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Tissue Inhibitor of Metalloproteinase-2 (TIMP-2) mRNA Is Constitutively Expressed in Bovine, Human Normal, and Osteoarthritic Articular Chondrocytes

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Abstract Tissue inhibitors of metalloproteinases (TIMPs) inhibit the extracellular matrix (ECM) metalloproteinases (MMPs). To determine the source of TIMPs in synovial fluids of patients with osteoarthritis (OA), the ability of chondrocytes to express TIMP-2 and its regulation by agents found in inflammed joints was investigated. The constitutive TIMP-2 mRNA expression was demonstrated in chondrocytes from normal bovine, human OA and normal cartilage. The cross-hybridization of human and bovine TIMP-2 suggested its evolutionary conservation. Serum, IL-1, IL-6 and TGF-β were unable to augment considerably the basal expression of TIMP-2 mRNA. TIMP-1 RNA expression in chondrocytes from human OA cartilage was elevated compared to non-OA chondrocytes, while TIMP-2 mRNA levels were similar in both. IL-1β, IL-6 and TGF-β did not affect TIMP-2 expression but TGF-β induced TIMP-1 mRNA in human OA chondrocytes. TIMP-2 and TIMP-1 are therefore differentially regulated in chondrocytes and the basal TIMP-2 levels may be needed for the cartilage ECM integrity.

Key words: cartilage, osteoarthritis, metalloproteinases, inhibitors, mRNA

Articular cartilage provides animals with resilience and flexibility. Chondrocytes of cartilage synthesize a massive extracellular matrix (ECM), made up of mainly type II collagen and proteoglycans. Matrix metalloproteinases (MMPs) [Kleiner and Stetler-Stevenson, 1993; Matrisian, 1992; Woessner, 1991] are a Ca^{++} and Zn^{++}-dependent family of enzymes that, upon activation by other proteases, degrade cartilage ECM during its normal turnover and in arthritis [Hamerman, 1989; Brinckerhoff, 1992]. Recent work demonstrated a unique cell-mediated activation mechanism for MMP-2 [Kleiner and Stetler-Stevenson, 1993]. MMPs are inhibited by complexation with tissue inhibitors of metalloproteinases (TIMPs). So far, TIMP-1 [Docherty et al., 1985], TIMP-2 [Stetler-Stevenson et al., 1989], and TIMP-3 [Leco et al., 1994; Apte et al., 1994] are known in mammals and in chicken [Pavloff et al., 1992]. TIMP-1 is a 28-kDa glycoprotein that is expressed constitutively in many adult and embryonic tissues, which suggests its role in animal development [Nomura et al., 1989; Flenniken and Williams, 1990]. The human TIMP-1 has erythroid-potentiating activity, while both TIMP-1 and TIMP-2 have growth-promoting activity for human and bovine cells, independent of MMP inhibition [Hayakawa et al., 1994]. In human osteoarthritic (OA) cartilage, imbalance between excess MMPs and TIMPs results in ECM erosion [Dean et al., 1989; Martel-Pelletier et al., 1994]. Recently we showed increased stromelysin and TIMP-1 mRNA expression in synovial membranes of patients with OA relative to non-OA tissues, indicating inflammation and tissue remodeling [Zafarullah et al., 1993]. Moreover, cells from the synovial linings of rheumatoid arthritis (RA) patients express MMP and TIMP-1 mRNAs at elevated levels relative to OA [Gravallese et al., 1991; Spence McCachren, 1991; Firestein et al., 1991]. TIMP-1 is up-regulated by IL-6 in human chondrocytes [Lotz and Guerne,

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1991] and rheumatoid synovial fibroblasts [Ito et al., 1992], by oncostatin M, leukemia inhibitory factor, interleukin-1α (IL-1α) [Richards et al., 1993], transforming growth factor-β (TGF-β) [Edwards et al., 1987], and IL-11 [Maier et al., 1993]. TIMP-1 inhibits degradation of cartilage explants by MMPs [Valhmu and Ratcliff, 1992], a notion questioned by Andrews et al. [1992].

