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Up-Regulation of Tissue Inhibitor of Metalloproteinases-3 Gene Expression by TGF-β in Articular Chondrocytes Is Mediated by Serine/Threonine and Tyrosine Kinases

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Abstract The balance between matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) regulates extracellular matrix turn-over in normal animal development, cancer cell metastasis, atherosclerotic plaque rupture and erosion of arthritic cartilage. Transforming growth factor beta (TGF-β), an inducer of matrix synthesis, potently enhances mRNA and protein of a recently characterized MMP inhibitor, TIMP-3, in bovine articular chondrocytes. We examined the implication of protein kinases in the TGF-β-mediated induction of TIMP-3 expression by utilizing activators and inhibitors of these enzymes. Protein kinase A activators, dibutyryl cyclic AMP, or forskolin had little or no effect, respectively, while phorbol 12-myristate 13-acetate (PMA), a PKC activator, increased TIMP-3 gene expression. H7, a serine/threonine protein kinase inhibitor, markedly reduced the response of TIMP-3 gene to TGF-β. Furthermore, two protein tyrosine kinase inhibitors, genistein and herbimycin A, inhibited TGF-β induction of TIMP-3. H7 and genistein also suppressed TGF-β-induced TIMP-3 protein expression. These results suggest that TGF-β signaling for TIMP-3 gene induction involves H7-sensitive serine/threonine kinase as well as herbimycin A- and genistein-sensitive protein tyrosine kinases. J. Cell. Biochem. 70:517–527, 1998.

Key words: arthritis; cartilage; gene regulation; kinases; signaling; tissue inhibitors of metalloproteinases; transforming growth factor beta

Matrix metalloproteinases (MMPs) are Ca^{++} and Zn^{++} dependent enzymes, comprising collagenases, gelatinases, and stromelysins, which digest connective tissue extracellular matrix (ECM) and whose activities are believed to be inhibited physiologically by tissue inhibitors of metalloproteinases (TIMPs) [Birkedal-Hansen, 1995; Matrisian, 1992]. MMPs and TIMPs balance plays a vital role in physiological ECM turn-over during animal development. Furthermore, enzyme-inhibitor imbalance induced by certain cytokines and growth factors could contribute to pathological processes such as metastasis of cancer cells, retinal degeneration, atherosclerotic plaque rupture [Galis et al., 1994], and arthritis [Dean et al., 1989]. TIMP gene family consists at present of TIMP-1, -2, -3, and -4 in mammals, which are known to have tumor suppressing abilities [Khokha, 1994] and could potentially protect animals from the pathological conditions caused by overexpression of active MMPs, such as arthritis [Vincenti et al., 1994]. Any therapeutic manipulation of TIMP levels, however, must be preceded by a detailed knowledge of signal transduction mechanisms associated with the regulation of gene expression.

TIMP-3 is a novel inhibitor of MMPs first discovered in chicken as a 21 kDa protein (ChIMP-3) from the ECM of transformed chicken embryo fibroblasts [Blenis and Hawkes, 1983] which is related but distinct from other TIMPs [Pavloff et al., 1992]. TIMP-3 promotes detachment of transforming cells from the ECM and stimulates proliferation of nontransformed

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cells [Yang and Hawkes, 1992]. Human TIMP-3 cDNA was isolated from a placenta cDNA library and its gene was localized on human chromosome 22 [Apte et al., 1994b]. Its possible function in trophoblastic invasion of uterus has been suggested [Apte et al., 1994b; Leco et al., 1996]. TIMP-3 expression pattern in cartilage and skeletal tissue of developing mouse embryo was distinct relative to other TIMPs [Apte et al., 1994a]. High levels of TIMP-3 transcripts were found in the mouse decidua close to implanting embryo, suggesting its role in mammalian development [Harvey et al., 1995; Reponen et al., 1995]. In contrast with other TIMPs, TIMP-3 mRNA remained elevated during pseudopregnancy in rats, which suggests its distinct role in this process [Nothnick et al., 1995]. Human fetal kidney [Silbiger et al., 1994] and breast tumors [Uría et al., 1994] also express TIMP-3. Human TIMP-3 is induced by bacterial lipopolysaccharide in THP-1 cells and is expressed in several embryonic and adult tissues [Apte et al., 1994b; Wilde et al., 1994]. Murine TIMP-3 has an 80% identity with ChIMP-3 and is expressed in fibroblasts and several mouse tissues, and is found in ECM but not in conditioned medium [Leco et al., 1994]. TIMP-3 and TIMP-1 were inducible with different kinetics by PMA, epidermal growth factor and TGF-β. TIMP-3 was up-regulated by dexamethasone in murine fibroblasts and is also regulated by mitogens at the G1 phase of the cell cycle [Leco et al., 1994; Wick et al., 1994]. It is also implicated in the inhibition of tumorigenesis and tumor invasion [Sun et al., 1994]. Furthermore, point mutations of the human TIMP-3 gene in patients with inherited Sorsby's fundus dystrophy result in disruption of the tertiary structure of the protein, possibly disabling its inhibitory activity, leading to progressive degeneration of the central retina and visual loss [Weber et al., 1994]. The protein can inhibit several MMPs implicated in cartilage resorption [Apte et al., 1995].

