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TIMP-1 protein expression is stimulated by IL-1β and IL-6 in primary rat hepatocytes

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Abstract

Degradation of extracellular matrix proteins is performed by metalloproteinases which are inhibited by tissue inhibitors of metalloproteinases (TIMP). We expressed the murine TIMP-1 protein in *E. coli* and prepared a polyclonal antiserum against the recombinant protein. Using this antiserum we studied the biosynthesis and glycosylation of murine TIMP-1 protein in COS-7 cells transfected with a TIMP-1 expression plasmid by metabolic labeling and indirect immunofluorescence studies. In primary rat hepatocytes we show for the first time that TIMP-1 protein expression is up-regulated upon stimulation with IL-1β and IL-6. Since TIMP-1 is induced during the acute phase reaction it could possibly be involved in the pathogenesis of liver fibrosis.

Key words: TIMP-1; Antibody; IL-6; IL-1; Liver; Hepatocyte

1. Introduction

Alterations in the balance between synthesis and degradation of extracellular matrix may result in tissue sclerosis and fibrosis. The interaction between metalloproteinases and their inhibitors modulates the rate of matrix degradation and accumulation for example in glomerulosclerosis [1] or liver fibrosis [2] as well as in rapid matrix destruction occuring during arthritis (for review see [3–5]). Furthermore it is widely accepted that collagenolytic enzymes and their inhibitors are involved in the invasion and spread of tumor cells into host tissues [6,7]. In some cell lines, however, TIMP-1 has the properties of a tumor-suppressor gene or anti-oncogene [8,9].

Murine TIMP-1 is a secreted glycoprotein of 184 amino acids (M_r = 28 kDa) with a high sequence identity to rat and human TIMP-1 [10]. By forming a tight complex of 1:1 stoichiometry with the target matrix metalloproteinases, TIMP-1 regulates the breakdown of collagen and basement membrane components [11,12]. In murine fibroblast cells the expression of TIMP-1 mRNA is induced by serum, 4β-phorbol-12-myristate-13-acetate, fibroblast growth factor and platelet derived growth factor [13–15]. Oncostatin M, Interleukin-6 (IL-6), leukemia inhibitory factor (LIF), and IL-1α elevate the TIMP-1 expression at the RNA level in human lung fibroblasts [16]. In previous reports we demonstrated that TIMP-1 mRNA is up-regulated in hepatic cells by inflammatory cytokines such as IL-1, IL-6, IL-11, ciliary neurotrophic factor (CNTF) and LIF [17,18].

Most animal models of tumor growth, metastasis, arthritis or fibrosis have been established with mice or rats. Therefore we produced a polyclonal antiserum specific for murine TIMP-1 which cross reacts with rat TIMP-1 but not with human TIMP-1. We show that the expression of secreted TIMP-1 protein can be up-regulated by the cytokines IL-1β and IL-6. Taken together with our finding that TIMP-1 mRNA is induced during the acute phase reaction in liver cells, our results emphasize the involvement of TIMP-1 in liver fibrosis and cirrhosis.

2. Materials and methods

2.1. Reagents

Restriction enzymes and PNGase F were obtained from Boehringer Mannheim (Mannheim, Germany). Tran[^{35}S]label was purchased from ICN (Meckenheim, Germany), [α-^{32}P]dATP were from Amersham International (Amersham, GB) and pansorbin from Calbiochem (La Jolla, CA). DMEM was obtained from Gibco (Eggenstein, FRG) and FCS from Seromed (Berlin, Germany). The murine TIMP-1 cDNA was a kind gift from Dr. D.R. Edwards (Calgary, Canada). The bacterial expression plasmid pRSET 5d and the host bacteria BL21 (DE3) were a gift from Dr. R. Schoepfer [19]. Recombinant Human IL-1β with a specific activity of 2×10^7 units/μg protein was a generous gift of Dr. A.R. Shaw (Glaxo Institut for Molecular Biology, Geneva, Switzerland). Recombinant human IL-6 with a specific activity of 1.5×10^6 B-cell stimulatory factor 2 units/μg protein was generously supplied by Drs. T. Hirano and T. Kishimoto (Osaka, Japan).

2.2. Cells and cell treatment

COS-7 cells were cultured to 70–90% confluency in DMEM containing 10% FCS streptomycin (100 mg/l) and penicillin (60 mg/l), at 5% CO_2 in a water-saturated atmosphere. Rat hepatocytes in primary culture were prepared as described in [17] after perfusion of rat livers with collagenase. Hepatocytes were cultured serum-free for 48 h before the experiments.