TIMP-2 is expressed in human melanoma cell lines and tissues [Stetler-Stevenson et al., 1990], which preferentially inhibits MMP-2 compared to MMP-9 [Goldberg et al., 1989; Howard et al., 1991]. These MMPs are expressed in human cartilage [Mohtai et al., 1993]. TGF-β induces TIMP-1 but down-regulates TIMP-2 in certain tumor cell lines [Stetler-Stevenson et al., 1990]. Serum augments TIMP-1 but not TIMP-2 expression in murine fibroblasts [Leco et al., 1992]. The human RA synovial cells secrete MMP-2/TIMP-2 complexes [Kolkenbrock et al., 1991] in RA synovial fluid [Osthues et al., 1992; Cawston et al., 1993]. Compared to TIMP-1, little is known about the TIMP-2 expression and regulation in mammalian cartilage. We investigated the potential of normal bovine and human OA articular chondrocytes to express TIMP-2 mRNA and demonstrate constitutive expression of TIMP-2 in primary chondrocytes as well as differential TIMP-1 and TIMP-2 regulation.

MATERIALS AND METHODS

Cartilage and Chondrocytes Culture

Human cartilage was obtained either immediately after total knee replacement surgery of patients with OA of the knee or from nonarthritic patients at autopsy within 12 h of death. Bovine articular cartilage was obtained from the femoral heads, condyles, and tibial plateaus of freshly slaughtered normal adult animals through a local slaughterhouse. Chondrocytes were released by digestion of cartilage with pronase (1 mg/ml) for 90 min and collagenase (Sigma type II) for 12 h in DMEM alone or with 10% fetal calf serum (FCS) (Gibco BRL) at 37°C. Viability by trypan blue exclusion test was about 80%. The cells were pelleted and washed three times with phosphate-buffered saline (PBS) and plated at high density. In some cases, RNA was extracted directly from the pellet. The cells were first allowed to attach to the plates in DMEM alone for 4 h [Pearson and Sasse, 1992] and then supplemented with 10% serum for confluent growth (up to 3 days). Prior to different treatments, cells were kept in serum-free DMEM for 24 h. The human recombinant interleukin-1β (hrIL-1β), rhIL-6 and TGF-β were all from R&D Systems (Minneapolis, MN), which were reconstituted as recommended.

RNA Extraction and Northern Hybridization Analysis

Total RNA from primary cultures and freshly released chondrocytes was extracted by a single-step procedure [Chomczynski and Sacchi, 1987] and 5-μg aliquots analyzed by fractionation in 1.2% formaldehyde-agarose gels, transferred and hybridized as previously described [Zafarullah et al., 1992]. The human TIMP-2 probe used was a 791 bp *Eco*RI–*Xba*I cDNA fragment cloned in the plasmid pGEM-1 (named pSS38). This vector was linearized with *Eco*RI and RNA probe synthesized from T7 polymerase according to the protocols of Promega Biotech. (Madison, WI). The TIMP-1 antisense probe was a 633-bp *Eco*RI–*Kpn*I segment (isolated by Dr. Stetler-Stevenson) cloned in vector Bluescript, and made from T7 polymerase promoter after linearization with *Eco*RI. The glyceraldehyde-3-phosphate dehydrogenase (ATCC) probe was described earlier [Zafarullah et al., 1993]. The probes were labeled to high-specific activity (1×10^8 cpm/μg) with [α-P^{32}]CTP (3,000 Ci/mmol; Dupont Canada, Inc., Mississauga, Ontario).

RESULTS

TIMP-2 mRNA Expression in Bovine Chondrocytes and Cartilage Explants

To determine the ability of cartilage to express TIMP-2 mRNA, chondrocytes were released after enzymatic digestion of bovine articular cartilage from several animals (n = 5) in medium with or without 10% FCS. Equal amounts of RNA (5 μg) were hybridized with a human TIMP-2 complementary RNA (cRNA) probe; the representative results are depicted in Figure 1A. The probe cross-hybridized with the bovine TIMP-2 transcripts of 3.5- and 1.0-kb length; the sizes similar to those described for human tumor cells [Stetler-Stevenson et al., 1990]. The TIMP-2 RNA was expressed constitutively under serum-deficient and serum-rich conditions. Very low-level TIMP-1 expression could also be detected. Chondrocytes grown as primary monolayer culture under both conditions showed the similar levels of TIMP-2 and TIMP-1 steady-state mRNA levels that were unaffected by serum (Fig. 1B). The RNA specimen from