Due to their their role in cartilage remodeling, a better elucidation of regulatory mechanisms of TIMPs in chondrocytes is needed. They are unique cells whose primary function is to maintain cartilage ECM [Muir, 1995]. We recently cloned the bovine TIMP-3 cDNA and demonstrated induction of its message by TGF-β and suppression by dexamethasone in chondrocytes [Su et al., 1996]. Current knowledge on TGF-β signaling is confined to cell-types where the factor inhibits growth. Both type I and type II receptors have cytoplasmic serine/threonine kinase domains that are required for TGF-β signaling. Type III receptor, a betaglycan, facilitates binding of TGF-β to type II receptor. This is followed by phosphorylation of type I receptor and propagation of downstream signals [Moustakas et al., 1993]. In cartilage, TGF-β initiates chondrogenesis in vivo [Joyce et al., 1990] and is a major anabolic factor [Lotz et al., 1995]. The aims of the present study were to elucidate the signaling pathways involved in TIMP-3 expression and to test the hypothesis that its induction by TGF-β in chondrocytes may involve protein kinases.

RESULTS

Temporal Induction of TIMP-3 Gene Expression by TGF-β

To examine TIMP-3 gene expression in response to TGF-β in chondrocytes, RNA was analyzed by Northern hybridization with bovine TIMP-3 cRNA probe, which yielded three transcripts of 5.5, 2.8, and 2.4 Kb sizes. Primary cultures of bovine articular chondrocytes have low-level basal expression of the three TIMP-3 transcripts. Treatment of high-density primary monolayer cultures with TGF-β resulted in a time-dependent induction of TIMP-3 mRNA, which started at 4 h and continued to elevate up to 24 h. (Fig. 1A). The levels of the control GAPDH gene RNA, remained constant. To investigate if the RNA was translated into the corresponding protein, Western immunoblot analysis was performed. A mouse TIMP-3 antibody raised in rabbits [Sun et al., 1995] cross-reacted with a single band (between 19 to 28 kDa prestained markers) corresponding to the bovine TIMP-3 in total chondrocyte extra- and intracellular protein extract. The basal TIMP-3 expression and time-dependent induction seen for RNA was also observed at the protein level with considerable induction at 48 h (Fig. 1B).

Protein Kinase Activators and TIMP-3 Expression

To elucidate the possible signal transduction pathways in TGF-β-induced TIMP-3 gene expression in bovine chondrocytes, we first assessed the effect of protein kinase activators on TIMP-3 expression. Cells were either kept un-

