidogen; Fibronectin; Fibrillin; Gelatin; Laminin; Link Protein; Osteonectin; Tenascin; Vitronectin	
	MMP-9/Gelatinase-B	Aggrecan; Collagen type IV, V, XI; Decorin; Elastin; Fibrillin; Gelatin; Laminin; Link protein; Osteonectin; Vitronectin	
MMP-type MMPs	MMP-14/MT-1	Aggrecan; Collagen type I, II, III, IV; Entactin/Nidogen; Fibrillin; Fibronectin; Gelatin; Laminin; Vitronectin	
	MMP-16/MT-3	Collagen type III; Fibronectin	
Aggrecanase	ADAMTS-4/ Aggrecanase-1	Aggrecan	
	ADAMTS-5/ Aggrecanase-2	Aggrecan	

S: signal peptide; P: propeptide; C: catalytic domain; Zn: zinc-binding active site; h: hinge region; Hem: hemopexin-homology domain; Fib: fibronectin-type repeats (gelatin-binding domain); tm: transmembrane domain; ic: intracellular domain; D: Disintegr in domain Cy: Cystein-rich domain; R: Spacer Region; t:TSP-1 motif

(Based on Ref: Cawston et al., 1998; Stamenkovic, 2000; Casterson et al., 2000 ; Sternlicht and Werb 2001; Tang, 2001)

Table B. Properties of tissue inhibitors of metalloproteinases (TIMPs)

	TIMP-1	TIMP-2	TIMP-3	TIMP-4
MMP inhibition	All	All	All	?
Aggrecanase inhibition	Yes	Yes	Yes (strong)	Yes (weak)
Mature protein size (kDa)	21	22	22	23
Glycosylation	Yes	No	Yes	?
Localization	Diffusible	Diffusible	ECM bound	?
Location of gene	Xp11.23-11.4	17q2.3-2.5	22q12.1-13.2	3p25
Transcripts (kb)	0.9	3.5, 1.0	4.5-5.0 (2.8, 2.4)	1.2-1.4
Expression	Inducible	Constitutive	Inducible	?
Major tissue sites	Bone, ovary	Lung, ovarues, brain, testis, heart, placenta, joint	Kidney, brain, Lung, heart, Ovary, joint	Kidney, Placenta, Colon, testis, Brain, heart, Ovary, joint, Skeletal muscle

*ECM: extracellular matrix

(Based on Ref: Cawston, 1998; Hashimoto et al., 2001; Huang et al., 2002)

II. ARTICLE

ARTICLE 1.

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Tissue Inhibitor of Metalloproteinases-4 (TIMP-4) Gene Expression is Increased in Human Osteoarthritic Femoral Cartilage

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Running title: TIMP-4 Expression in Joints

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ABSTRACT

Tissue inhibitor of metalloproteinases-4 (TIMP-4), the newest member of the TIMP family, blocks the activities of several matrix metalloproteinases (MMPs) implicated in the arthritic cartilage erosion. By utilizing semi-quantitative RT-PCR, immunoblotting and immunohistochemistry, we investigated whether the TIMP-4 gene is expressed in human non-arthritic and osteoarthritic (OA) cartilage. Directly analyzed femoral head cartilage showed TIMP-4 RNA expression in 2 of 9 non-arthritic and 12 of 14 OA patients. Femoral head cartilage from 6 of 9 OA patients had elevated TIMP-4 protein compared to the low-level expression in 3 of 8 non-arthritic controls. In most patients, there was correlation between TIMP-4 RNA and protein expression. TIMP-4 protein was also detected immunohistochemically in the upper zone of OA cartilage. The widespread TIMP-4 RNA and protein expression and augmentation in femoral OA cartilage suggests its important role in joint tissue remodeling and pathogenesis of OA. Increased TIMP levels in arthritic cartilage may not be a sufficiently effective defense against cartilage resorption by excessive multiple MMPs and aggrecanases.

Key words: Osteoarthritis, cartilage, matrix metalloproteinases, tissue inhibitor of metalloproteinases-4, cytokines, articular chondrocytes, gene expression

INTRODUCTION

Osteoarthritis (OA) and rheumatoid arthritis (RA) are the common forms of arthritis, and knee and hip OA occurs in 6.1 and 0.7-4.4% of adults over 30 respectively [Felson and Zhang, 1998]. Multiple factors contribute to the development of OA. These include joint injuries, obesity, age, gender (higher prevalence in women over 50) and genetic susceptibility [reviewed in Felson and Zhang, 1998; Hamerman, 1995]. OA joints exhibit impaired cartilage repair, excessive proinflammatory cytokines and activated matrix metalloproteinases (MMPs) compared to their natural inhibitors, the TIMPs, leading to the erosion of articular cartilage [Poole, 1999; Pelletier et al., 2001]. MMPs include collagenase-1 and -3, stromelysin-1, gelatinases and membrane-type MMPs, which cleave cartilage extracellular matrix (ECM) during its physiological and pathological turn-over [Westermarck and Kähäri, 1999; Nagase and Woessner, 1999]. MMPs and aggrecanases digest various components of the cartilage ECM including major collagens and aggrecan at distinct sites [Tortorella et al., 1999]. The TIMP gene family consists of four members named TIMP-1, -2, -3 and –4 which have multiple activities in different systems [Gomez et al., 1997]. MMP inhibitory and growth promoting activities of TIMPs may be beneficial for protecting degenerating OA cartilage. Their pro-apoptotic and antiangiogenic activities may be useful for treating rheumatoid synovial hyperplasia and angiogenesis.

TIMP-1 and MMP-3 protein or RNA were found increased in the serum of RA and synovia of OA patients [Yoshihara et al., 1995; Zafarullah et al., 1993]. MMP-TIMP-1 balance is altered in favor of MMPs in arthritic synovium, human OA cartilage and in experimental models of OA [McCachren, 1991; Dean et al., 1989; Pelletier et al., 1990]. RA and OA synovium had similar TIMP-1 levels [Firestein et al., 1991]. Despite its MMP inhibitory activity, TIMP-1 overexpression did not protect mice from collagen-induced arthritis possibly due to its multiple activities [Apparailly et al., 2001]. The human RA cells and synovial fluid contain an MMP-2 inhibitory TIMP-2 and pro-MMP-2 complex [Cawston et al., 1993]. Normal bovine and human OA chondrocytes,

and synovia express TIMP-2 mRNA constitutively [Zafarullah et al., 1996]. While TIMP-1 and TIMP-2 are secreted, TIMP-3 distinctly binds with the sulfated glycosaminoglycans of ECM [Yu et al., 2000]. Like TIMP-1, TIMP-3 inhibits major MMPs [Apte et al., 1995]. The embryonic cartilage expresses TIMP-3 RNA [Apte et al., 1994]. It is induced by mitogens and up-regulated at the G1 phase of the cell cycle progression [Wick et al., 1994]. TIMP-3 inhibits TNF-α converting enzyme (TACE) and aggrecanases *in vitro* and may potentially protect articular cartilage from these mediators *in vivo* [Amour et al., 1998; Hashimoto et al., 2001; Kashiwagi et al., 2001]. We previously demonstrated the expression of TIMP-3 mRNA in bovine and human OA chondrocytes [Su et al., 1996], its *in vivo* increase in arthritic synovial linings and its induction by TGF-ß, a major inducer of cartilage regeneration [Su et al., 1999].

TIMP-4 is the latest member of the TIMP gene family whose expression is selectively elevated in human heart and in mouse brain, heart, ovary and skeletal muscle, suggesting its tissue-specificity [Greene et al., 1996; Leco et al., 1997]. TIMP-4 inhibits the major MMPs implicated in arthritis [Liu et al., 1997]. Like TIMP-2, TIMP-4 binds strongly with the carboxy hemopexin-domain of gelatinase A (MMP-2) and inhibits its activation by membrane-type MMP-1, the enzymes also expressed in arthritic cartilage and synovial membranes [Bigg et al., 2001]. Rat TIMP-4 mRNA is expressed in several tissues except cartilage [Wu and Moses, 1998]. TIMP-4 is reduced in patients with ischemic cardiomyopathy [Li et al., 1998]. It is transiently induced by IL-1 and TNF-α in cardiac cells [Li et al., 1999]. During vascular remodeling after balloon injury, TIMP-4 expression is increased [Dollery et al., 1999]. Since the levels of TIMPs relative to MMPs are critical for cartilage integrity, understanding the role of TIMPs in joints is of profound importance. We have previously shown that MMP-3, TIMP-1 and TIMP-3 (but not TIMP-2) mRNA is increased in human OA synovium, and articular chondrocytes express TIMP-1, TIMP-2 and TIMP-3 genes [Zafarullah et al., 1993 and 1996; Su et al., 1996 and 1999]. Besides one report of differential TIMP-4 expression relative to

other TIMPs in the healing rabbit ligaments [Reno et al., 1998] and non-inducibility of TIMP-4 in rheumatoid synovial fibroblasts by calcium pentosan polysulfate [Takizawa et al., 2000], no information is available about the expression of the novel TIMP-4 in human joints. We investigated the physiologic and pathophysiologic role of TIMP-4 in joints by studying the expression of its RNA and protein in human cartilage.

