Journal of Cellular Biochemistry 85:295–303 (2002)

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

Wensheng Huang,[1] Wen Qing Li,[1] Faramaze Dehnade,[2] and Muhammad Zafarullah[1]*

[1]Departments of Medicine, Research Center and Notre-Dame Hospital of the Centre hospitalier de l'université de Montréal (CHUM), Montreal, Quebec, Canada H2L 4M1
[2]Departments of Medicine, Surgery, Research Center and Notre-Dame Hospital of the Centre hospitalier de l'université de Montréal (CHUM), Montreal, Quebec, Canada H2L 4M1

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. J. Cell. Biochem. 85: 295–303, 2002.

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

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 Hamerman, 1995; Felson and Zhang, 1998]. 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 turnover [Nagase and Woessner, 1999; Westermarck and Kähäri, 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

Grant sponsor: Canadian Institutes of Health (Research grant); Grant number: MT-12867; Grant sponsor: Arthritis Society of Canada; Grant sponsor: Canadian Arthritis Network (CAN).

*Correspondence to: Dr. Muhammad Zafarullah, K-5255 Mailloux, Hôpital Notre-Dame du CHUM, 1560 Sherbrooke est, Montréal, Québec, Canada H2L 4M1.
E-mail: Muhammad.Zafarullah@UMontreal.ca

Received 14 March 2001; Accepted 13 December 2001
DOI 10.1002/jcb.10138

of OA patients [Zafarullah et al., 1993; Yoshihara et al., 1995]. MMP-TIMP-1 balance is altered in favor of MMPs in arthritic synovium, human OA cartilage, and in experimental models of OA [Dean et al., 1989; Pelletier et al., 1990; McCachren, 1991]. 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 upregulated 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-β, a major inducer of cartilage regeneration [Su et al., 1999].

Tissue inhibitor of metalloproteinases-4 (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, 1996; Su et al., 1996, 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 I) or from OA patients

TABLE I. Characteristics of the Patients Analyzed for TIMP-4 RNA Expression

	Patient no.	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

F, female; M, male; OA, osteoarthritis; RA, rheumatoid arthritis.

(9 female, 5 male, ranging from 50 to 79 years, mean age 61.6 years; see Table I) 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. (Palo 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 1 min, 35 cycles of 94°C for 1 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 2 M guanidine hydrochloride, pH 7.5. The homogenate was incubated overnight at 4°C with gentle shaking. After centrifugation at 3,000*g* for 10 min, extracts were dialyzed against 50 mM Tris-HCl, pH 7.5 with three 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 1:1,000 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, -2, and -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 1–20 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 1:1,000 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, and dehydrated with 70 and 100% ethanol. The slides were cleared with three xylene washes, mounted with Permount (Fisher Scientific) and photographed. For negative controls, 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