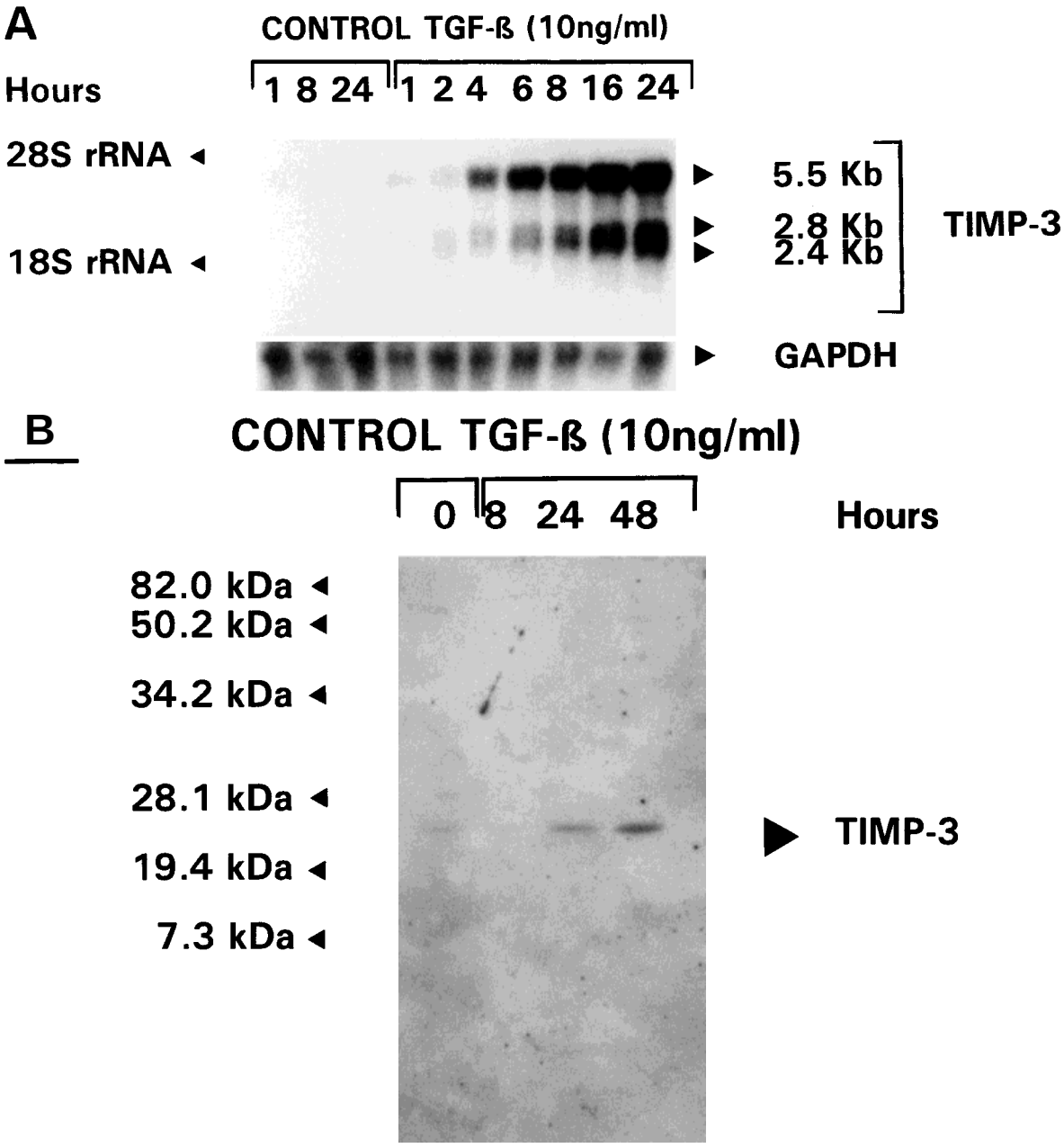

Fig. 1. Time-course of TIMP-3 mRNA and protein expression in bovine chondrocytes following TGF-β exposure. **A: Lanes 1 to 3** show RNA from control cells maintained in serum-free medium for 1, 8, and 24 h, respectively. **Lanes 4 to 10** depict RNA from cells treated with TGF-β (10 ng/ml) for 1, 2, 4, 6, 8, 16, and 24 h. Following hybridization of the Northern blot with the TIMP-3 probe, the membrane was rehybridized with loading control GAPDH probe. **B:** Total cellular protein (20 µg) was isolated either from untreated (control) cells or chondrocytes exposed to TGF-β for indicated time periods, subjected to SDS-PAGE and Western blot analysis with a rabbit anti-mouse TIMP-3 antibody. Only one band cross-reacting with the bovine TIMP-3 (indicated by an arrow) was observed.

treated or exposed for either 8 or 16 h to TGF-β (10 ng/ml), phorbol ester (PMA) (200 ng/ml, PKC activator), PMA and TGF-β, dibutyryl cyclic AMP (250 µg/ml) alone, cAMP and TGF-β, forskolin (activator of adenylate cyclase) (40 µg/ml) alone or with TGF-β. These doses were shown to be effective for connective tissue cells in earlier published reports [DiBattista et al., 1995]. TGF-β potently induced TIMP-3 gene expression while PMA and cAMP were relatively weaker inducers. Forskolin and db cAMP had minimal (db cAMP) or no effect (forskolin)

in three different experiments. PMA and TGF-β together appeared to increase TIMP-3 RNA expression relative to individual treatments. The RNA loading control, constitutive GAPDH mRNA, was unaffected by the treatments (Fig. 2). These results suggested that certain protein kinase A and PKC activators moderately increased TIMP-3 gene expression.

Inhibition of TIMP-3 Gene Expression by H7

To evaluate the role of protein kinases in TGF-β signaling leading to inducible TIMP-3 expression, we next used protein kinase inhibitors, H7 and staurosporine. As shown in Figure 3, staurosporine (50 ng/ml) or serine/threonine protein kinase inhibitor H7 (10 μg/ml) alone had no effect on basal TIMP-3 expression. Simultaneous exposure with TGF-β and staurosporine also did not influence TIMP-3 induction by TGF-β. Combined treatment with H7 and TGF-β, however, significantly down-regulated TIMP-3 gene expression (Fig. 3A). Even more drastic suppression was observed when cells were pretreated for 30 min with H7 followed by TGF-β treatment (Fig. 3B). The internal control GAPDH RNA was unaffected by the inhibitors. We further assessed the role of PKC in the transduction of the TGF-β signal by using a highly specific inhibitor of PKC, Calphostin C, and by down-regulation of protein kinase C after prolonged pretreatment with PMA [Collins and Rozengurt, 1984]. None of the treatments had any effect on TIMP-3 mRNA induction by TGF-β (data not shown), further supporting the lack of PKC involvement. These results imply a H7-sensitive protein kinase in the TGF-β-stimulated TIMP-3 gene expression.

Implication of Tyrosine Kinases in TGF-β-Inducible TIMP-3 Gene Expression

We further investigated the role of tyrosine kinases in the TGF-β regulation of TIMP-3. Bovine chondrocytes were pretreated with different concentrations of a tyrosine kinase inhibitor, genistein (25–50 μM) alone for 30 min or followed by additional treatment with TGF-β for 24 h. As depicted in the Northern blot, genistein at 50 μM concentration, inhibited induction of the TIMP-3 mRNA by TGF-β in three separate experiments (Fig. 4A). In additional experiments, higher genistein concentrations (ranging from 100–400 μM) completely sup-