2.3. Construction of a bacterial TIMP-1 expression plasmid

Standard cloning procedures were performed as described in Sambrook et al. [20]. A cDNA insert coding for murine TIMP-1 without signal peptide was constructed by polymerase chain reaction using the following primers: 5′-CCGGCCATGGCCTGTAGCTGGCCCCA-3′, 5′-TCATCGGGCCCCAAGGG-3′, thus replacing the signal peptide

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with the start codon of translation (underlined). Finally the 540 bp *Bam*HI/*Hin*dIII fragment was inserted into the *Nco*I/*Hin*dIII site of the expression vector pRSET. All polymerase chain reaction-generated sequences were verified by DNA sequencing [21] using T7 DNA polymerase (Pharmacia, Freiburg, Germany).

2.4. Preparation of rabbit polyclonal anti-murine-TIMP-1

A rabbit was immunized subcutaneously with 200 μg of recombinant murine TIMP-1 emulsified with Freund's complete adjuvant. Immunizations were made in Freund's complete and incomplete adjuvant in 4-week intervals for a total of three months. The antiserum called antiserum A was obtained 10 days after the last injection.

2.5. Western blots

Supernatants were separated on 15% SDS polyacrylamide gel and transferred to nitrocellulose. Protein bands were localized by staining with Ponceau S. Blots were blocked with PBS, containing 2% BSA washed and incubated with the polyclonal antiserum against murine TIMP-1. After washing the blot was incubated with an anti-rabbit antibody (Serva). Immunocomplexes were visualized by peroxidase staining.

2.6. Construction of a eucaryotic TIMP-1 expression plasmid

The mammalian TIMP-1 expression vector was constructed by inserting the entire coding region of the murine TIMP-1 exised from the Bluescript-vector KS- by the restriction endonucleases *Bam*HI/*Xho*I and cloned into the *Xho*I and filled *Hin*dIII site of the expression vector pCDM8.

2.7. Transfection of mammalian cells

Transfections were performed using the calcium phosphate technique [22]. COS-7-cells for transfection were subcultured 16 h prior to the addition of the calcium phosphate precipitate; 18 h after transfection the precipitate was removed and cells were cultured for 24 h.