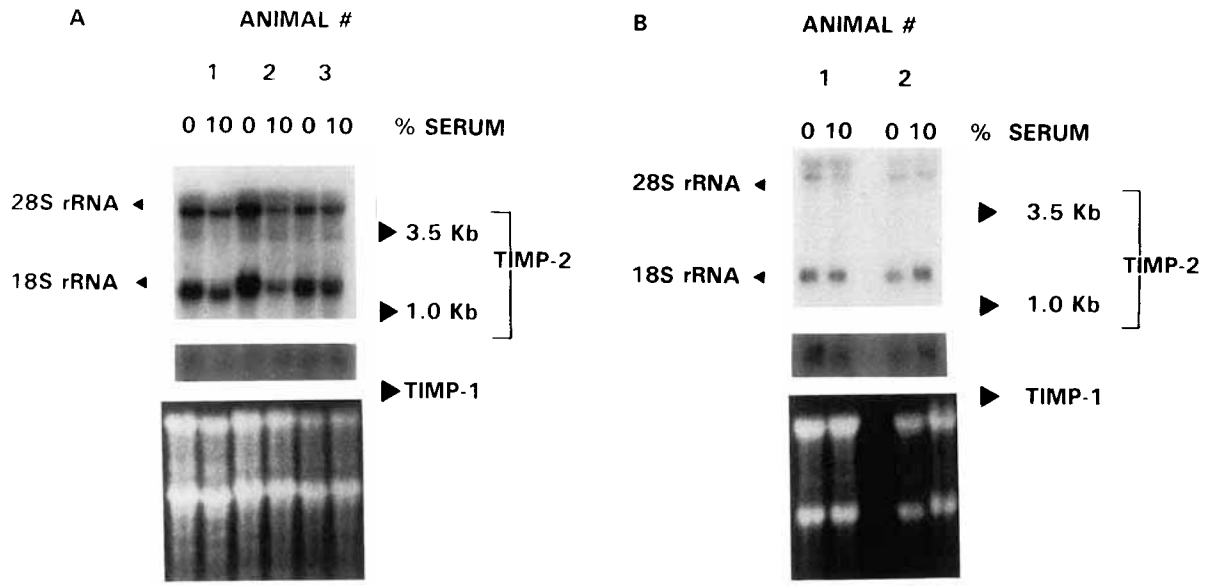

Fig. 1. Expression of TIMP-2 mRNA in freshly released and primary bovine chondrocytes. **A:** Bovine articular cartilage from three different animals was digested with pronase and clostridial collagenase in the absence or in the presence of 10% fetal calf serum (FCS) and chondrocyte RNA subjected to Northern analysis. The positions of 28S and 18S ribosomal RNA as well as 3.5- and 1.0-kb TIMP-2 transcripts are shown. The ribosomal RNA bands in the lower panel demonstrate the integrity and quality of RNA. The autoradiography for TIMP-2 was for 7 days. **B:** Primary chondrocytes from two animals were grown to confluency in DMEM plus 10% FCS, cells washed with PBS, and maintained in FCS-free or FCS-containing medium for 48 h. Northern analysis of TIMP-2 mRNA is shown in the upper, TIMP-1 mRNA in the middle and photograph of the gel is shown in the bottom panel. Film exposure time was 4 days for TIMP-2 and 6 days for TIMP-1.

animal #2 in 10% serum is slightly overloaded, as seen by ethidium bromide stained RNA gel. In summary, the freshly released and primary cultures of articular chondrocytes express TIMP-2 mRNA constitutively.

To assess the capacity of bovine chondrocytes to express TIMP-2 steady-state mRNA within their native matrix, cartilage slices (explants) were kept in DMEM or in DMEM plus 10% serum for 48 h; chondrocytes were then released for RNA extraction and Northern analysis. The amount of applied RNA under 10% serum condition was slightly higher than serum-free conditions as seen by GAPDH (not shown) mRNA measurement and by ethidium bromide-stained gel (Fig. 2, lower panel). Serum itself induced GAPDH mRNA (not shown). Nevertheless, chondrocytes under these conditions also expressed TIMP-2 and low levels of TIMP-1 mRNA constitutively (Fig. 2).

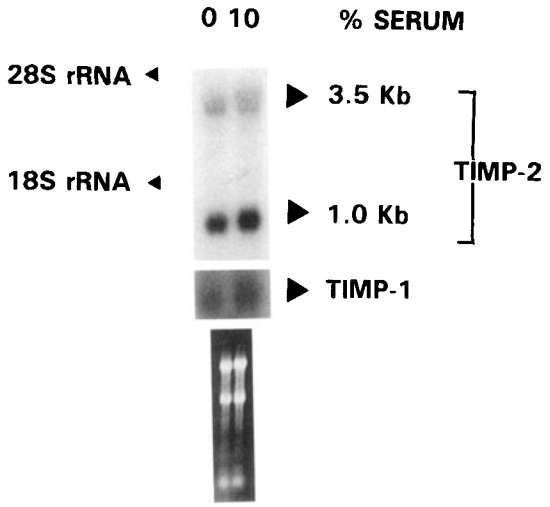

Fig. 2. Steady-state TIMP-2 mRNA levels in bovine cartilage slices. Cartilage explants were maintained in serum-free (0) or 10% serum-containing medium for 24 h and RNA analyzed as in Fig. 1. The film was exposed for 4 (TIMP-2) and 6 (TIMP-1) days.