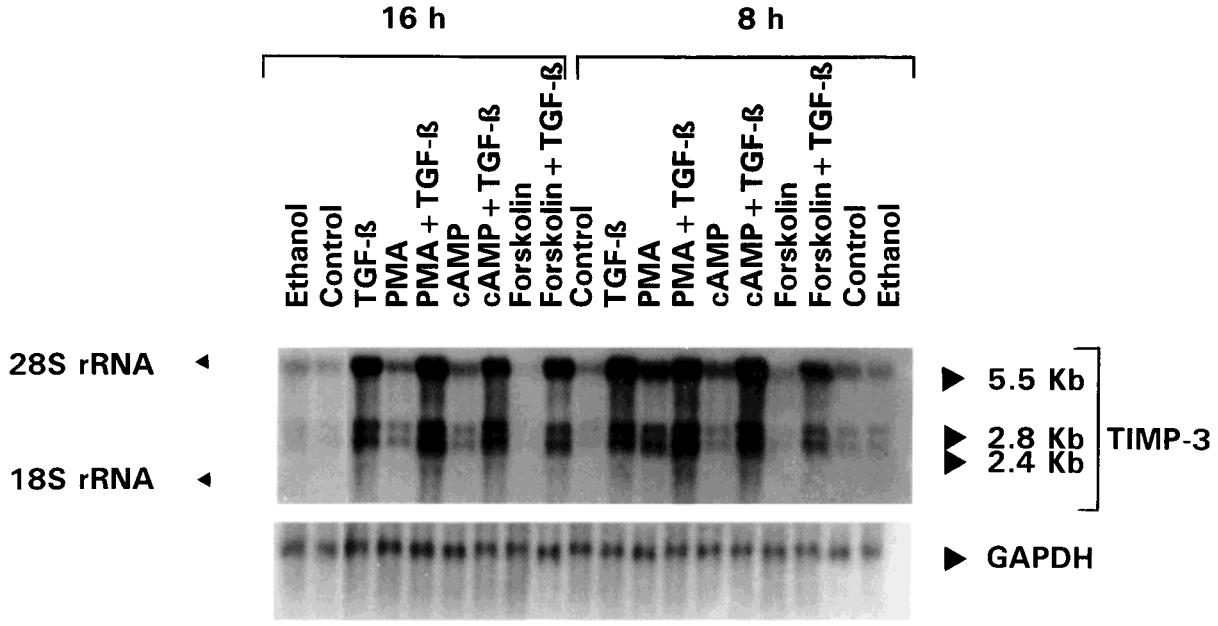

Fig. 2. Effect of protein kinase activators and TGF-β on TIMP-3 gene expression in bovine chondrocytes. Primary monolayer cultures of chondrocytes were either kept untreated in DMEM (control), in ethanol (vehicle only control) or exposed to TGF-β (10 ng/ml), PKC activator, phorbol ester (PMA) (200 ng/ml), PMA and TGF-β, PKA activator, dibutyryl cyclic AMP (250 μg/ml) alone, cAMP and TGF-β, forskolin (adenylate cyclase activator) (40 μg/ml) alone or with TGF-β. These treatments were done either for 16 h (**lanes 1–9**) or for 8 h (**lanes 10–19**) and RNA analysed by Northern hybridization with digoxigenin (DIG)-labeled bovine TIMP-3 cRNA probe. The RNA loading control, constitutive, GAPDH mRNA is shown in the bottom panel. The film was exposed for 30 min.