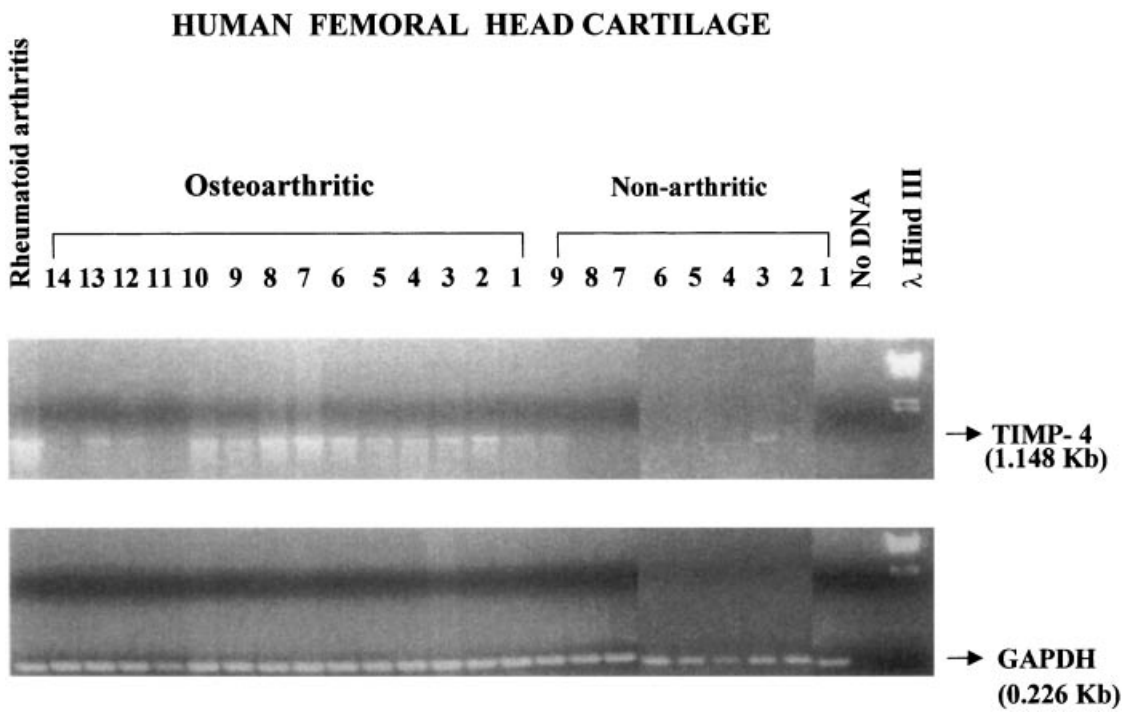

Fig. 1. Expression of the TIMP-4 mRNA in human non-arthritic and OA femoral head cartilage. Total RNA (2 μg) extracted directly from 9 non-arthritic (femoral head fractures), 14 femoral head OA, and 1 RA cartilage was reverse transcribed into cDNA and 5 μl aliquots amplified with the TIMP-4- or GAPDH-specific 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. 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 eight non-arthritic (corresponding to the non-arthritic samples 2–9, respectively in Fig. 1) and nine OA (corresponding to the OA samples 7–14, 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 Figure 1, we were able to compare RNA and protein expression in the same tissues. RNA results from Figure 1 are depicted at the bottom of Figure 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,D,F) that was absent in sections where antibody was pre-adsorbed with the purified total TIMP-4 protein (Fig. 3A,C) or upon omission of the primary antibody (Fig. 3E,G). 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

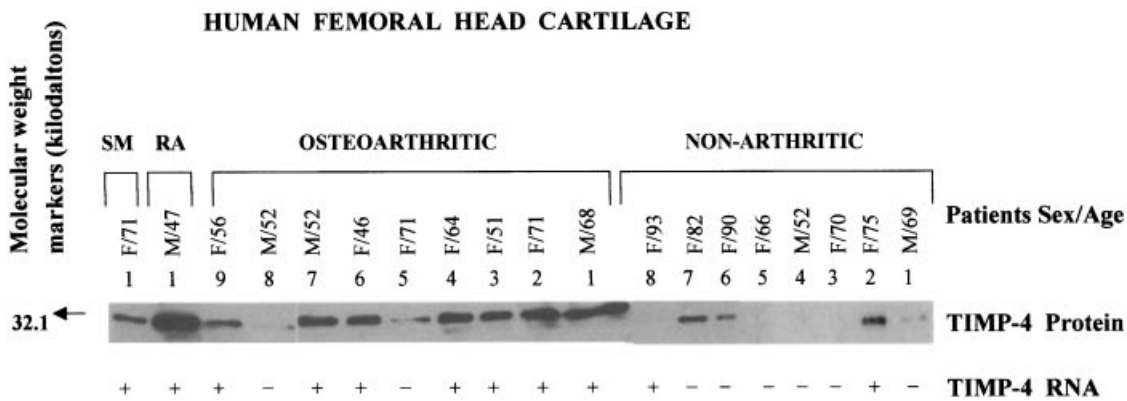

Fig. 2. TIMP-4 protein expression in human non-arthritic and OA femoral head cartilage. Total protein was extracted directly from the femoral head cartilage of 8 patients with fractures, 9 with OA, 1 with RA, and 1 OA synovial membrane (SM), subjected to SDS–PAGE, probed with the 1:1,000 dilution of 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 1 min. 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 Figure 1 is also included for comparison.