Regulation of TIMP-2 mRNA by Cytokines and Growth Factors

Human TIMP-1 expression was previously shown to be induced by IL-1α in fibroblasts [Richards et al., 1993], by IL-6 in chondrocytes [Lotz and Guerne, 1990], and by TGF-β in fibroblasts [Edwards et al., 1987]. We investigated if these factors regulated another member of this gene family, the TIMP-2. Primary bovine chondrocytes were exposed to recombinant human IL-1β, IL-6, and TGF-β (Fig. 3A) in minimal

serum (2%) conditions at the concentrations and time periods previously shown to up-regulate TIMP-1 [Richards et al., 1993]. As depicted in Figure 3A, when corrected for loading differences with GAPDH mRNA and ribosomal RNA bands intensity, none of these agents was able to influence significantly the constitutive expression of TIMP-2 mRNA. No major changes were observed in the time course with IL-1β, IL-6, and TGF-β. In addition, rhIL-1β induced stromelysin mRNA in bovine chondrocytes (our unpublished results) but failed to affect TIMP-2 expression. When chondrocytes in cartilage explants were subjected to IL-1β, IL-6, or TGF-β treatments for 36 h, TIMP-2 mRNA basal levels were unaffected (Fig. 3B). The apparent increase by TGF-β appeared to be due to overloading of RNA, as judged by the intensity of ribosomal RNA bands and GAPDH RNA level. Three separate experiments gave similar results. A moderate qualitative increase in TIMP-1 mRNA by TGF-β could also be seen in Figure 3A,B.

Differential TIMP-2 and TIMP-1 mRNA Expression in Human Nonarthritic and Osteoarthritic Chondrocytes

We investigated expression of TIMP-2 in human normal and OA cartilage. To that end, we released chondrocytes from the intact cartilage of three patients without arthritis and from the five different patients with OA. The chondrocytes released in the presence of serum expressed TIMP-2 in a constitutive fashion in both normal and OA specimens (Fig. 4). In one of the five OA specimens, the 1.0-kb transcript was elevated. Expression of the 3.5-kb transcript was low in both normal and OA. Two additional OA specimens showed similar results (not shown). In contrast with constant TIMP-2 levels, TIMP-1 mRNA levels were elevated approximately 3.6-fold in OA chondrocytes compared to normal cells.

Response of Human TIMP-2 Gene to IL-1β, IL-6, and TGF-β

We tested the ability of known inducers of TIMP-1 to regulate TIMP-2 mRNA expression in four separate lines of human OA chondrocytes. None of these agents was able to influence the minimal expression of TIMP-2 transcripts in either line under these experimental conditions (Fig. 5, upper panel). The TIMP-1 mRNA expression was, however, markedly increased by TGF-β after 36-h exposure without any alteration in GAPDH mRNA levels (Fig. 5, middle panel).

DISCUSSION

This study demonstrates the capacity of bovine and human chondrocytes to express TIMP-2