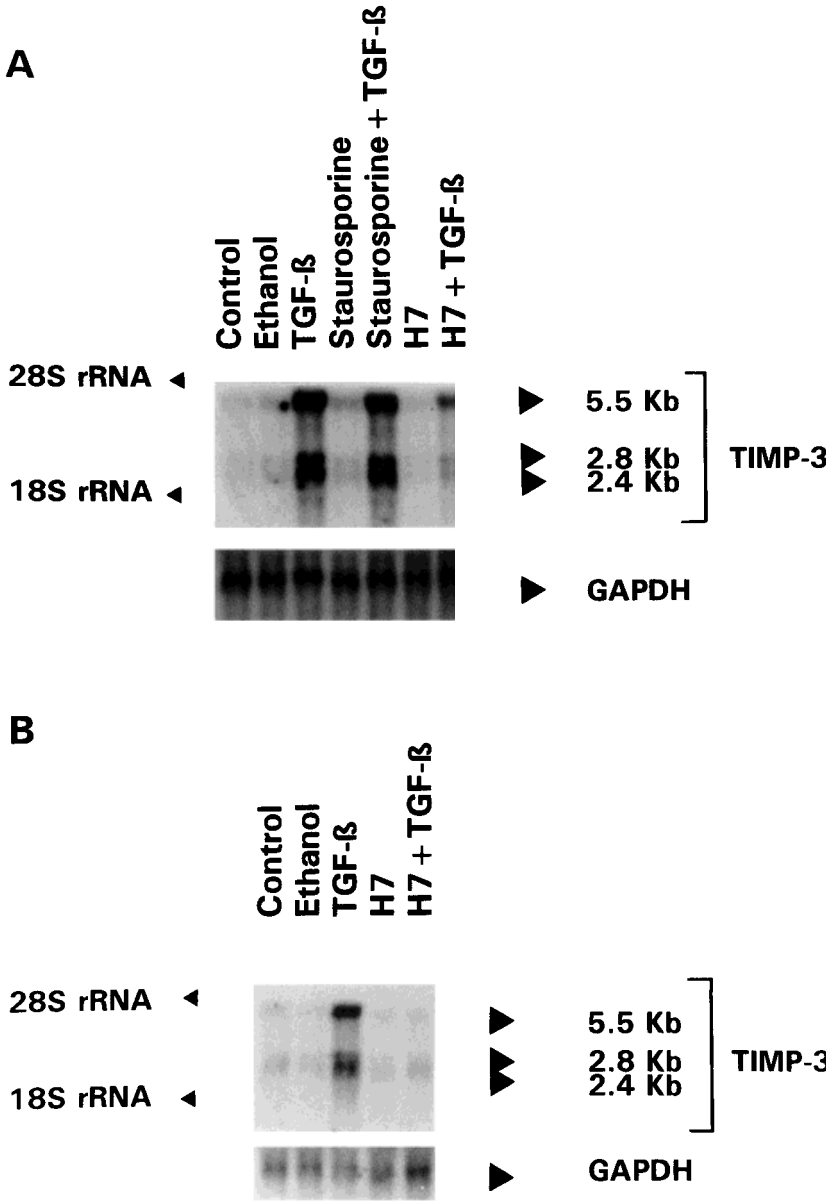

Fig. 3. Influence of protein kinase inhibitors, H7 and staurosporine on TGF-β-induced TIMP-3 gene expression. **A:** Primary monolayers of chondrocytes were maintained in DMEM only (control), medium with ethanol vehicle, TGF-β (10 ng/ml), protein kinase inhibitor, staurosporine (50 ng/ml) alone or simultaneously with TGF-β, serine/threonine protein kinase inhibitor H7 (10 μg/ml) alone, or with TGF-β for 16 h. **B:** Cells were pretreated with H7, for 30 min and then exposed to TGF-β for 6 h. TIMP-3 and GAPDH RNAs were analyzed as in previous figures. The film was exposed for 1 h.

pressed TIMP-3 gene expression (not shown). Pretreatment with another tyrosine kinase inhibitor, herbimycin A (0.5–5 μM) for 30 min followed by TGF-β treatment also resulted in strong inhibition of TIMP-3 steady-state mRNA (Fig. 4B). The GAPDH RNA expression was mostly unaffected by the treatments. These results suggest that TGF-β induces TIMP-3 gene expression by mediation of protein tyrosine kinases.

Suppression of TIMP-3 Protein Expression by Kinase Inhibitors

To examine a possible correlation between the results attained with mRNA and corresponding protein, TIMP-3 protein levels were