MATERIALS AND METHODS

Patients and Tissues

For direct analysis of TIMP-4 expression in cartilage, femoral head cartilage was obtained from patients with hip fractures (7 female, 2 male ranging from 52 to 93 years, mean age 74.4 years, see table 1) or from OA patients (9 female, 5 male, ranging from 50 to 79 years, mean age 61.6 years, see table 1) who underwent hip replacement surgery due to OA at the Notre-Dame Hospital. The excised human cartilage slices were frozen at -80°C until the extraction of RNA or protein.

RNA Extraction and RT-PCR

For direct RNA extraction, fresh human femoral head cartilage was rapidly frozen in guanidinium isothiocynate solution at -80°C until extraction. Cartilage tissues were first ground in solution D [Chomczynski and Sacchi, 1987] with a homogenizer (Kinetica, Switzerland) and subsequently with pestle and mortar. The homogenates were extracted once with equal volume of water-saturated phenol, precipitated with ethanol and nucleic acids resuspended in 450 µl of solution D. Subsequent purifications were with the RNeasy Plant Mini kit and spin columns (Qiagen Inc., Mississauga, ON) according to the manufacturer's protocols. Spin columns of these kits eliminate the interfering glycosaminoglycans in cartilage, whose analogues are also found in plants. RNA was quantified and its integrity verified by agarose-formaldehyde gel electrophoresis.

For RT-PCR, 2 µg RNA aliquots were heated for 5 min at 65°C and reverse transcribed in the reaction mixture consisting of oligo d(T) 12-18mer, dNTPs, RNase inhibitor (Pharmacia), acetylated BSA (Promega) with Moloney murine leukemia-virus reverse transcriptase (MMLV-RT) (GIBCO/BRL) according to the protocols of Clontech Laboratories Inc. (Paolo Alto, CA). Aliquots of 5 µl from the 30 µl RT reaction were subjected to PCR with TIMP-4, or GAPDH primers. The forward and reverse primers specific for TIMP-4

were designed from the published sequence of the human TIMP-4 cDNA [Greene et al., 1996] whose sequences were: 5' AGA CCT CAC AGG CTC AGT CG 3' (from nucleotide 25 to 44) and 5' CAT TCC TGC CAG TCA GCC TG 3' (from nucleotides 1151 to 1170) respectively (synthesized by GIBCO/BRL, Burlington, ON). The amplification profile was one cycle of 94°C for one min, 35 cycles of 94°C for one min, hybridization at 60°C for 2 min and extension at 72°C for 3 min. A final extension cycle of 7 min at 72°C was also included. The PCR amplifications were performed in the Gene E thermal cycler (Techne, Cambridge, England) in a 50 µl reaction with 1.25 mM dNTPs, Taq DNA polymerase (Pharmacia), respective primers and overlaid with mineral oil. The GAPDH cDNA amplification kit and primers were from Maxime Biotech. Inc. (South San Francisco, CA) whose sequences were (forward) 5' GAA GGT GAA GGT CGG AGT C 3' and (reverse) 5' GAA GAT GGT GAT GGG ATT TC 3' which were utilized according to the recommendations of the suppliers. Aliquots of 10 µl from the 50 µl PCR reaction were analyzed on 1.2 (TIMP-4) or 1.4 (GAPDH) % agarose gels to detect TIMP-4 and GAPDH cDNA amplification products of 1148 and 226 bp respectively. Negative controls included either all the RT-PCR reagents except cDNA or additionally, RT was omitted in the reaction mix before PCR. None of these controls gave any bands. The TIMP-4 cDNA product was cloned in pGEM-4Z and the identity of the amplification product was confirmed by DNA sequence analysis (Sheldon Biotechnology Centre, McGill University) which completely matched with the published sequences [Greene et al, 1996].

Extraction of Cartilage Proteins and Western Immunoblot Analysis

For the analysis of TIMP-4 protein expression in human cartilage, total protein was extracted by homogenization of cartilage slices in a buffer consisting of 50 mM Tris.HCl, 10 mM $CaCl_2.2H_2O$, 0.05% Brij 35 and 2M guanidine hydrochloride, pH 7.5. The homogenate was incubated overnight at 4°C with gentle shaking. After centrifugation at 3000 rpm for 10 min, extracts were

dialyzed against 50 mM Tris.HCl pH 7.5 with 3 buffer changes. Protein was measured with Bio-Rad protein assay system. The protein extracts were fractionated by 5% stacking, 12% separating SDS-PAGE using Bio-Rad (Mississauga, ON) mini-gel system, transferred to nitrocellulose by electroblotting, membranes washed three times with TBS buffer, blocked by 10% carnation milk and reacted with 1:1000 dilution of rabbit anti-human TIMP-4 polyclonal antibody (Chemicon International Inc. Temecula, CA) for 3 h at room temperature or overnight at 4°C. This antibody has no cross-reactivity with TIMP-1, TIMP-2 and TIMP-3. The membranes were then washed, blocked for 20 min and reacted with peroxidase-conjugated anti-rabbit secondary IgG (Roche Molecular Biochemicals, Laval, QC) and the TIMP-4 band revealed for 1 to 20 min by chemiluminescence system of Roche according to the manufacturer's protocols.

Immunohistochemistry

Cartilage blocks were frozen in OCT medium, 7-μm thick sections cut with a cryostat, placed on silane-covered slides and treated with 0.25 U/ml chondroitinase ABC (Sigma) for 1 h in PBS. The samples were treated with 0.3% H_2O_2 in methanol for 30 min to quench the endogenous peroxidase activity, washed with PBS for 5 min and blocked by incubating in normal goat serum (ABC Elite kit, Vector Labs. Inc. Burlington, ON) for 20 min. The slides were incubated with 1:1000 dilution of the anti-human TIMP-4 primary antibody in PBS overnight at 4°C, washed with PBS for 5 min, incubated for 30 min. with biotinylated anti-rabbit IgG in goat (secondary antibody), washed, reacted with ABC stain reagent for 30 min., incubated with 3,3'-diaminobenzidine substrate (Vector Labs) for 10 min., counterstained the nuclei with hematoxylin for 1 min., dehydrated with 70% and 100% ethanol. The slides were cleared with 3 xylene washes, mounted with Permount (Fisher Scientific) and photographed. For negative conrtols, primary antibody was incubated with the competing purified TIMP-4 protein for 1 h at room temperature or overnight at 4°C and then reacted with tissue slices. Additional

negative controls were processed without the addition of primary antibody. Such controls did not yield positive staining. Nuclei appeared blue and TIMP-4-specific staining was brown.

RESULTS

TIMP-4 mRNA Expression in Human Femoral Head Articular Cartilage

We initially found that primary cultures of non-arthritic and OA chondrocytes as well as human synovial membranes constitutively expressed TIMP-4 RNA (data not shown). To investigate the *in vivo* expression of TIMP-4 gene in human joints, RNA was extracted directly from the femoral head cartilage without any prior treatment or culturing. Only 2 of 9 (3 and 9) cartilage specimens from femoral head fractures expressed TIMP-4 RNA. The other 7 either did not express or expressed this gene at very low levels. In contrast, 12 of 14 femoral head OA and the only available RA cartilage specimens expressed this mRNA (Fig. 1). The constitutive GAPDH RNA levels were constant (Fig. 1, lower panel). These results suggest an increased TIMP-4 mRNA expression in the cartilage of OA patients.

Expression of TIMP-4 Protein in Human Femoral Head Articular Cartilage

To further evaluate the *in vivo* expression of TIMP-4 in human joints, protein was extracted directly from the 8 non-arthritic (corresponding to the non-arthritic samples 2 to 9 respectively in Fig.1) and 9 OA (corresponding to the OA samples 7 to 14 respectively in Fig. 1) femoral heads as well as 1 RA cartilage and 1 OA synovial membrane without any prior treatment or culturing. The Western blots containing 30 μg/lane of total protein were probed with anti-human TIMP-4 antibody. The controls had undetectable or low, while 6 of 9 OA and 1 RA femoral cartilage as well as 1 OA synovial membrane had elevated levels of TIMP-4 protein (Fig. 2). The band of 29 kDa size protein is comparable in size with that from human heart, the major tissue expressing this protein [Greene et al., 1996; Dollery et al., 1999]. This band co-migrated with the purified human synovial fibroblast TIMP-4 protein (results not shown). Since most of these samples were also analyzed for TIMP-4 RNA expression in Fig. 1, we were able to compare RNA and protein expression in the same tissues. RNA results from Fig. 1 are depicted at the bottom of Fig. 2. In general,

samples with low TIMP-4 protein levels also had low or undetectable TIMP-4 RNA and tissues with elevated TIMP-4 protein were positive for respective RNAs. Thus, human cartilage clearly synthesizes TIMP-4 protein (and RNA) whose levels are increased in most OA patients.