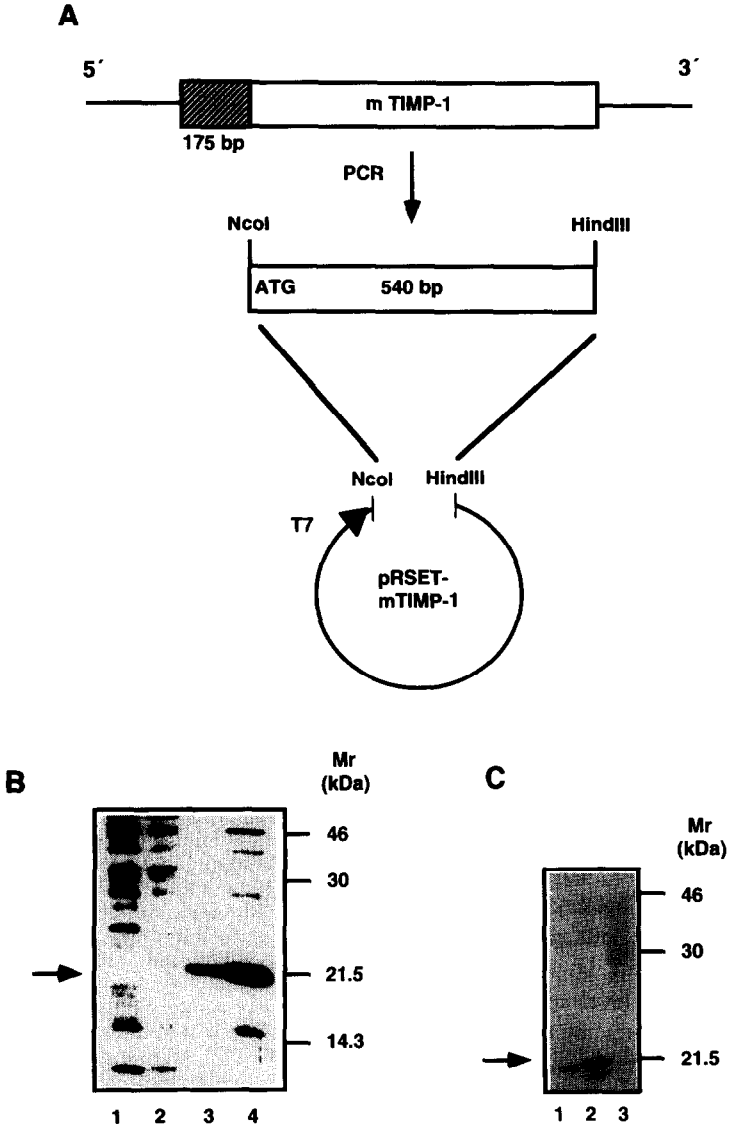

Fig. 1. Bacterial expression of murine TIMP-1. (A) Construction of the bacterial murine TIMP-1 expression vector: A cDNA insert coding for murine TIMP-1 without signal peptide was constructed by polymerase chain reaction (as described in section 2) and inserted into the expression vector pRSET5d. (B) SDS-PAGE of proteins from bacteria transformed with the empty expression vector pRSET (lane 1), the murine TIMP-1 vector before induction with IPTG (lane 2) and 1 μg (lane 3) and 5 μg (lane 4) inclusion bodies of the same cells. The arrow indicates the position of TIMP-1 expressed by transformed bacteria. (C) Detection of murine TIMP-1 inclusion bodies by Western blotting. 1 μg (lane 1) and 5 μg (lane 2) of inclusion bodies in lysis buffer were electrophoresed on a 13% SDS-polyacrylamide gel and analyzed by Western blotting using a polyclonal murine-TIMP-1-antiserum obtained as described in section 2. The arrow indicates the position of TIMP-1 expressed by transformed bacteria.

2.8. Immunoprecipitation

Cells were metabollically labeled with [^{35}S]methionine/cysteine under the conditions indicated in the figure legends. Cell lysis was performed in 10 mM Tris-HCl, pH 7.4, 60 mM EDTA, 1% Nonidet P40 and 0.4% sodium deoxycholate. Supernatants and lysates were pretreated with pansorbin (Calbiochem, La Jolla, CA) and subsequently incubated with the appropriate antisera for 3 h at 4°C. The immunocomplexes were precipitated with protein A-Sepharose, separated on a 15% SDS-polyacrylamide gel [23] and visualized by fluorography [24]. Rat hepatocytes in primary culture were stimulated 12 h before immunoprecipitation.

2.9. Indirect immunofluorescence

Procedures for indirect immunofluorescence of transfected COS-7 cells were done as described [25]. Cells grown on cover glasses for 24 h were fixed with 0.2% paraformaldehyde and treated with a 1/100 dilution of an antiserum for 20 min. Detection of the antibodies bound to membrane proteins was carried out by treatment with a 1/200 dilution of a rhodamine-conjugated anti-rabbit IgG antibody for 20 min. Cover glasses were mounted on slides with Moviol 4-88 (Calbiochem, La Jolla, CA) and analyzed using fluorescence microscopy. A 200-fold magnification was used to photograph the cells.

3. Results and discussion

A cDNA coding for the murine TIMP-1 lacking the signal peptide (mTIMP-1) was cloned into the expression plasmid pRSET5d as described in section 2 (Fig. 1A).