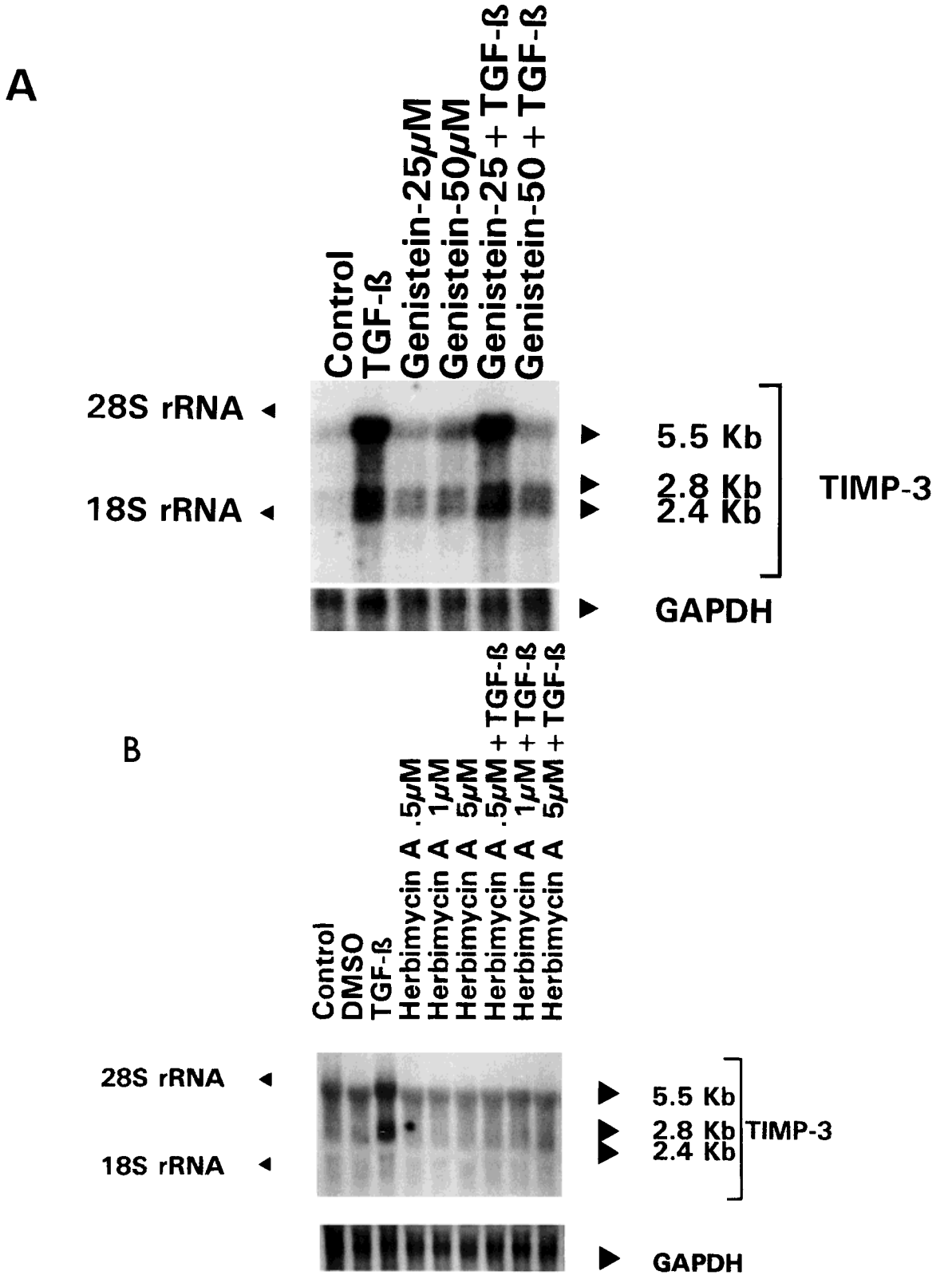

Fig. 4. Inhibition of TGF-β-inducible TIMP-3 gene expression by genistein and herbimycin A. **A:** Bovine chondrocytes were pretreated with different concentrations of genistein (25–50 μM) alone for 30 min or followed by additional treatment with TGF-β for 24 h and RNA levels measured by Northern hybridization analysis. The GAPDH RNA expression is shown at the bottom. Film was exposed for 24 h. **B:** Bovine chondrocytes were pretreated with different concentrations of herbimycin A (0.5 to 5 μM) alone for 30 min or followed by additional treatment with TGF-β for 24 h and RNA levels determined by Northern hybridization analysis. The GAPDH RNA expression is shown at the bottom.

measured in total cellular (extra- and intracellular) proteins by Western immunoblot analysis with a mouse TIMP-3 antibody [Sun et al., 1995]. As shown in Figure 5, this antibody detected a single protein band between 19–28 kDa relative molecular mass markers, which is close to the sizes of other mammalian TIMP-3s (22 kDa). TGF-β induced TIMP-3 at the protein level while H7 and genistein potently inhibited this induction; a result very similar to the one for TIMP-3 mRNA shown in Figures 3 and 4.

DISCUSSION

Since TIMP-3 is a cell-cycle regulated, potentially cartilage protective, tumor suppressor and antimacular degenerative protein, it is of great interest to a variety of disciplines. In this paper, using a mammalian chondrocyte model system, we have attempted, as a primary approach, to dissect signal transduction pathways involved in the regulation of this multi-functional and novel gene. To our knowledge, this is the first report demonstrating that TIMP-3 induction by TGF-β can be suppressed by protein kinase inhibitors H7, genistein and herbimycin A, which suggests involvement of multiple kinases in the signaling.

Minimal or no TIMP-3 induction by adenylate cyclase and protein kinase A activators (cAMP) suggest that this pathway may not be a major player in TIMP-3 gene regulation. Although cyclic AMP response element has been detected in the mouse TIMP-3 promoter by computer analysis, the gene did not respond to forskolin in our system and in mouse epidermal cells (Sun, unpublished observation). Bovine TIMP-3 gene promoter characterization is currently underway in our laboratory. Moderate induction by phorbol ester alone suggests that the activation of PKC may be partly involved in

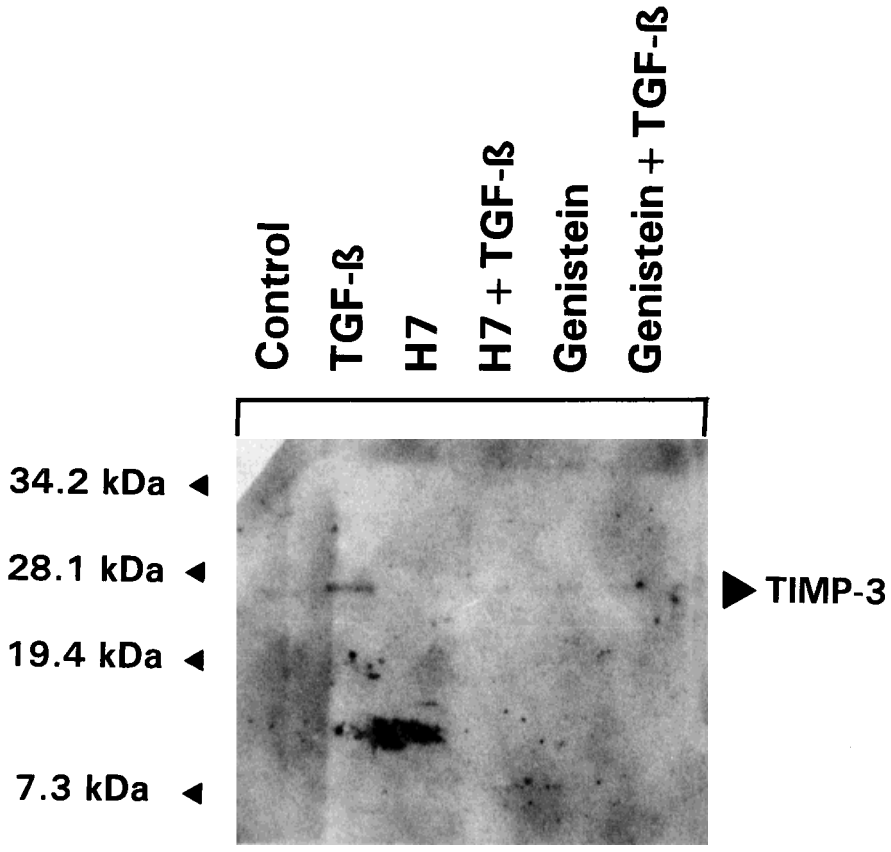

Fig. 5. Inhibition of TGF-β-inducible TIMP-3 protein expression by H7 and genistein. Chondrocytes were maintained without any treatment or exposed to TGF-β alone or along with H7 or genistein for 24 h. Total cellular protein (20 μg) was isolated and subjected to SDS-PAGE under non-reducing conditions and Western blot analysis performed with a rabbit anti-mouse TIMP-3 antibody. The TGF-β induced TIMP-3 band revealed by chemiluminescence is suppressed by the two inhibitors.