Detection of TIMP-4 Expression in Cartilage and Chondrocytes by Immunohistochemistry

To further confirm the above results and to investigate spatial expression of TIMP-4 in human OA and normal cartilage, tissue sections were analyzed by immunohistochemistry with peroxidase-based ABC system. TIMP-4-positive brown staining was observed predominantly in the upper zone and minimally in the middle zone of the OA cartilage from three different patients (Fig. 3B, 3D and 3F) that was absent in sections where antibody was pre-adsorbed with the purified total TIMP-4 protein (Fig. 3A, and 3C) or upon omission of the primary antibody (Fig. 3E and 3G). The TIMP-4 expression was relatively much lower in the upper zone of normal human cartilage (Fig. 3H). Cells in control tissues showed only blue stained nuclei as a result of hematoxylin counterstaining while the TIMP-4 antibody-treated cells displayed both blue-stained nuclei and TIMP-4-specific brown staining intracellularly and in the ECM of chondrocytes. Primary chondrocytes in culture were also positive for TIMP-4 (results not shown).

DISCUSSION

Hip and knee are the most commonly affected joints in obese and elderly patients suffering from osteoarthritis that ultimately require joint replacement surgery. It is important to understand the molecular mechanisms contributing to this disease. TIMP-4, the newest member of the TIMP gene family, blocks the activities of several enzymes (MMP-1, MMP-2, MMP-3, MMP-7 and MMP-9) known to be implicated in the arthritic artilage erosion [Liu et al., 1997]. We have shown here for the first time that TIMP-4 RNA and protein expression is increased in the cartilage of most patients with femoral head OA. Additionally, it is widely expressed in the other major joint tissue, synovium, under non-arthritic and arthritic conditions (results not shown). Thus, TIMP-4 gene has widespread expression in human joints and may contribute to the pathogenesis od OA.

TIMP-4 mRNA levels are generally low in all tissues including those from joints but could nevertheless be detected by RT-PCR using as low as 1 μg of total RNA. This is in contrast with other TIMPs which can be detected by Northern hybridizations [Zafarullah et al., 1993 and 1996, Su et al., 1999]. In other tissues poly A^+ mRNA from 150-210 μg of total RNA was used to detect its expression [Greene et al., 1996; Li et al., 1998 and 1999]. Such amounts are unachievable from the limited human joint tissues. Similar levels of TIMP-4 RNAs in non-arthritic and OA synovial membranes (data not shown) suggests a pattern analogous to that of TIMP-2 [Su et al., 1999] which may also be indicative of lesser involvement of synovial linings in OA. In contrast, TIMP-1 and TIMP-3 mRNAs are mostly increased [Su et al., 1999] due to possible altered metabolism of arthritic joints. The continuous TIMP-4 expression may be related to its important role and persistent requirement in both physiologic and pathologic situations such as protection of synovial ECM integrity, anti-angiogenic, growth promoting or anti-apoptotic activities [Gomez et al., 1997].

Contrary to the expression of TIMP-4 RNA in all non-OA and OA femoral head-derived primary chondrocytes (our unpublished data), analysis of the RNA directly from the femoral head revealed low-level expression of TIMP-4 in non-arthritic cartilage and increased expression in 85% of the OA cases. Thus there is considerable disparity in TIMP-4 gene expression under the *ex vivo* and *in vivo* conditions. Low-level TIMP-4 expression in normal human cartilage may have physiological roles such as protection of its matrix from MMPs, growth promotion or apoptosis [Gomez et al., 1997, Tummalapalli et al., 2001]. The expression of TIMP-4 protein in directly analyzed human non-OA and OA cartilage suggests that despite some variation between different individuals, there is a clear tendency of increased TIMP-4 expression in most OA cases. This is consistent with the observed altered phenotype of metabolically hyperactive OA chondrocytes [Aigner and Dudhia, 1997] and supports the proposed role of TIMPs in OA cartilage matrix remodeling. Expression in the superficial zone of OA cartilage further supports such a role. Curiously, the only RA sample analyzed (due to non-availability) had higher TIMP-4 RNA and protein levels relative to OA which may be due to more aggressive nature of RA and merits further investigation. TIMP-4 augmentation observed here in the remodeling OA cartilage is analogous to the vascular remodeling after balloon-induced injury [Dollery et al, 1999]. While cartilage tissues analyzed here are from the end-stage OA, it is possible that TIMP-4 expression is increased temporally in the earlier phases where type II collagen and aggrecan core protein synthesis are known to be increased [Matyas et al., 1995]. In the early inflammatory phase of healing rabbit ligaments, proteinases and all TIMPs were increased except TIMP-4, which was repressed, suggesting distinct functions of TIMPs. Since collagenase-3 is a major enzyme implicated in type II collagen cleavage in arthritic cartilage [Shlopov et al., 1997; Mitchell et al., 1996; Reboul et al., 1996; Billinghurst et al., 1997], we also investigated its mRNA expression in the femoral head specimens in Fig. 1. None of the control patients expressed collagenase-3 message but 71% of OA and 1 RA patients did (our

unpublished results). Expression of the MMP-13 and TIMP-4 genes was mostly but not always coordinate suggesting differential *in vivo* regulatory mechanisms.

The signals implicated in the pathogenesis of arthritis include IL-1, TNF-α, oncostatin M and TGF-β among others [Poole, 1999]. However, our preliminary results (not shown) suggest that TIMP-4 protein is not upregulated by the proinflammatory cytokines, IL-1 and TNF-α which induce MMPs [Shlopov et al., 1997; Mitchell et al, 1996; Reboul et al., 1996]. Thus factors which increase TIMP-4 expression *in vivo* need to be identified by more work. IL-1β and TGF-β upregulate TIMP-3 and TIMP-1 in synovial fibroblasts [DiBattista et al., 1995, Gatsios et al., 1996]. Contrary to TIMP-1 and TIMP-3 induction by TGF-ß [Zafarullah et al., 1996; Su et al., 1996 and 1999, Günther et al., 1994], TIMP-2 [Zafarullah et al., 1996] and TIMP-4 RNA (results not shown) and protein are not regulated by TGF-β which is involved in cartilage repair and osteophyte formation in arthritic joints [Guerne et al., 1995; Van den Berg, 1999]. Oncostatin M, a factor that synergizes with IL-1 to degrade cartilage [Cawston et al., 1998], induces TIMP-1 in human chondrocytes [Nemoto et al., 1996] and TIMP-3 in bovine chondrocytes [Li and Zafarullah, 1998]. Non-induction of TIMP-4 by OSM suggests that TIMP-1, TIMP-3 and TIMP-4 may be differentially regulated by this signal. The coordinate or differential regulation may be due to similarities and differences in the promoter composition of inducible (TIMP-1 and TIMP-3) and non-inducible (TIMP-2, TIMP-4) TIMPs. Differential expression of TIMPs may also suggest their distinct functions. Characterization of the TIMP-4 promoter may shed light on the observed patterns of its expression and enable to identify *in vivo* regulatory mechanisms.

In summary, previously undocumented expression and elevation of the TIMP-4 RNA and protein was shown directly in the OA femoral head cartilage by several approaches. These patterns of TIMP-4 expression may be related to its possible role in tissue matrix protection, growth promotion, anti-angiogenic

activities and pathologic ECM remodeling [Brew et al., 2000]. Although TIMPs such as TIMP-4 are increased in arthritic cartilage as a defense mechanism against MMPs, such levels may not be enough to prevent cartilage erosion from overwhelmingly excessive levels of multiple MMPs and aggrecanases from chondrocytes and other sources. Additional studies are needed to define the functions of TIMPs in joints and their therapeutic potential for inhibiting cartilage resorption.