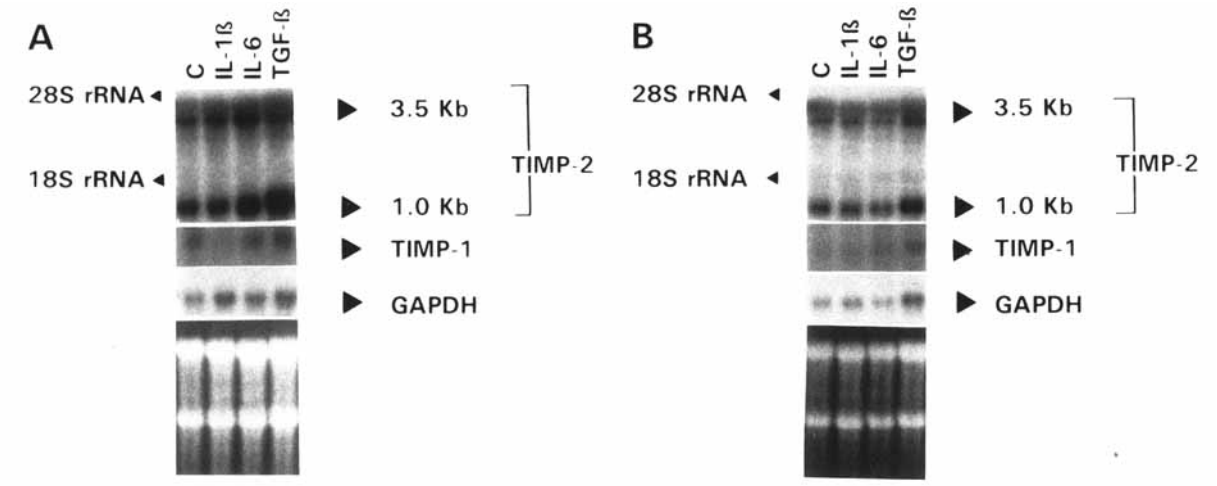

Fig. 3. Effect of cytokines and growth factors on TIMP-2 mRNA expression in bovine chondrocytes. **A:** Primary bovine articular chondrocytes were grown to confluency, maintained in 2% serum-DMEM for 24 h (C, control) and treated with rhIL-1β (20 ng/ml), rhIL-6 (50 ng/ml), and TGF-β (10 ng/ml) for 48 h. Lower panel, RNA gel, depicting the quality of application of RNA. X-ray films were exposed for autoradiography for 4 days (TIMP-2 and GAPDH) and 6 days (TIMP-1). **B:** Cartilage explants were kept in serum-free medium (**C**) or exposed to rhIL-1β, rhIL-6, and TGF-β separately for 36 h in serum-free conditions and the RNA from released chondrocytes subjected to Northern analysis. Autoradiography was for 7 days (TIMP-2), 6 days (TIMP-1), and 4 days (GAPDH).

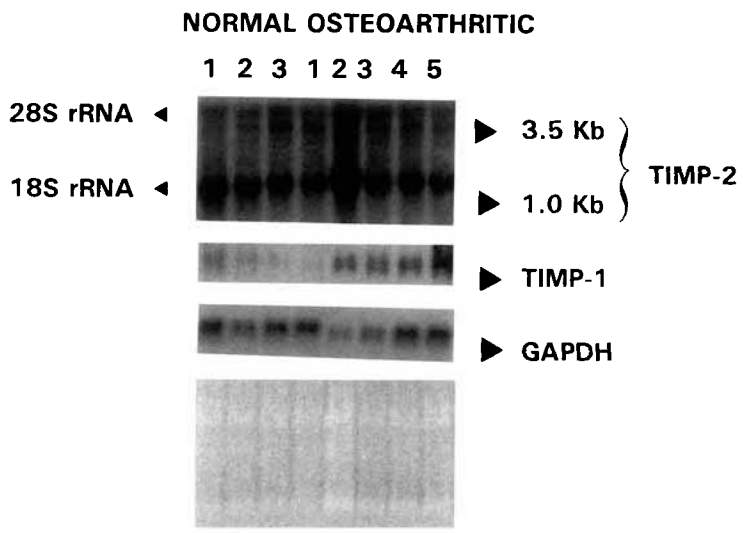

Fig. 4. Constitutive expression of TIMP-2 mRNA in human osteoarthritic (OA) and normal chondrocytes. Cartilage was obtained from 3 non-OA male (mean age, 78.6 years) and 5 OA (3 female and 2 male; mean age, 70.8 years) patients. Following enzymatic release of chondrocytes and RNA extraction, mRNA was analysed sequentially by Northern analysis with the TIMP-2 (*upper*), TIMP-1 (*middle*), and GAPDH (*lower*) probes. At the bottom, photograph of the gel is also shown. The time of autoradiography was 6 days for TIMP-2 and 20 h for TIMP-1 and GAPDH.