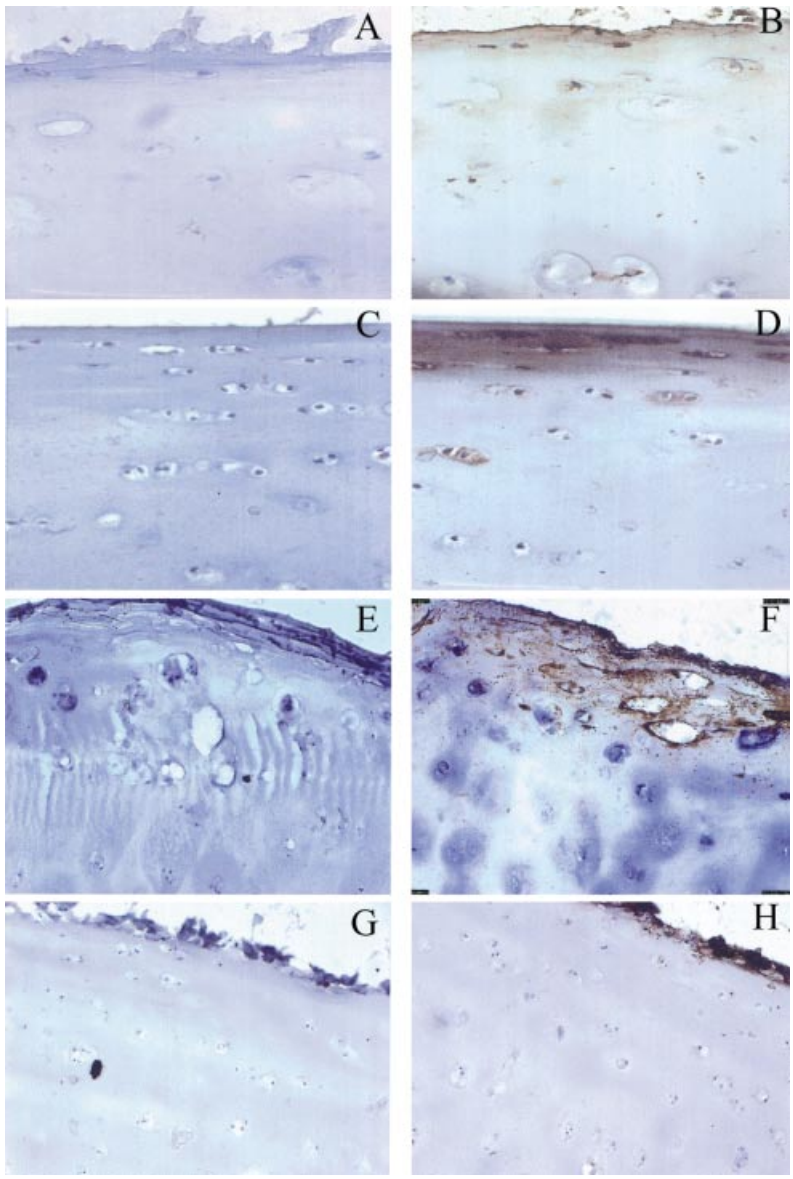

Fig. 3. Detection of TIMP-4 expression in human femoral head cartilage. In **panels A–F**, cartilage sections from three OA patient were stained after preincubation with the competing pure TIMP-4 protein and TIMP-4 antibody (A and C), without TIMP-4 antibody (**E**) (negative controls) or with TIMP-4 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.

to this disease. TIMP-4, the newest member of the TIMP gene family, blocks the activities of several enzymes (MMP-1, -2, -3, -7, and -9) known to be implicated in the arthritic cartilage 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 of 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, 1996; Su et al., 1999]. In other tissues, poly A^+ mRNA from 150 to 210 μg of total RNA was used to detect its expression [Greene et al., 1996; Li et al., 1998, 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 -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 [unpublished communications], 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 [Mitchell et al., 1996; Reboul et al., 1996; Billinghurst et al., 1997; Shlopov et al., 1997], we also investigated its mRNA expression in the femoral head specimens in Figure 1. None of the control patients expressed collagenase-3 message, but 71% of OA and 1 RA patients did [unpublished communications]. 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 [Mitchell et al., 1996; Reboul et al., 1996; Shlopov et al., 1997]. 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 -1 in synovial fibroblasts [DiBattista et al., 1995; Gatsios et al., 1996]. Contrary to TIMP-1 and -3 induction by TGF-β [Günther et al., 1994; Su et al., 1996, 1999; Zafarullah et al., 1996], 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, -3, and -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 -3) and non-inducible (TIMP-2, -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.