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Figure Legends

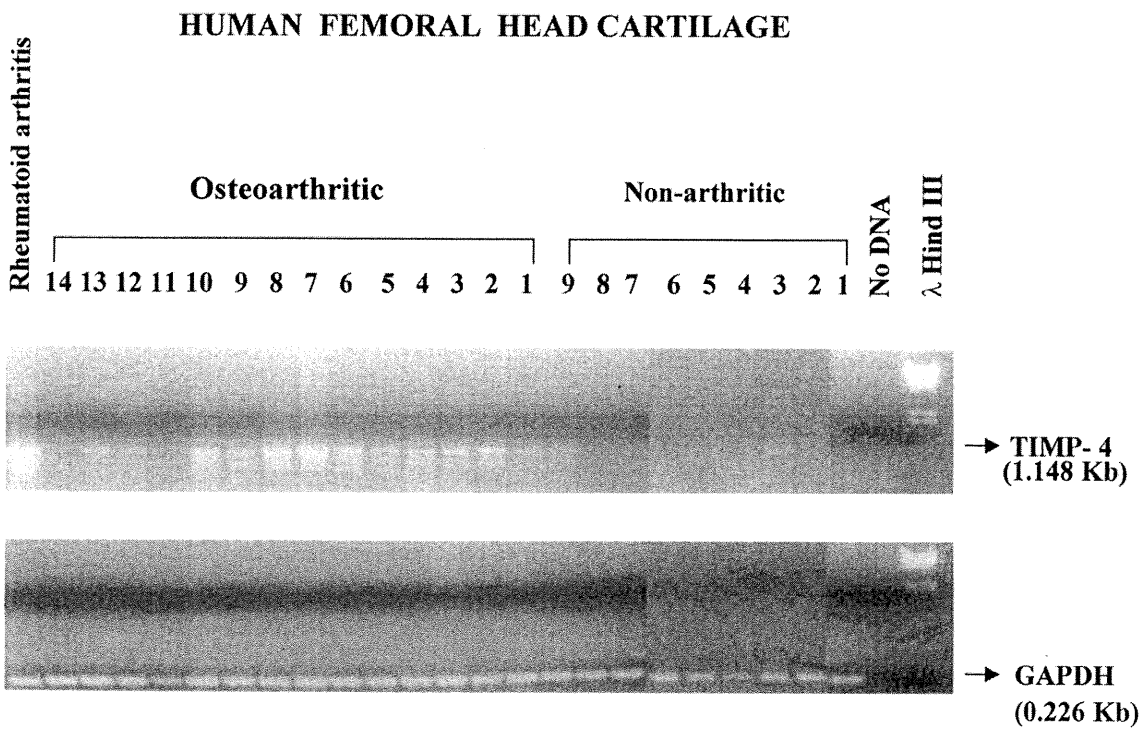

Fig. 1. Expression of the TIMP-4 mRNA in human non-arthritic and OA femoral head cartilage. Total RNA (2 μg) extracted directly from 9 non-arthritic (femoral head fractures), 14 femoral head OA and 1 rheumatoid arthritis cartilage was reverse transcribed into cDNA and 5 μl aliquots amplified with the TIMP-4- or GAPDH-specific primers for 35 cycles. Aliquots of 10 μl from a 50 μl reaction were analyzed by agarose gel electrophoresis. The specific amplification products of 1.148 Kb (upper panel), and 0.226 Kb (lower panel) along with the lambda HindIII markers are depicted. The 'No DNA' control lane represents PCR reactions with primers but without cDNA, and did not yield any products.

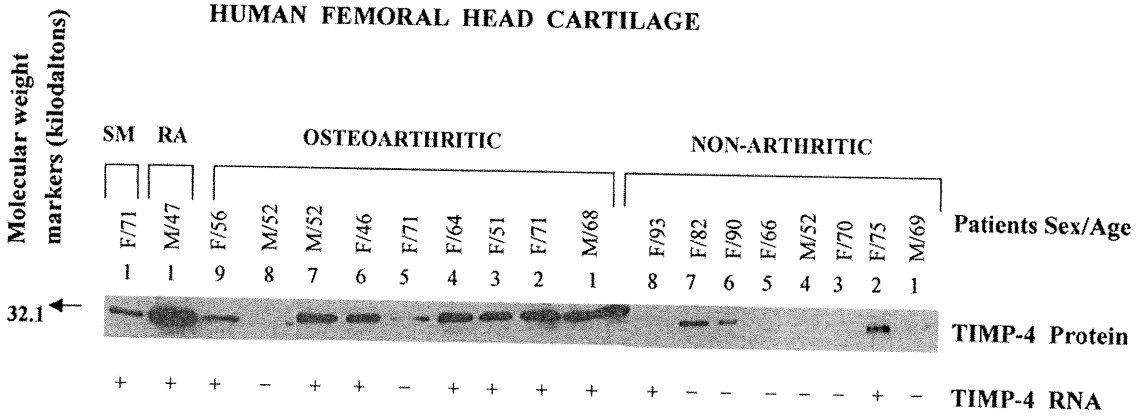

Fig. 2. TIMP-4 protein expression in human non-arthritic and OA femoral head cartilage. Total protein was extracted directly from the femoral head cartilage of 8 patients with fractures, 9 with OA, 1 with rheumatoid arthritis (RA) and 1 OA synovial membrane (SM), subjected to SDS-PAGE, probed with the 1:1000 dilution of polyclonal human TIMP-4 antibody, reacted with the 1:400 dilution of anti-rabbit secondary antibody and revealed by chemiluminescence. The film was exposed for one minute. The position of TIMP-4 band relative to the molecular weight markers is shown. On the top, age and gender of each patient is shown. Additionally, the result of TIMP-4 RNA expression from Fig. 1 is also included for comparison.

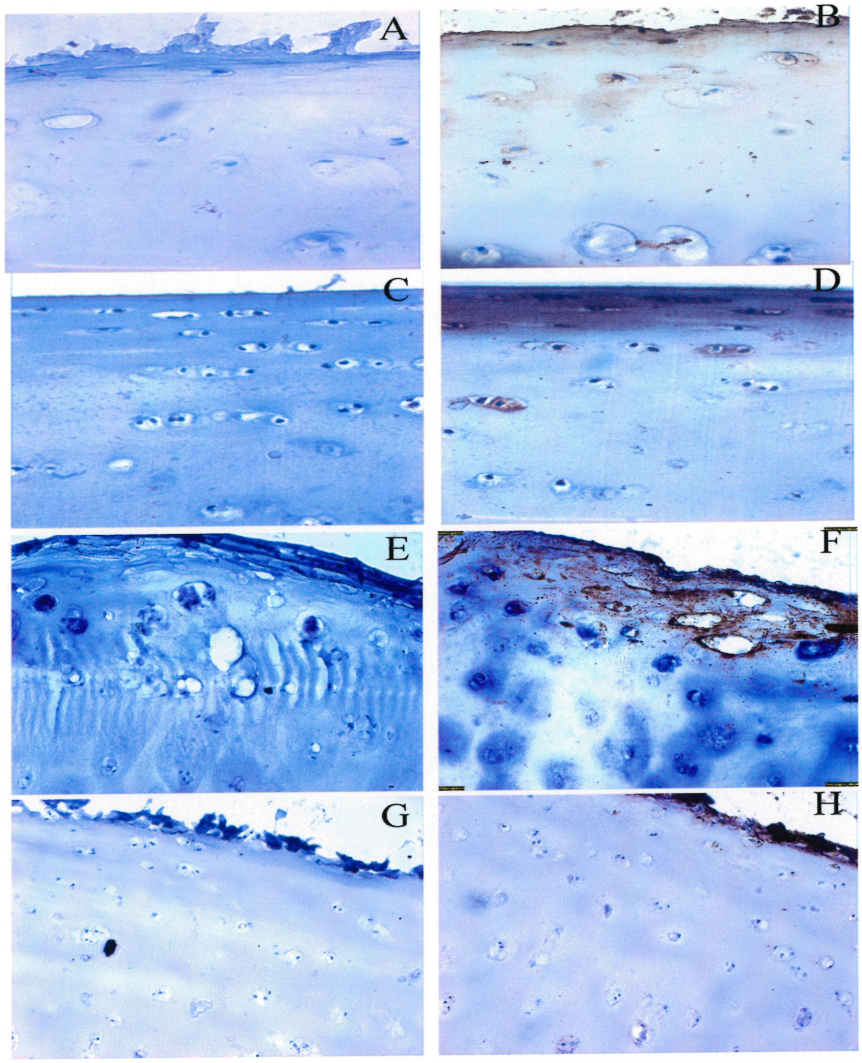

Fig. 3. Detection of TIMP-4 expression in human femoral head cartilage. In panels A-F, cartilage sections from three OA patient were stained after preincubation with the competing pure TIMP-4 protein and TIMP-4 antibody (A and C), without TIMP-4 antibody (E) (negative controls) or with TIMP-4 antibody (B, D, F) using an avidin/biotin horse radish peroxidase-based (ABC elite) kit, nuclei counterstained with hematoxylin and photographed. Only blue nuclear staining with hematoxylin is seen in A, C, E and G (negative controls) while additional brown TIMP-4 staining can be observed in B, D and F. In panels G and H, staining of a non-OA cartilage specimen is shown without (G) and with (H) TIMP-4 antibody.

Table 1. Characteristics of the patients analyzed for TIMP-4 RNA expression. Abbreviations: F, female; M, male; OA, osteoarthritis; RA, rheumatoid arthritis.

Patient #		*Age/Sex*	*Disease*
Non-arthritic patients	1	73/F	hip fracture
	2	69/M	hip fracture
	3	75/F	hip fracture
	4	70/F	hip fracture
	5	52/M	hip fracture
	6	66/F	hip fracture
	7	90/F	hip fracture
	8	82/F	hip fracture
	9	93/F	hip fracture
Arthritis patients	1	57/F	OA
	2	79/M	OA
	3	69/M	OA
	4	71/F	OA
	5	50/F	OA
	6	61/F	OA
	7	68/M	OA
	8	71/F	OA
	9	51/F	OA
	10	64/F	OA
	11	71/F	OA
	12	46/F	OA
	13	52/M	OA
	14	52/M	OA
	15	47/M	RA

ARTICLE 2.