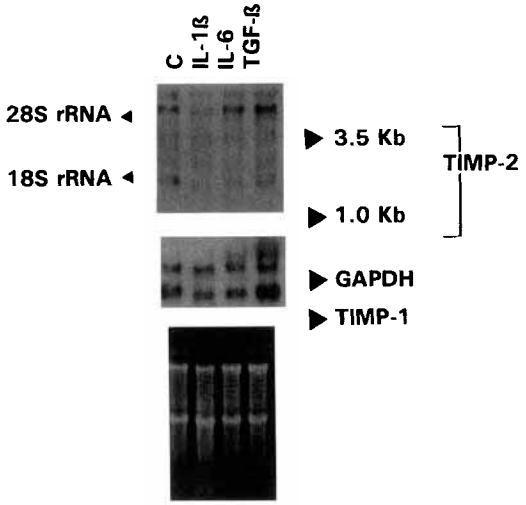

Fig. 5. Effect of IL-1β, IL-6, and TGF-β on TIMP-2 expression in human OA chondrocytes. Primary chondrocyte cultures were established from surgically removed osteoarthritic cartilage from a patient who underwent total knee replacement surgery. The cells were grown to confluence in DMEM with 10% serum. After washing the cells with PBS, the medium was replaced with serum-free DMEM for 24 h and exposed to rhIL-1β (20 ng/ml), IL-6 (50 ng/ml), and TGF-β (5 ng/ml) for 36 h. The RNA blot was hybridized sequentially with digoxigenin labelled (Boehringer Mannheim) TIMP-2, TIMP-1, and GAPDH probes. C (control) represents untreated cells. The resulting autoradiogram exposed for 16 h at room temperature is shown. The photograph of the RNA gel is shown at the bottom.

mRNA constitutively, which resisted regulation by proinflammatory and anti-inflammatory cytokines and growth factors found in joints during normal and disease states. The cross-hybridization of the human TIMP-2 probe with the bovine TIMP-2 RNA of the similar sizes demonstrates conservation and important function for this gene. Indeed, human and bovine TIMP-2 have 94% amino acid identity [Boone et al., 1990]. Human [Stetler-Stevenson et al., 1990], murine [Leco et al., 1992], and bovine (this work) TIMP-2 have two transcripts—possibly a consequence of differential splicing. The lack of or minimal serum-responsiveness of TIMP-2 in bovine and human chondrocytes was also noted in murine fibroblasts [Leco et al., 1992]. Basal TIMP-2 mRNA expression under various conditions of chondrocytes culture suggested its vital physiological function, which is most likely to inhibit the 72-kDa gelatinase and to maintain the integrity of cartilage ECM.

First, the inability of human IL-1β, IL-6, and TGF-β to induce TIMP-2 significantly in bovine chondrocytes is not due to species differences as these agents are well conserved among different species. Second, we routinely use IL-β for induction of stromelysin gene expression. Third, a lack of TIMP-2 induction was observed when human cytokines were applied to human OA chondrocytes. The lack of TIMP-2 response by

proinflammatory (IL-1β) and anti-inflammatory (IL-6, TGF-β) factors suggests a different function for TIMP-2 compared to TIMP-1. At the protein level, IL-1 was shown to decrease both TIMP-1 and TIMP-2, causing an imbalance relevant to collagenase and stromelysin [Martel-Pelletier et al., 1994].

The TIMP-2 in joint fluid [Kolkenbrock et al., 1991; Osthues et al., 1992; Cawston et al., 1993] is associated with MMP-2. In arthritic joints, lining cells, monocytes, and macrophages synthesize TIMPs [Spence McCachren, 1991]. This study at the RNA and our recent study [Martel-Pelletier et al., 1994] at the protein levels demonstrate chondrocytes as an additional source of TIMP-2. Basal levels of MMP-2 in chondrocytes from normal human cartilage [Mohtai et al., 1993] may be expressed coordinately along with TIMP-2 to prevent autodegradation of MMP-2 [Kleiner and Stetler-Stevenson, 1993]. TIMP-2 and MMP-2 genes may share regulatory elements responding to common stimuli. Analogously, TIMP-1 and stromelysin promoters have AP-1 and PEA-3 responsive elements [Campbell et al., 1991; Edwards et al., 1992; Buttice and Kurkinen, 1993]. The TIMP-2 promoter has high GC content, a TATA-like element, multiple initiation sites, SP-1 elements, one AP-2 motif, and an inactive AP-1 sequence. In TIMP-1 promoter, the AP-1 site is at a different location and is associated with a PEA-3 element [DeClerck et al., 1994]. Such promoter differences could yield differential TIMP-2 and TIMP-1 regulation, which may indicate gene-specific function. TIMP-2 may serve for chronic protection of ECM against degradation by MMPs, while TIMP-1 may function as an acute protective response to remodeling pressure [Leco et al., 1992]. This notion is supported by increased TIMP-1 mRNA in actively remodeling arthritic cartilage (Fig. 4). The co-evolution of MMP and inhibitor gene families may be a natural mechanism of checks and balances. TIMPs could serve as therapeutic agents for rheumatic diseases [Cawston, 1993].

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