ACKNOWLEDGMENTS

We thank Dr. Julio Fernandes and Dr. Nicolas Duval of the Orthopaedic Surgery for some of the tissue specimens utilized in this study. Ms. Anna Chelchowska and Mr. Pierre Cambron kindly prepared the figures.

REFERENCES

Aigner T, Dudhia J. 1997. Phenotypic modulation of chondrocytes as a potential therapeutic target in osteoarthritis: a hypothesis. Ann Rheum Dis 56:287–291.

Amour A, Slocombe PM, Webster A, Butler M, Knight CG, Smith BJ, Stephens PE, Shelley C, Hutton M, Knauper V, Docherty AJ, Murphy G. 1998. TNF-alpha converting enzyme (TACE) is inhibited by TIMP-3. FEBS Lett 435: 39–44.

Apparailly F, Noel D, Millet V, Baker AH, Lisignoli G, Jacquet C, Kaiser MJ, Sany J, Jorgensen C. 2001. Paradoxical effects of tissue inhibitor of metalloproteinases 1 gene transfer in collagen-induced arthritis. Arthritis Rheum 44:1444–1454.

Apte SS, Hayashi K, Seldin MF, Mattei MG, Hayashi M, Olsen BR. 1994. Gene encoding a novel murine tissue inhibitor of metalloproteinases (TIMP), TIMP-3, is expressed in developing mouse epithelia, cartilage, and muscle, and is located on mouse chromosome 10. Dev Dyn 200:177–197.

Apte SS, Olsen BR, Murphy G. 1995. The gene structure of tissue inhibitor of metalloproteinases (TIMP)-3 and its inhibitory activities define the distinct TIMP gene family. J Biol Chem 270:14313–14318.

Bigg HF, Morrison CJ, Butler GS, Bogoyevitch MA, Wang Z, Soloway PD, Overall CM. 2001. Tissue inhibitor of metalloproteinases-4 inhibits but does not support the activation of gelatinase A via efficient inhibition of membrane type 1-matrix metalloproteinase. Cancer Res 61:3610–3618.

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

Brew K, Dinakarpandian D, Nagase H. 2000. Tissue inhibitors of metalloproteinases: evolution, structure and function. Biochim Biophys Acta 1477:267–283.

Cawston TE, Bigg HF, Clark IM, Hazleman BL. 1993. Identification of tissue inhibitor of metalloproteinase-2 (TIMP-2)-progelatinase complex as the third metalloproteinase inhibitor peak in rheumatoid synovial fluid. Ann Rheum Dis 52:177–181.

Cawston TE, Curry VA, Summers CA, Clark IM, Riley GP, Life PF, Spaull JR, Goldring MB, Koshy PJ, Rowan AD, Shingleton WD. 1998. The role of oncostatin M in animal and human connective tissue collagen turnover and its localization within the rheumatoid joint. Arthritis Rheum 41:1760–1771.

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

Dean DD, Martel-Pelletier J, Pelletier JP, Howell DS, Woessner JF, Jr., 1989. Evidence for metalloproteinase and metalloproteinase inhibitor imbalance in human osteoarthritic cartilage. J Clin Invest 84:678–685.

DiBattista JA, Pelletier J-P, Zafarullah M, Iwata K, Martel-Pelletier J. 1995. Interleukin-1β induction of tissue inhibitor of metalloproteinase (TIMP-1) is functionally antagonized by prostaglandin E_2 in human synovial fibroblasts. J Cell Biochem 57:619–629.

Dollery CM, McEwan JR, Wang M, Sang QA, Liu YE, Shi YE. 1999. TIMP-4 is regulated by vascular injury in rats. Circ Res 84:498–504.

Felson DT, Zhang Y. 1998. An update on the epidemiology of knee and hip osteoarthritis with a view to prevention. Arthritis Rheum 41:1343–1355.

Firestein GS, Paine MM, Littman BH. 1991. Gene expression (collagenase, tissue inhibitor of metalloproteinases, complement, and HLA-DR) in rheumatoid arthritis and osteoarthritis synovium. Quantitative analysis and effect of intraarticular corticosteroids. Arthritis Rheum 34: 1094–1105.

Gatsios P, Haubeck HD, Van de Leur E, Frisch W, Apte SS, Greiling H, Heinrich PC, Graeve L. 1996. Oncostatin M differentially regulates tissue inhibitors of metalloproteinases TIMP-1 and TIMP-3 gene expression in human synovial lining cells. Eur J Biochem 241:56–63.