Annals of Rheumatic Diseases (under revision)

Tissue Inhibitor of Metalloproteinases-4 (TIMP-4) RNA is Constitutively Expressed in Human Synovial Membranes and Primary Chondrocytes

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Running title: TIMP-4 expression in synovium and chondrocytes

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ABSTRACT

Objective- To investigate whether tissue inhibitor of metalloproteinases-4 (TIMP-4), the newest member of the TIMP gene family is expressed in human joints.

Methods- TIMP-4-specific primers were utilized for monitoring its RNA expression in human non-arthritic and osteoarthritic (OA) synovial membranes and primary femoral head chondrocytes by RT-PCR analysis in comparison with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. TIMP-4 protein levels from cytokine-treated chondrocytes were measured by western blotting.

Results- Synovial membranes from 7 non-arthritic and 8 OA patients constitutively expressed TIMP-4 RNA. Femoral head chondrocytes from 2 non-arthritic and 15 patients with hip OA also exhibited substantial constitutive expression of the TIMP-4 mRNA. TIMP-4 protein was not inducible by transforming growth factor β1 (TGF-β1), oncostatin M (OSM), tumor necrosis factor α (TNF-α) and interleukin-1 (IL-β) in human and bovine chondrocytes.

Conclusion- TIMP-4 gene is constitutively expressed in synovial membranes and chondrocytes in culture, which suggests its physiological role in joints. TIMP-4 detection may be useful for monitoring its expression and function during the pathogenesis of arthritis.

Key words: Osteoarthritis, synovium, chondrocytes, TIMPs

INTRODUCTION

The TIMP gene family consists of TIMP-1, -2, -3 and –4 which have matrix metalloproteinases (MMPs) inhibiting, anti-metastatic, anti-angiogenic, growth promoting and proapoptotic activities in different *in vitro* and *in vivo* systems.[1 2] MMPs and TIMP-1 are increased in several patients with arthritis. [3] Ability of TIMPs to inhibit MMPs, aggrecanases and TNF-α converting enzyme (TACE) (TIMP-3) makes them candidate proteins for blocking resorption of cartilage in arthritis.[4 5 6] Despite *in vitro* inhibition of cartilage collagen breakdown by TIMP-1 and TIMP-2, *in vivo* TIMP-1 overexpression did not block collagen-induced arthritis. [7 8] TIMP-4 is the latest member of the TIMP gene family whose expression is elevated in human heart and in certain mouse tissues, suggesting its tissue-specificity. [9 10] TIMP-4 inhibits the major MMPs implicated in arthritis.[11] Since TIMPs-MMPs balance is critical for cartilage integrity, understanding the role of TIMPs in joints is important. We have previously shown that MMP-3, TIMP-1 and TIMP-3 (but not TIMP-2) mRNA is increased in human OA synovium, and articular chondrocytes express TIMP-1, TIMP-2 and TIMP-3 genes.[12] Besides reported lack of expression in synovial fibroblasts,[13] no information is available about the expression of the novel TIMP-4 in human joints. We investigated its expression in the main joint tissues affected by arthritis, synovial membranes and cartilage cells.

MATERIALS AND METHODS

PATIENTS, TISSUES AND CELL CULTURE

For TIMP-4 expression in synovial membranes, 7 non-arthritic post-mortem patients (within 12 h of death; 3 female and 4 male, mean age 53.6 years) with no knee joint disease were analyzed. OA synovial membranes obtained at knee replacement surgery were from eight patients (4 female and 4 male, mean age 71.3 years) with clinically and radiologically defined OA of the knee. Chondrocytes were released from human femoral head cartilage after digestion with pronase and bacterial collagenase, grown in DMEM supplemented with 1X penicillin-streptomycin solution and 10% fetal calf serum as primary cultures, maintained without serum for 24 h and RNA extracted. Chondrocytes were also treated individually for 24 h with TGF-β1, OSM, TNF-α and IL-1β, and conditioned media and cells harvested for protein analysis.

RNA EXTRACTION AND RT-PCR

Primary chondrocyte RNA was isolated by the method of Chomczynski and Sacchi.[13] RNA from human knee synovial membranes was extracted by homogenization in guanidinium isothiocyanate solution and cesium chloride ultracentrifugation, and quantified by spectrophotometry. RNA was quantified and its integrity verified by agarose-formaldehyde gel electrophoresis.

For RT-PCR, 2 μg RNA aliquots were heated for 5 min at 65°C and reverse transcribed in the reaction mixture consisting of oligo d (T) 12-18mer, dNTPs, RNase inhibitor (Pharmacia), acetylated BSA (Promega) with reverse transcriptase (MMLV-RT) (GIBCO/BRL) according to the protocols of Clontech Laboratories Inc. (Paolo Alto, CA). Aliquots of 5 μl from the 30 μl RT reaction were subjected to PCR with TIMP-4, or GAPDH primers. The forward and reverse primers specific for TIMP-4 were designed from the published sequence of the human TIMP-4 cDNA[9] whose sequences were: 5' AGA CCT CAC AGG CTC AGT CG 3' (from nucleotide 25 to 44) and 5' CAT

TCC TGC CAG TCA GCC TG 3' (from nucleotides 1151 to 1170) respectively (synthesized by GIBCO/BRL, Burlington, ON). The amplification profile was one cycle of 94°C for one min, 35 cycles of 94°C for one min, hybridization at 60°C for 2 min and extension at 72°C for 3 min. A final extension cycle of 7 min at 72°C was also included. The amplifications were performed in the Gene E thermal cycler (Techne, Cambridge, England) in a 50 µl reaction with 1.25 mM dNTPs, Taq DNA polymerase (Pharmacia), respective primers and overlaid with mineral oil. The GAPDH cDNA amplification kit and primers were from Maxime Biotech. Inc. (South San Francisco, CA) whose sequences were (forward) 5' GAA GGT GAA GGT CGG AGT C 3' and (reverse) 5' GAA GAT GGT GAT GGG ATT TC 3' which were utilized as recommended by the suppliers. Aliquots of 10 µl from the 50 µl PCR reaction were analyzed on 1.2 (TIMP-4) or 1.4 (GAPDH) % agarose gels to detect TIMP-4 and GAPDH cDNA amplification products of 1148 and 226 bp respectively. Negative controls included either all the RT-PCR reagents except cDNA or additionally, RT was omitted in the reaction mix before PCR. None of these controls gave any bands. The TIMP-4 cDNA product was cloned in pGEM-4Z and the identity of the amplification product confirmed by DNA sequencing, which completely matched with the published sequences.[9]

PROTEIN ANALYSIS

The protein extracts were fractionated by 5% stacking, 12% separating SDS-PAGE using Bio-Rad (Mississauga, ON) mini-gel, transferred to nitrocellulose by electroblotting, membranes washed with TBS buffer, blocked by 10% carnation milk and reacted with 1:1000 dilution of rabbit anti-human TIMP-4-specific polyclonal antibody (Chemicon International Inc. Temecula, CA) for 3 h at room temperature. The membranes were washed, blocked for 20 min and reacted with peroxidase-conjugated anti-rabbit secondary IgG (Roche Biochemicals, Laval, QC) and the TIMP-4 band revealed by its chemiluminescence system.

RESULTS

TIMP-4 mRNA EXPRESSION IN HUMAN NON-ARTHRITIC AND OA SYNOVIAL MEMBRANES

To investigate the ability of human synovium to express the TIMP-4 gene, total RNA was analyzed by Northern hybridization, however, no TIMP-4 RNA could be detected by this technique, suggesting rather low message levels. RT-PCR analysis of 7 control subjects and 8 patients with the OA of knee revealed that both non-arthritic and OA patients expressed easily detectable levels of TIMP-4 mRNA. One normal and one OA synovium had lower but detectable levels of TIMP-4 RNA relative to the other samples. The identity of the band as TIMP-4 cDNA was confirmed by cloning and DNA sequence analysis of the PCR product at both ends. The control GAPDH RNA levels were consistent in all patients (fig 1).

EXPRESSION OF TIMP-4 mRNA IN PRIMARY HUMAN FEMORAL HEAD ARTICULAR CHONDROCYTES

To examine if human hip joint chondrocytes expressed TIMP-4 gene *ex vivo*, RNA from the quiescent (under serum-free conditions) chondrocytes of two patients with femoral fracture and 15 patients with hip OA were analyzed. As depicted in fig 2, all specimens expressed variable but clearly detectable levels of TIMP-4 mRNA along with consistent GAPDH control RNA (fig 2).

IMPACT OF CYTOKINES AND GROWTH FACTORS ON TIMP-4 PROTEIN EXPRESSION IN CHONDROCYTES

We investigated TIMP-4 regulation by the cytokines and growth factors found in arthritic joints. Treatment of three OA patient chondrocytes with TGF-β1, OSM, TNF-α and IL-1 and TIMP-4 protein analysis revealed that none of these factors significantly upregulated TIMP-4 expression in the cells or secreted medium (fig. 3A). TIMP-4 expression was also not inducible in bovine chondrocytes (fig. 3B).