TIMP-3 gene expression. This is supported by the localization of AP-1 elements in the promoter of murine TIMP-3 gene [Sun et al., 1995].

TGF-β is a very important growth factor in cartilage as it induces chondrogenesis [Joyce et al., 1990] and thereby is a potential candidate for stimulating cartilage repair in joint degenerative diseases such as osteoarthritis. This is the only factor that stimulates in vitro proliferation of chondrocytes from older patients and those with arthritis whose cartilage is generally unresponsive to anabolic growth factors [Guerne et al., 1995]. A detailed knowledge of TGF-β target genes and mechanisms of molecular signaling involved in their induction is of profound interest, as defective signaling may be a cause of impaired cartilage repair. TGF-β type I and type II receptors have intrinsic ligand-activated serine/threonine kinase motifs in their cytoplasmic domains. TGF-β first binds with type II receptors and then phosphorylates and activates type I receptor on serine and threonine leading to transcription via Smads [Wrana and Pawson, 1997; Massagué et al., 1997]. H7 is an inhibitor of a protein kinase C or cyclic-nucleotide-dependent protein kinase, myosin light chain kinase, and protein kinase G [Kawamoto and Hidaka, 1984]. Since H7 inhibited TIMP-3 induction without affecting GAPDH mRNA level (an indicator of cell metabolic activity), suppression by H7 suggests the implication of a H7-sensitive serine/threonine protein kinase in the signal transduction of TGF-β leading to induced TIMP-3 gene expression. H7 may have either inhibited receptor serine/threonine domain or subsequent steps involving serine/threonine phosphorylation. Another broad-spectrum inhibitor, staurosporine (which preferentially inhibits PKC but has high affinity for PKA and PKG) failed to suppress induction by TGF-β. However, lack of inhibition by down-regulation of PKC by prolonged pretreatment with PMA, or by a highly specific PKC inhibitor, calphostin C [Kobayashi et al., 1989] suggests that PKC may not be involved in TGF-β-induced TIMP-3 expression. In contrast with this observation, TGF-β was shown to increase protein kinase C alpha isoform in different zones of rat costochondral chondrocytes, which is related to state of cell maturation [Sylvia et al., 1994]. Articular chondrocytes utilized in our study may behave differently from costochondral chondrocytes. Interestingly, expression of TGF-β inducible plasminogen activator inhibitor-1 (PAI-1) gene, another regulator of proteolytic cascades, was inhibited by H7 but was unaffected by staurosporine or prolonged pre-incubation with PMA in mink lung epithelial cells [Thalacker and Nilsen-Hamilton, 1992], a pattern very similar to that of TIMP-3 observed here [Ohtsuki and Massagué, 1992]. In TGF-β-inhibited mouse osteoblastic cells, H7 inhibits a TGF-β-inducible early-response gene, which encodes a leucine zipper structure [Shibanuma et al., 1992]. The signaling pathways for transcriptional induction may be different from those of growth-related responses [Franzen et al., 1993].

Genistein is an inhibitor of autophosphorylation of epidermal growth factor receptor kinase as well as pp60src and pp110gag-fes tyrosine kinases. Treatment of the cells with a wide range of concentrations resulted in TIMP-3 suppression, providing strong support for the implication of tyrosine kinases in this signaling. The inhibition of TIMP-3 expression by another inhibitor, herbimycin A, reinforced tyrosine kinase involvement and further suggested implication of src-related oncogene in TGF-β signaling. So far serine/threonine kinase activity was implicated in TGF-β signaling, and our results support involvement of tyrosine kinases as well. A similar degree of inhibition at both the RNA and protein levels suggests that inhibitors exert their effect at the pre-translational levels. These inhibitors may ultimately work through inhibition of transcription factor activation and genes associated with cell-cycle. Interestingly, TIMP-3 itself is a cell-cycle induced gene [Wick et al., 1995]. Recently, mitogen-activated protein kinase family member, TAK1 (TGF-β-activated kinase 1) was identified, which is a mediator of TGF-β signaling [Shibuya et al., 1996]. Furthermore, down-stream effectors of TGF-β signaling, SMADs have been described that either activate or inhibit TGF-β signaling [Whitman, 1997]. It will be interesting to examine if TIMP-3 expression in chondrocytes is mediated by these pathways, where certain steps involve serine/threonine and tyrosine phosphorylation. In addition to TGF-β-inducible fibronectin and PAI-1 genes, TIMP-3 could serve as a novel target for this purpose.

In summary, our results suggest a lack of protein kinase C involvement and implication of a H7-sensitive serine/threonine protein kinase in the TGF-β induction of TIMP-3. Furthermore, TGF-β may induce TIMP-3 gene expres-

sion by mediation of genistein- and herbimycin A-sensitive tyrosine specific protein kinases. Therefore, in chondrocytes, TGF-β signal leading to TIMP-3 up-regulation may be propagated via multiple kinases.