Gomez DE, Alonso DF, Yoshiji H, Thorgeirsson UP. 1997. Tissue inhibitors of metalloproteinases: structure, regulation and biological functions. Eur J Cell Biol 74:111–122.

Greene J, Wang M, Liu YE, Raymond LA, Rosen C, Shi YE. 1996. Molecular cloning and characterization of human tissue inhibitor of metalloproteinase 4. J Biol Chem 271: 30375–30380.

Guerne P-A, Blanco F, Kaelin A, Desgeorges A, Lotz M. 1995. Growth factor responsiveness of human articular chondrocytes in aging and development. Arthritis Rheum 38:960–968.

Günther M, Haubeck HD, van de Leur E, Blaser J, Bender S, Gutgemann I, Fischer DC, Tschesche H, Greiling H, Heinrich PC. 1994. Transforming growth factor beta 1 regulates tissue inhibitor of metalloproteinases-1 expression in differentiated human articular chondrocytes. Arthritis Rheum 37:395–405.

Hamerman D. 1995. Clinical implications of osteoarthritis and ageing. Ann Rheum Dis 54:82–85.

Hashimoto G, Aoki T, Nakamura H, Tanzawa K, Okada Y. 2001. Inhibition of ADAMTS4 (aggrecanase-1) by tissue inhibitors of metalloproteinases (TIMP-1, 2, 3 and 4). FEBS Lett 494:192–195.

Kashiwagi M, Tortorella M, Nagase H, Brew K. 2001. TIMP-3 is a potent inhibitor of aggrecanase 1 (ADAM-TS4) and aggrecanase 2 (ADAM-TS5). J Biol Chem 276: 12501–12504.

Leco KJ, Apte SS, Taniguchi GT, Hawkes SP, Khokha R, Schultz GA, Edwards DR. 1997. Murine tissue inhibitor of metalloproteinases-4 (Timp-4): cDNA isolation and expression in adult mouse tissues. FEBS Lett 401:213–217.

Li WQ, Zafarullah M. 1998. Oncostatin M up-regulates tissue inhibitor of metalloproteinases-3 gene expression in articular chondrocytes via de novo transcription, protein synthesis, and tyrosine kinase- and mitogen-activated protein kinase-dependent mechanisms. J Immunol 161:5000–5007.

Li YY, Feldman AM, Sun Y, McTiernan CF. 1998. Differential expression of tissue inhibitors of metalloproteinases in the failing human heart. Circulation 98: 1728–1734.

Li YY, McTiernan CF, Feldman AM. 1999. Proinflammatory cytokines regulate tissue inhibitors of metalloproteinases and disintegrin metalloproteinase in cardiac cells. Cardiovascular Res 42:162–172.

Liu YE, Wang M, Greene J, Su J, Ullrich S, Li H, Sheng S, Alexander P, Sang QA, Shi YE. 1997. Preparation and characterization of recombinant tissue inhibitor of metalloproteinase 4 (TIMP-4). J Biol Chem 272:20479–20483.

Matyas JR, Adams ME, Huang D, Sandell LJ. 1995. Discoordinate gene expression of aggrecan and type II collagen in experimental osteoarthritis. Arthritis Rheum 38:420–425.

McCachren SS. 1991. Expression of metalloproteinases and metalloproteinase inhibitor in human arthritic synovium. Arthritis Rheum 34:1085–1093.

Mitchell PG, Magna HA, Reeves LM, Lopresti-Morrow LL, Yocum SA, Rosner PJ, Geoghegan KF, Hambor JE. 1996. Cloning, expression, and type II collagenolytic activity of matrix metalloproteinase-13 from human osteoarthritic cartilage. J Clin Invest 97:761–768.

Nagase H, Woessner JF, Jr., 1999. Matrix metalloproteinases. J Biol Chem 274:21491–21494.

Nemoto O, Yamada H, Mukaida M, Shimmei M. 1996. Stimulation of TIMP-1 production by oncostatin M in human articular cartilage. Arthritis Rheum 39:560–566.

Pelletier JP, Mineau F, Faure MP, Martel-Pelletier J. 1990. Imbalance between the mechanisms of activation and inhibition of metalloproteases in the early lesions of experimental osteoarthritis. Arthritis Rheum 33:1466–1476.