DISCUSSION

TIMP-4, the newest member of the TIMP gene family, blocks the activities of MMP-1, MMP-2, MMP-3, MMP-7, MMP-9 and aggrecanase-1 known to be implicated in the arthritic cartilage erosion.[6 11] We showed here for the first time its constitutive expression in synovium and primary chondrocytes from cartilage under non-arthritic and arthritic conditions. Thus, TIMP-4 gene may have a physiological role in human joints.

Other TIMPs can be detected by Northern hybridizations.[12] In contrast, except heart, TIMP-4 RNA levels are low in all tissues including those from joints but could nevertheless be detected by RT-PCR. Similar TIMP-4 RNA levels in non-arthritic and OA synovial membranes suggest a pattern analogous to that of TIMP-2[12] which indicates lesser involvement of synovial linings in OA. In contrast, TIMP-1 and TIMP-3 mRNAs are increased possibly to counteract excessive MMP-driven destruction.[12] Demonstrated TIMP-4 expression in synovial membrane differs from a recent study which failed to detect any TIMP-4 protein in rheumatoid synovial fibroblasts.[13] This may be due to higher sensitivity of our RT-PCR technique. The constitutive TIMP-4 expression may be related to its important role and persistent requirement in both physiologic and pathologic situations such as protection of synovial ECM integrity, anti-angiogenic, growth promoting or anti-apoptotic activities.[1 2]

Human femoral head normal and OA chondrocytes clearly have the capacity to synthesize TIMP-4 message under *ex vivo* conditions suggesting its physiological roles such as maintenance of balance with MMPs to protect its matrix. As TIMP-4 is a weak inhibitor of aggrecanase-1 activity[6], chondrocyte expression may be related to inhibition of this major proteoglycan degrading enzyme. Since TIMP-4 also has either growth promoting or proapoptotic activities in other systems,[14 15] it remains to be examined if the observed expression is related to these activities. Lack of

TIMP-4 protein induction by arthritis-associated cytokines suggests that TIMPs may be differentially regulated and could have distinct functions. TGF-β induces TIMP-1 and TIMP-3 in chondrocytes.[12] So far, TIMP-1 and TIMP-3 are inducible while TIMP-2, TIMP-4 appear non-inducible. Thus factors regulating TIMP-4 expression need to be identified by more work.

In summary, previously undocumented constitutive expression of TIMP-4 in human synovium and primary chondrocytes was shown. Due to multiple activities of TIMPs in other tissues, additional studies with TIMP-4-specific primers may be useful for defining its functions in joints and potential for inhibiting cartilage resorption.

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Figure Legends

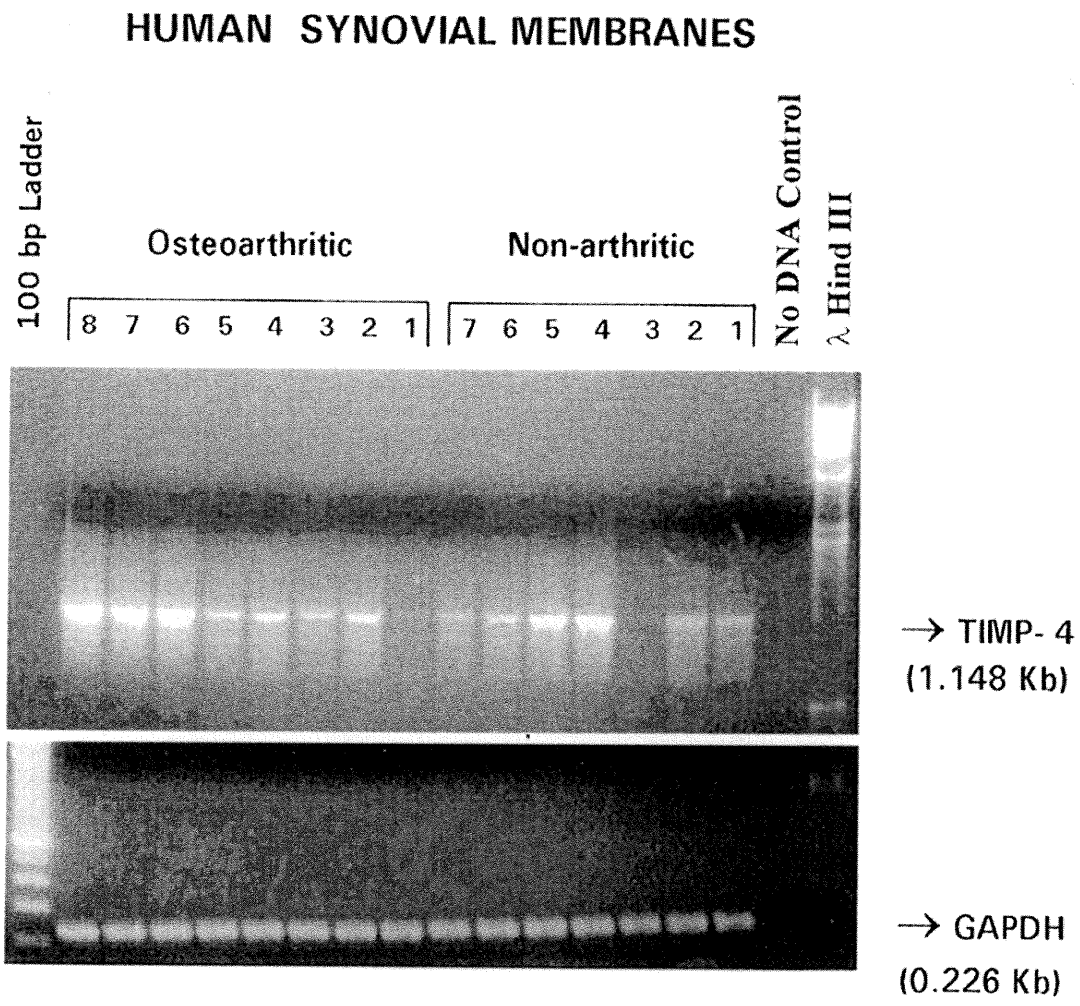

Fig. 1. Constitutive expression of TIMP-4 gene in human synovial membranes from non-arthritic and osteoarthritic patients. The specific amplification TIMP-4 and GAPDH ethidium bromide-stained fragments (arrows) along with the lambda HindIII or 100 bp ladder markers are shown. The 'No DNA' control lane represents PCR reactions with primers but without cDNA, and did not yield any products.

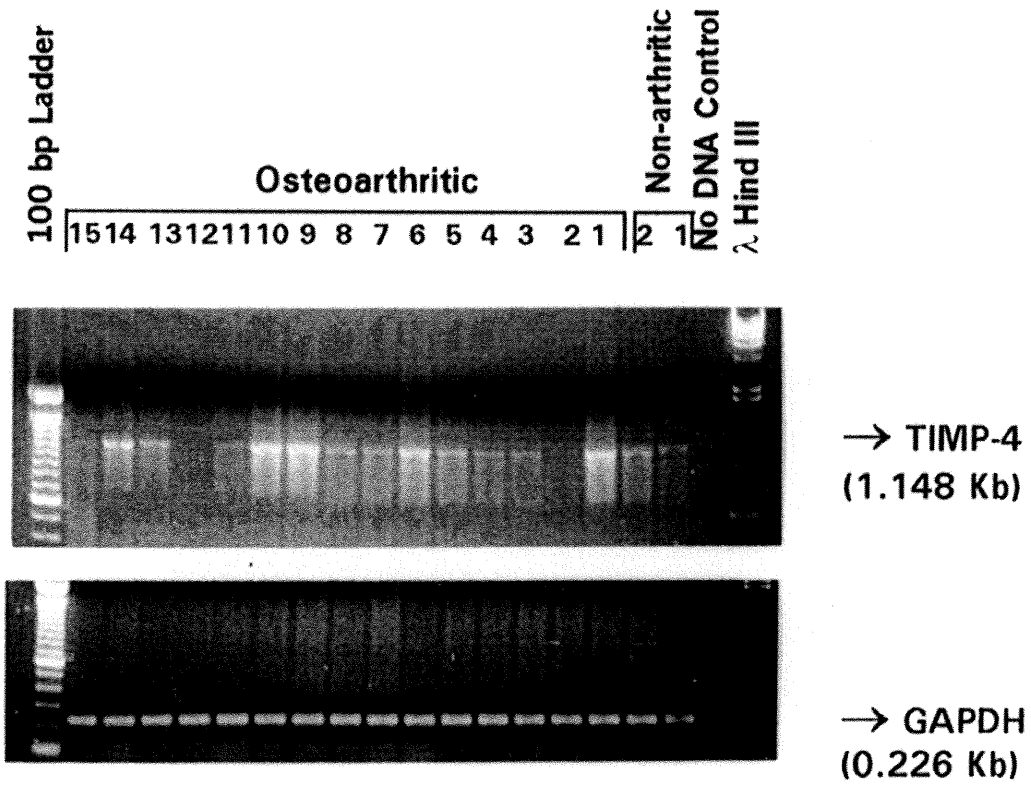

Fig. 2. Expression of the TIMP-4 message in primary human non-arthritic and OA femoral head articular chondrocytes. All conditions were same as in Fig. 1.