EXPERIMENTAL PROCEDURES

Materials

Cell culture supplies such as Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), antibiotic-antimycotic agents, trypan blue, and agarose were from Canadian Life Technologies Inc. (Gibco-BRL, Burlington, Ontario). Plastiware such as 6-well plates and T-75 flasks were from Nunc (Roskilde, Denmark). Pronase, collagenase type II, and protein kinase activators phorbol 12-myristate 13-acetate (PMA), dibutyryl cyclic AMP, forskolin and inhibitors, H7 and staurosporine, were from Sigma Chemical Company (St. Louis, MO). Protein tyrosine kinase inhibitors, herbimycin A and genistein, were from Calbiochem (San Diego, CA). Digoxigenin (DIG) RNA labeling and chemiluminescence systems were from Boehringer Mannheim (Laval, Québec). Hybond nylon membrane was from Amersham Canada (Oakville, ON). The human platelet transforming growth factor (TGF-β1) was from R&D Systems (Minneapolis, MN), and was reconstituted as recommended. RNA probe labeling kits were from Promega (Madison, WI). Restriction endonucleases were from Pharmacia Biotech. Inc. (Baie d'urfé, Québec) and Boehringer Mannheim (Laval, PQ). Other common laboratory reagents were from Fisher Scientific (Montreal).

Chondrocyte Culture

Normal bovine articular cartilage was obtained from the knee and hip joints of freshly slaughtered adult animals from a local abattoir. The cartilage-containing bones were kept briefly in 1% proviodine (Rougier Inc., Chambly Quebec) for sterilization and washed extensively in large volumes of 0.9% NaCl. The slices of cartilage were dissected out, kept for 1–2 h at 4°C in 5 X antibiotic-antimycotic solution, and washed five times with large volumes of phoshpate-buffered saline (PBS) containing 5 X penicillin-streptomycin and 1 X fungizone (Gibco-BRL Burlington, Ontario). Chondrocytes were released by dissociation of cartilage with pronase (1 mg/ml) for 90 min and collagenase (Sigma type II) for 12 h in DMEM at 37°C. Viability of cells by trypan blue exclusion test was about 80%. The cells were pelleted and washed three times with PBS and plated as high-density primary monolayer cultures. The cells were first allowed to adhere to the plates in DMEM alone for 4 h [Pearson and Sasse, 1992] and then supplemented with 10% serum for confluent growth (up to 3–7 days). Prior to different treatments, cells were kept in serum-free DMEM for 24 h and then exposed to different reagents in FCS-free medium for various periods of time as described in figure legends and text.

RNA Extraction and Northern Hybridization Analysis

Total RNA from primary cultures of chondrocytes was extracted by the procedure of Chomczynski and Sacchi [1987] and aliquots of 5 µg analyzed by electrophoretic fractionation in 1.2% formaldehyde-agarose gels, transferred to nylon membranes and hybridized as previously described [Zafarullah et al., 1993]. The integrity and quantity of applied RNA were verified by ethidium bromide staining of the gels and photography of 28S and 18S ribosomal RNA bands. The RNA was electroblotted onto Hybond nylon membrane using a Bio-Rad (Mississauga, ON) Transblot in the presence of 1 X TAE buffer at a current of 500 mAmp for 12 h. Complete transfer was ascertained by ethidium bromide staining of the gel where no ribosomal RNA bands were visible. Northern blot analysis of RNA was performed with a recently cloned bovine TIMP-3 cDNA [Su et al., 1996]. The probe was isolated by screening the plaques of a bovine cDNA library with a human TIMP-3 cDNA probe [Apte et al., 1994b] generously provided by Dr. Suneel Apte (Dept. of Biomedical Engineering, Cleveland Clinic Foundation, Cleveland, OH). This probe was a 2.042 Kbp Eco RI-Eco RI cDNA fragment cloned in the plasmid pGEM-4Z (Promega Biotech., Madison, WI). The vector was linearized with SacI and RNA probe synthesized from SP6 polymerase according to the protocols of Promega Biotech. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (from ATCC, Rockville, MD) probe was described earlier [Zafarullah et al., 1993]. The probes were labeled with the digoxigenin (DIG) RNA labeling kit containing DIG-11-UTP followed by pre-hybridization, hybridization, and detection of Northern blots with

the DIG Luminiscent Detection Kit of Boehringer Mannheim according to their protocols.

Western Blot Analysis

Cells in 6-well plates were washed with PBS, lysed in the sample buffer (62.5 mM Tris.HCl, pH 6.8, 20% glycerol, 2% SDS without β-mercaptoethanol), and protein quantified by the Bio-Rad protein assay reagent. Equal amounts of total protein extract (20 µg) and broad-range known molecular mass markers (Bio-Rad Canada) were fractionated on a minigel by SDS-PAGE (15% separating, 4% stacking gel) in a running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3) and electroblotted in a buffer (containing 39 mM glycine, 48 mM Tris base, 20% methanol) onto nitrocellulose membrane with the Bio-Rad apparatus. For subsequent steps, BM Chemiluminescence Western Blotting kit and instructions were used. Briefly, the membrane was blocked for nonspecific binding with 1% blocking reagent overnight at 4°C and incubated with a anti-mouse TIMP-3 primary antibody raised in rabbit [Sun et al., 1995] overnight at 4°C in 0.5% blocking solution at 1:800 dilution. After washings, membrane was incubated with secondary anti-rabbit peroxidase (POD)-conjugated IgG (from sheep) (Boehringer Mannheim) at room temperature in 0.5% blocking reagent. Subsequently, protein band was detected with the chemiluminescence detection reagents of Boehringer Mannheim.

All the experiments reported in this paper were performed at least twice with different batches of chondrocytes and the reported results were reproducible.

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