Pelletier JP, Martel-Pelletier J, Abramson SB. 2001. Osteoarthritis, an inflammatory disease: potential implication for the selection of new therapeutic targets. Arthritis Rheum 44:1237–1247.

Poole AR. 1999. An introduction to the pathophysiology of osteoarthritis. Front Biosci 4:D662–D670.

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

Reno C, Boykiw R, Martinez ML, Hart DA. 1998. Temporal alterations in mRNA levels for proteinases and inhibitors and their potential regulators in the healing medial collateral ligament. Biochem Biophys Res Commun 252:757–763.

Shlopov BV, Lie WR, Mainardi CL, Cole AA, Chubinskaya S, Hasty KA. 1997. Osteoarthritic lesions: involvement of three different collagenases. Arthritis Rheum 40:2065–2074.

Su S, Dehnade F, Zafarullah M. 1996. Regulation of tissue inhibitor of metalloproteinases-3 gene expression by transforming growth factor-beta and dexamethasone in bovine and human articular chondrocytes. DNA Cell Biol 15:1039–1048.

Su S, Grover J, Roughley PJ, DiBattista JA, Martel-Pelletier J, Pelletier JP, Zafarullah M. 1999. Expression of the tissue inhibitor of metalloproteinases (TIMP) gene family in normal and osteoarthritic joints. Rheumatol Int 18:183–191.

Takizawa M, Ohuchi E, Yamanaka H, Nakamura H, Ikeda E, Ghosh P, Okada Y. 2000. Production of tissue inhibitor of metalloproteinases 3 is selectively enhanced by calcium pentosan polysulfate in human rheumatoid synovial fibroblasts. Arthritis Rheum 43:812–820.

Tortorella MD, Burn TC, Pratta MA, Abbaszade I, Hollis JM, Liu R, Rosenfeld SA, Copeland RA, Decicco CP, Wynn R, Rockwell A, Yang F, Duke JL, Solomon K, George H, Bruckner R, Nagase H, Itoh Y, Ellis DM, Ross H, Wiswall BH, Murphy K, Hillman MC, Jr., Hollis GF, Arner EC. 1999. Purification and cloning of aggrecanase-1: a member of the ADAMTS family of proteins. Science 284:1664–1666.

Tummalapalli CM, Heath BJ, Tyagi SC. 2001. Tissue inhibitor of metalloproteinase-4 instigates apoptosis in transformed cardiac fibroblasts. J Cell Biochem 80:512–521.

Van den Berg WB. 1999. The role of cytokines and growth factors in cartilage destruction in osteoarthritis and rheumatoid arthritis. Z Rheumatol 58:136–141.

Westermarck J, Kähäri V-M. 1999. Regulation of matrix metalloproteinase expression in tumor invasion. FASEB J 13:781–792.

Wick M, Bürger C, Brüsselbach S, Lucibello FC, Müller R. 1994. A novel member of human tissue inhibitor of metalloproteinases (TIMP) gene family is regulated during G1 progression, mitogenic stimulation, differentiation, and senescence. J Biol Chem 269:18953–18960.

Wu I, Moses MA. 1998. Molecular cloning and expression analysis of the cDNA encoding rat tissue inhibitor of metalloproteinase-4. Matrix Biol 16:339–342.

Yoshihara Y, Obata K, Fujimoto N, Yamashita K, Hayakawa T, Shimmei M. 1995. Increased levels of stromelysin-1 and tissue inhibitor of metalloproteinases-1 in sera from patients with rheumatoid arthritis. Arthritis Rheum 38:969–975.

Yu WH, Yu S, Meng Q, Brew K, Woessner JF, Jr., 2000. TIMP-3 binds to sulfated glycosaminoglycans of the extracellular matrix. J Biol Chem 275:31226–31232.

Zafarullah M, Pelletier JP, Cloutier JM, Martel-Pelletier J. 1993. Elevated metalloproteinase and tissue inhibitor of metalloproteinase mRNA in human osteoarthritic synovia. J Rheumatol 20:693–697.

Zafarullah M, Su S, Martel-Pelletier J, DiBattista JA, Costello BG, Stetler-Stevenson WG, Pelletier JP. 1996. Tissue inhibitor of metalloproteinase-2 (TIMP-2) mRNA is constitutively expressed in bovine, human normal, and osteoarthritic articular chondrocytes. J Cell Biochem 60:211–217.