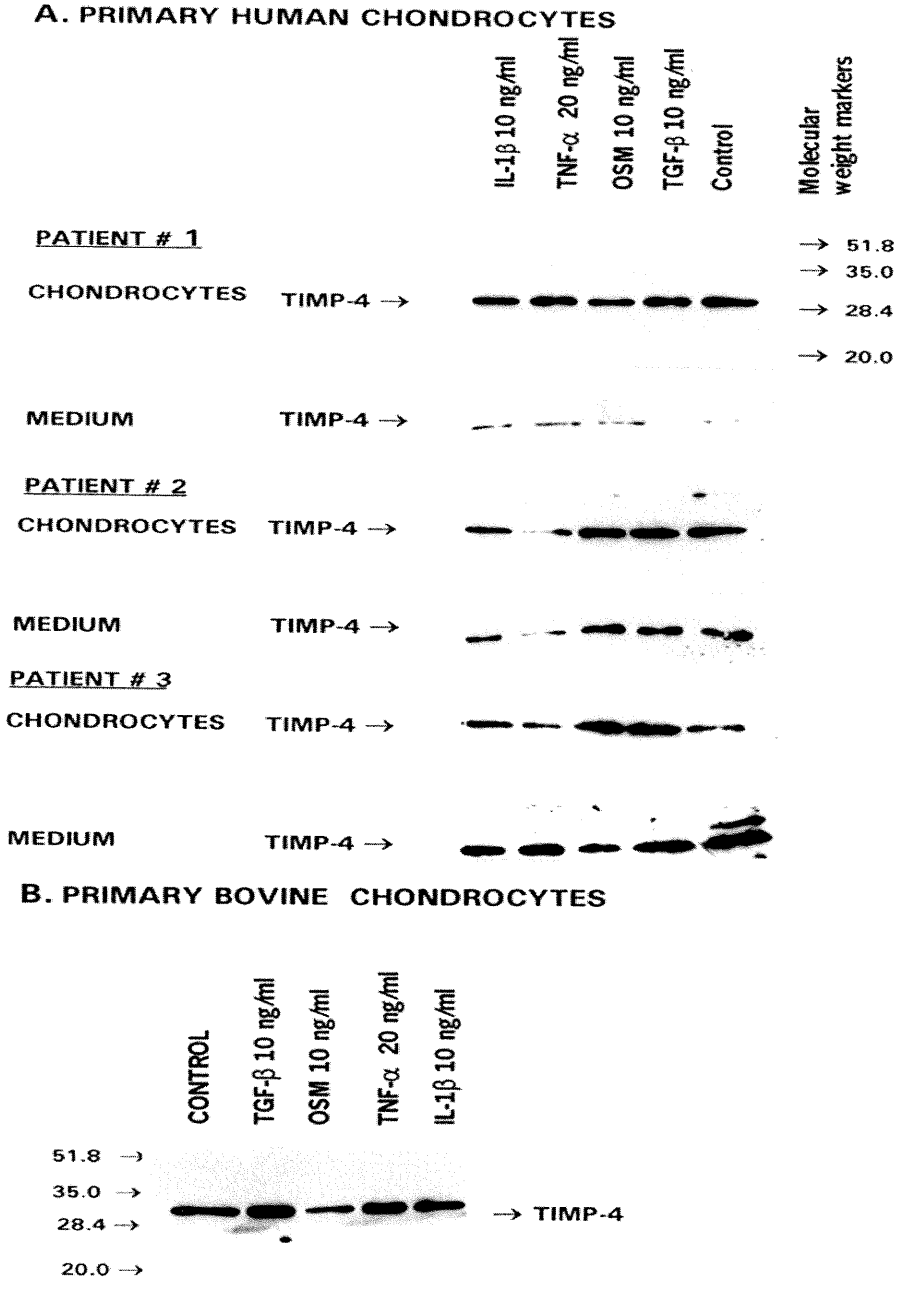

Fig. 3. Impact of TGF-β1, OSM, TNF-α and IL-1β on TIMP-4 protein expression in human and bovine chondrocytes. Primary chondrocyte from the human femoral head of OA (A) or normal bovine cartilage (B) were grown in culture, kept in serum-free medium for 24 h and subjected to the indicated treatments. Equal amount of protein (10 μg/lane) was subjected to western immunoblot analysis with the TIMP-4 antibody, reacted with secondary antibody and revealed by chemiluminescence. The position of TIMP-4 band relative to the known molecular weight standards is shown.

III. DISCUSSION

Discussion:

In this work, we demonstrated for the first time the expression of TIMP-4 in normal and osteoarthritic human cartilage as well as in synovial membrane. Osteoarthritis is a common disease affecting hip and knee joints in elderly patients. It is important to understand the molecular mechanisms contributing to this disease. Previous studies from our laboratory demonstrated that the expression of TIMP-1 and TIMP-3 gene was increased in human OA synovial membranes and articular chondrocytes while TIMP-2 levels were unchanged (DiBattista et al., 1995; Zafarullah et al., 1996; Su et al., 1996). Now we showed here that TIMP-4 RNA and protein were widely expressed in joint tissue. Expression of the both was increased in cartilage of most patients with femoral head OA disease but not in synovial membrane. TIMP-4 mRNA levels are low in all tissues as well as in joints with the exception of cardiac tissues. We could nevertheless detect TIMP-4 mRNA by RT-PCR with even 1μg of total RNA. So, all the TIMP family members are expressed in joint tissues. Except TIMP-2, all the other TIMPs are increased due to possible altered metabolism of arthritic joints. Such increase may also be a defense response of the tissue to the degenerating action of proteases (MMPs, aggrecanases).

For the TIMP-1 to TIMP-3, we could detect RNA expression by Northern blot analysis. For TIMP-4, due to its tissue-specificity, TIMP-4 RNA is expressed at a very low level in most tissues except heart. To detect TIMP-4 in cartilage, we had to use RT-PCR assay. The full length of TIMP-4 cDNA is 1189 bp. We designed a primer from both ends that enabled us to get almost the full-length 1145 bp of TIMP-4 cDNA from the low level of total RNA. These primers are specific to TIMP-4 cDNA. Another study showed that by using RT-PCR, one can detect TIMP-4 mRNA in IPF lungs (Selman et al., 2000). They designed a primer to get part of TIMP-4 cDNA as a 446 bp band. This showed that it is a convenient and sensitive way to detect the low-level TIMP-4 gene expression in human tissues. Using RT-PCR, we

found a tendency of increased TIMP-4 expression in OA patients. TIMP-4 RNA expression was present in 12 of 14 OA patients and only 2 of 9 non-arthritic patients (Fig. 1, article 1). TIMPs and MMPs are expressed at different levels in OA and RA. Expression of MMP-3, MMP-8 was elevated in the damaged cartilage compared to their very low level mRNA expression in normal cartilage (Chubinskaya et al., 1999). MMP-1, MMP-2, MMP-3 and MMP-9 were detected in higher concentration in the OA samples while TIMP-1 and TIMP-2 did not increase in the synovial fluid of OA patients (Kanyma et al., 2000). This suggests role for an imbalance between MMPs and TIMPs in the breakdown of cartilage. The tendency of increased TIMP-4 suggests TIMP-4 may be involved in the attempted repair of damaged articular cartilage. The similar augmentation of TIMP-4 protein expression is also observed during vascular remodelling after ballon-induced injury in rats (Dollery et al., 1999).

The only one RA sample we could obtain showed a much higher expression of TIMP-4 than the level in OA samples (Fig.1, article 1). Since RA presents more immunological etiology and more synovial inflammation, MMPs showed a markedly increased level in RA synovial fluid than in OA synovial fluid (Yoshihara et al., 2000). TIMP-1 is found to be increased in RA also, and TIMP-1 is inversely correlated with increased MMP-3 level (Ishiguro et al., 2001). Thus, increased level of TIMP-4 in OA and much higher level in RA suggests its anti-MMPs activity in the process of arthritis although the expression of TIMP-4 in RA needs more samples to be tested.

Immunohistochemistry methods confirmed the expression of TIMP-4 in OA cartilage. We found TIMP-4 protein is expressed in the superficial zone of cartilage (Figue.3, article1). In normal cartilage, TIMP-1, TIMP-2 and TIMP-3 are found mainly in the deep zone and in subchondral zone while TIMP-4 localization was not studied (Joronen et al., 2000). In our study, we included the negative control without TIMP-4 antibody or blocking TIMP-4 antibody with excessive recombinant TIMP-4 protein to confirm the specificity of

TIMP-4 protein detection. This result directly shows that TIMP-4 protein expression in OA cartilage is related to the cartilage damage, since the superficial zone is the location where the damage occurs first.