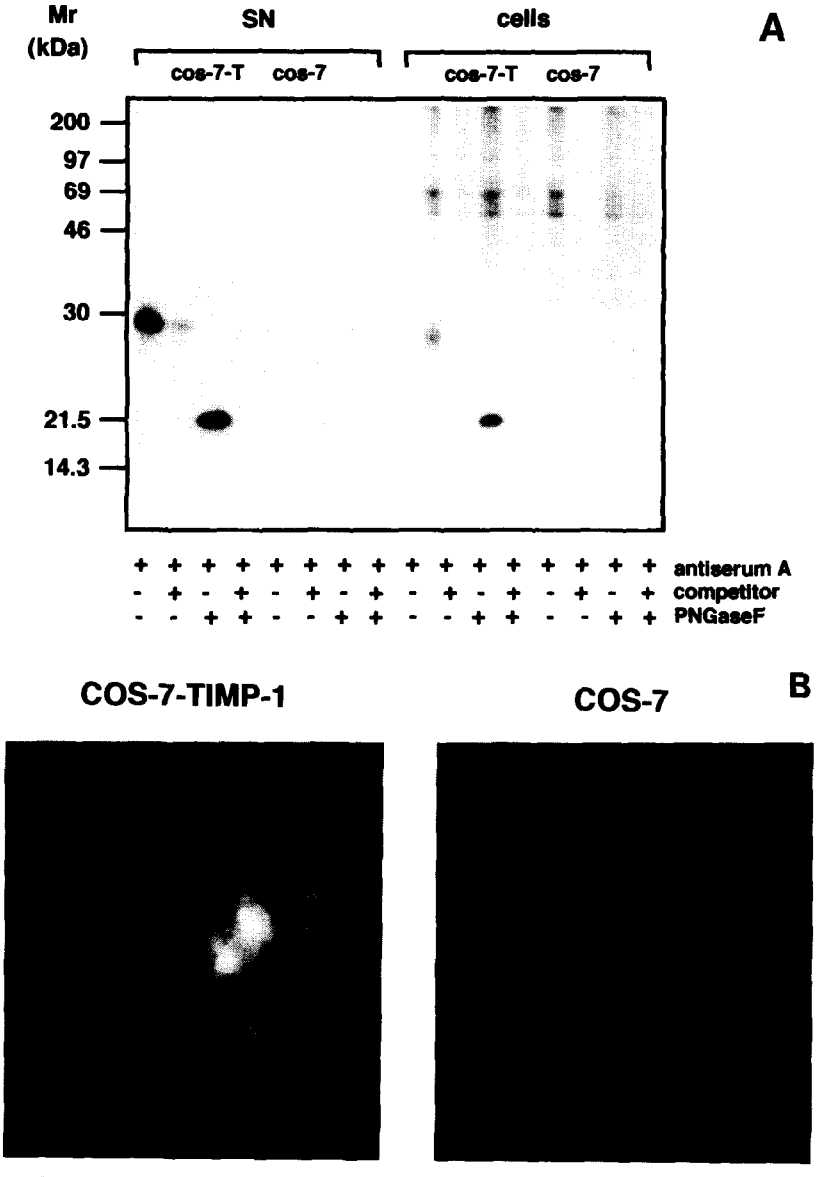

Fig. 2. Detection of murine TIMP-1 protein. (A) Immunoprecipitation of murine TIMP-1 from supernatants and cell lysates of transfected and non-transfected COS-7 cells with the polyclonal antiserum A. COS-7 (2×10^6) and COS-7-murine-TIMP-1 (2×10^6) cells were labeled with 80 μCi Tran[^{35}S]label in methionine/cysteine-free medium for 3 h. After 2 h of chasing supernatants (SN; left side) and cell lysates (Cells; right side) were immunoprecipitated with murine-TIMP-1-antiserum A and analyzed by SDS-PAGE and fluorography. 100 ng of murine TIMP-1 expressed in *E. coli* was added as competitor before immunoprecipitation as indicated in the figure. Immunoprecipitated proteins were deglycosylated with PNGase F (2U/100 μl) as indicated. (B) Immunofluorescence of transfected COS-7 cells transfected with murine TIMP-1 and untransfected COS-7 cells. Cells were stained with the polyclonal antiserum against murine TIMP-1 and a rhodamine-conjugated antirabbit IgG antibody. Magnification: × 200.

E. coli BL21 were transformed with this expression vector, induced with IPTG and proteins solubilized from inclusion bodies were separated by SDS-PAGE and visualized by silver staining (Fig. 1B). After induction with IPTG a protein of about 20–22 kDa is expressed (lane 3 and 4). In the absence of IPTG no protein of the corresponding size could be detected (lane 2). The recombinant protein was injected into rabbits and an antiserum was obtained. Using this antiserum against murine TIMP-1 (see section 2) the inclusion bodies could be visualized by Western blot analysis (Fig. 1C).

Human TIMP-1 protein from IL-6 stimulated human hepatoma cells (HepG2) [18] was not recognized by the antiserum A raised against the recombinant murine TIMP-1 protein, thus demonstrating that the murine TIMP-1 antiserum does not cross-react with human TIMP-1 protein (data not shown).