The expression of TIMP-4 protein was directly analyzed in cartilage and other major joint tissues, synovium, under non-arthritic and arthritic conditions. We detected TIMP-4 protein in the cartilage in 8 of 9 OA patients while their RNA were detected in 7 of 9. In non-arthritic patients, we detected very low level TIMP-4 protein in 4 of 8 samples. The RNA expression could be correlated with protein as it was detected in only one of them due to the very low RNA level. The only one RA sample showed a high TIMP-4 protein expression which correlated with the RNA expression. One synovial membrane sample also presented TIMP-4 protein expression (Figue 2, article 1). This suggested that TIMP-4 gene has widespread expression in human OA joints and TIMP-4 RNA was translated to the corresponding protein.

The expression of TIMP-4 protein is detected in both human and bovine cartilage, near 29 kDa molecular mass, in the range of 27~30 kDa (Tummalapalli et al., 2001; Riley et al., 2001). As a positive control, we detected the purified human recombinant TIMP-4 protein from CHEMICON at 27 kDa. With TIMP-4 antibody from another company, Santa-Cruz, we detected TIMP-4 band at the same place as Chemicon's. Other studies showed that TIMP-4 protein is found as two different forms, 21-23 kDa (Bigg et al., 2001; Hurst et al., 2001) and 27-30 kDa (Tummalapalli et al., 2001; Riley et al., 2001). This is difficult to be explained since TIMP-4 has no potential glycosylation site in the sequence. We deduced that TIMP-4 protein may have different forms in different tissue due to its tissue specificity. In vivo it may have possibility to be glycosylated. Future structural studies on TIMP-4 from different tissues are needed to clarify this issue.

The expression of TIMP-4 protein can be detected in bovine cartilage and bovine chondrocytes (Fig. 3, article 2). The cross-reactivity between human and bovine TIMP-4 protein suggested the strong homology and conservation in these mammals. However this homology may not extend at the level of RNA. TIMP-4 has a closer homology of sequence with TIMP-2 and TIMP-3 (51%) than that with TIMP-1 (37%) (Greene et al, 1996). In our laboratory, we had previously cloned bovine TIMP-3 cDNA by hybridization with a human probe. This further supports the conservation of the TIMP gene family. TIMP-4 protein expression in bovine cartilage showed that it may have physiological roles such as protection of its matrix from MMPs in animals. So we could utilize bovine cartilage as a non-arthritic cartilage control model for studying function of TIMP-4 as human cartilage is very difficult to obtain in large quantities. Recently, a partial bovine TIMP-4 cDNA has been cloned by Hosseini,GH. and Pepper,MS (PubMed, 4104631) although details are not available. Due to the abundant availability of bovine cartilage, for further experiment it will be possible to design a primer or DNA probe for bovine TIMP-4 for Northern Blot or RT-PCR to detect TIMP-4 RNA in bovine cartilage. By this approach it will be convenient to measure TIMP-4 RNA levels and TIMP-4 regulation in this animal model.

Many inflammatory cytokines and growth factors are implicated in the pathogenesis of arthritic diseases, such as interlukin-1 (IL-1), tumor necrosis factor-α (TNF–α), oncostatin M (OSM) and transforming growth factor-β (TGF–β) which regulate important genes in the chondrocytes from cartilage. Previously our laboratory has demonstrated the expression of all the other TIMP members in these cells and showed induction of TIMP-3 by TGF-β and OSM. This time, we demonstrated that TIMP-4 protein was not regulated by IL-1, TNF-α, OSM and TGF-β. Using both human and bovine chondrocytes from femoral head cartilage, we stimulated the cells with different cytokines and growth factors, but the TIMP-4 protein level did not differ from non-stimulated cells (Fig. 3, article 2). IL-1 and TNF-α are now

commonly accepted as pivotal mediators in cartilage matrix degradation in OA and RA joints (Martel-Pelletier et al., 1999; and Goldring, 2000). However in human and bovine chondrocytes, our data showed that TIMP-4 protein was not regulated by these two cytokines. OSM, which induced TIMP-1 in human chondrocytes (Nemoto et al., 1996) and TIMP-3 in bovine chondrocytes (Li et al., 1998), did not appear to regulate the TIMP-4 protein in chondrocytes. TIMP-4 protein, like TIMP-2, was not regulated by TGF-β which is involved in cartilage repair and osteophyte formation as well as induction of TIMP-1 and TIMP-3 in arthritic joints. Several Sp-1 and Ap-1 transcription factor binding sites were found in the TIMP-1 and TIMP-3 promoters which play important role in the gene regulation. Characterization of TIMP-4 promoter elements in the future may give us new clues about the observed results.

Two cartilage aggrecanases have been identified so far. Aggrecanase-1 and aggrecanase-2 can efficiently cleave soluble aggrecan at the Glu373-Ala374 site within the IGD domain (Tortorella et al., 2000 and 2001). Aggrecanases play a key role in arthritis disease, through the destruction of aggrecan. Aggrecanases is induced by IL-1 whereas aggrecanase-2 is constitutively expressed (Arner et al., 1999; Fosang et al., 2000; Sandy et al., 2001; Tortorella et al., 2001). Like MMPs, the activities of aggrecanases increase during the development of OA lesions (Chambers et al., 2001). TIMPs not only inhibit MMP activities but also inhibit avtivities of aggrecanases. It was demonstrated that TIMP-1, TIMP-2, TIMP-3, TIMP-4 can all inhibit aggrecanases to different degrees. Among all the TIMPs, TIMP-3 is a strong inhibitor of aggrecanase-1 and aggrecanase-2 (Kashiwagi et al., 2001; Hashimoto et al., 2001). TIMP-4 is the most inefficient inhibitor of aggrecases among all the TIMP family members (Hashimoto et al., 2001).

TIMPs have been shown to be multifunctional proteins in other systems. In addition to their anti-MMP activity, anti-aggrecanases activity, TIMPs also regulate cell growth. In normal heart, TIMP-4 is constitutively expressed

(Green et al., 1996), and is suppressed in heart disease (Tummalapalli et al., 2001). TIMP-4 controlled normal cardiac fibroblast transformation and induced apoptosis in transformed cells. Cardiospecific TIMP-4 plays a significant role in regulating the normal cardiac cell phenotype. The cardiac tumors are rarely malignant in part due to the presence of high levels of constitutively expressed TIMP-4. The reduced level of TIMP-4 elicit cellular transformation and may lend to adverse extracellular matrix degradation (remodeling), cardiac hypertrophy and failure. Similar result was obtained from an animal model as introduction of human TIMP-4 transfectants to a nude mice significantly inhibited the tumor growth as compared with control (Wang et al., 1997). In a study of systemic administration of TIMP-4 DNA, TIMP-4 inhibited the Wilms' tumor growth at a concentration lower than that required for MMP inhibition (Celiker et al., 2001). The biological function of TIMP-4 in joints is not known.

TIMP-1 and TIMP-2 can inhibit tumor growth, and suppress programmed cell death (PCD) independently of the MMP-inhibiting activity. TIMP-3 can induce PCD, overexpression also induces PCD (Mannello and Gazzanelli, 2001). The role of TIMP-4 in apoptosis may be complicated. Recent studies showed that TIMP-4 may favor tumor growth because of its lower anti-apoptosis activity (Tummalapalli et al., 2001). Thus, TIMP-4 has both anti-MMP activity and anti-apoptosis activity. Anti-MMP activity favors tumor-suppressing effect and anti-apoptosis and growth-stimulating activity favors the pro-tumor effect. At high level, TIMP-4 may inhibit tumor growth by inducing apoptosis whereas at low level, it may induce tumor growth. Recently apoptotic cell death has been reported in OA cartilage. Some studies showed that chondrocyte apoptosis occurs more frequently in OA cartilage compared to normal cartilage, and the apoptotic cells are mainly detected in the middle zone and in the superficial zone (Hashimoto et al, 1998; Heraud et al., 2000). Similar results also showed that apoptotic chondrocyte death increased in OA lesional compared to OA non-lesional cartilage (Kim et al., 2000). This suggests that apoptotic cell death correlates with the cartilage

degradation of OA. TIMP-4 protein is also present in the superficial zone of OA cartilage (Fig. 3, article 1). This may suggest that TIMP-4 could also play a role in chondrocytes apoptosis. In contrast, one study showed that apoptotic cell death is not a widespread phenomenon in normal aging and OA human knee articular cartilage (Aigner et al., 2001). Nevertheless, the apoptosis in cartilage is still very important and there is some potential for repairing articular cartilage in adults by blocking apoptosis. Whether TIMP-4 in cartilage induces or prevents apoptosis in cartilage and synovial cells remains to be investigated by future studies.

In summary, TIMP-4 is expressed in human cartilage and chondrocytes and is increased in OA and RA. It is a specific inhibitor protein of TIMP family. It may play an important role in tissue matrix protection, growth promotion, anti-angiogenic activities and pathologic ECM remodeling. Further studies are needed to define the TIMP-4 function for inhibiting MMPs and aggrecanases in joints, the functions relative to cell apoptosis and its therapeutic potential for controlling cartilage degradation. Future studies may also involve other cytokines and regulatory factors to investigate the regulatory mechanism in vitro and in vivo.

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