To investigate the biosynthesis and glycosylation of native TIMP-1 protein immunoprecipitations of simian kidney cells (COS-7) transiently transfected with an expression vector coding for murine TIMP-1 were carried out. Using the polyclonal antiserum A against murine TIMP-1 a 28–30 kDa protein was immunoprecipitated from supernatants and cell lysates of transfected COS-7 cells metabollically labeled with [^{35}S]cysteine/methionine (Fig. 2A). To show that the immunoprecipitation of murine TIMP-1 protein was specific, we performed competition experiments with non-radioactive labeled recombinant TIMP-1 protein. Immunoprecipitation of TIMP-1 could be prevented by the addition of murine TIMP-1 expressed in *E. coli* as competitor (Fig. 2A).

In order to explain the discrepancy in apparent molecular weight of the TIMP-1 expressed in bacterial and in mammalian cells we analyzed glycosylation of the murine TIMP-1 protein. TIMP-1 from metabollically labeled transfected COS-7 cells was immunoprecipitated from cell lysates and media and treated with PNGase F as indicated in Fig. 2A. Upon treatment with PNGase F the mobility of the TIMP-1 protein shifted from 28–30 kDa to 20–22 kDa indicating that N-glycosylation is the predominant post-translational modification of TIMP-1 expressed in COS-7 cells. It is not clear so far which of the two N-glycosylation sites are used in the protein [27]. Interestingly TIMP-1 could also be detected in cell lysates even after a chase time of 2 h (Fig. 2A, right side). We conclude that the mature form of TIMP-1 is either not secreted or the secreted protein is partially deposited in the ECM of cells. We therefore chose to analyse the cellular distribution of murine TIMP-1 by indirect immunofluorescence staining using the polyclonal antiserum A. In studies with transfected COS-7 cells murine TIMP-1 protein is detected on the surface of non-permeabilized cells (Fig. 2B). Untransfected control cells showed no specific cell-associated TIMP-1 staining. It is therefore possible that newly synthesized TIMP-1 protein associates with matrix proteinases.

In order to observe the regulation of TIMP-1 in a more physiological system and since we already now that TIMP-1 mRNA is up-regulated in liver cells under acute phase conditions [17,18] we tried to immunoprecipitate TIMP-1 from stimulated rat hepatocytes. Rat hepatocytes in primary culture were incubated 12 h with recombinant human IL-1β, IL-6, or conditioned medium from human monocytes stimulated with LPS as indicated in

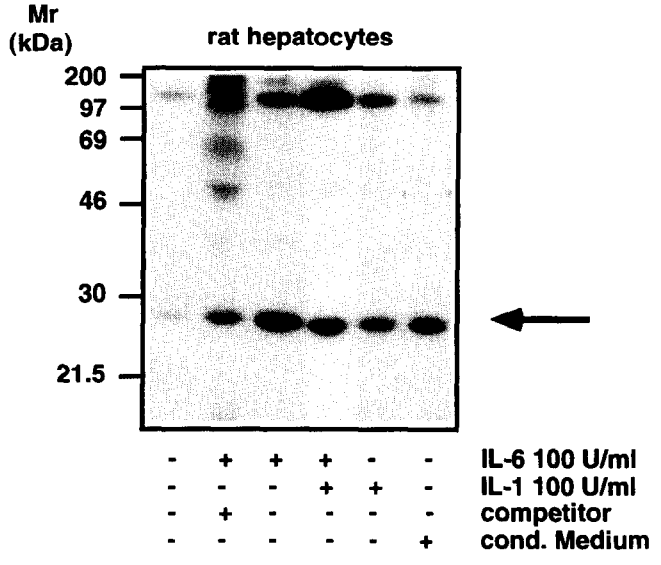

Fig. 3. Immunoprecipitation of TIMP-1 from supernatants of rat hepatocytes in primary cultures. Rat hepatocyte primary cultures (4×10^6 cells per dish) were incubated in the presence of IL-1β, IL-6 and 1 ml of conditioned medium from LPS-stimulated human monocytes (1 μg/ml for 18 h) for 12 h before labeling with 200 μCi Tran[^{35}S]label in methionine/cysteine-free medium for 3 h. After 2 h of chasing supernatants were immunoprecipitated with murine-TIMP-1-antiserum A and analyzed by SDS-PAGE and fluorography. 100 ng of murine TIMP-1 expressed in *E. coli* was added as competitor before immunoprecipitation as indicated in the figure. The arrow indicates the position of TIMP-1.

Fig. 3. The newly synthesized and secreted proteins were analyzed by SDS-polyacrylamide gel electrophoresis after immunoprecipitation with the antiserum A against murine TIMP-1. As we could already show on mRNA level [17] TIMP-1 protein is up-regulated after stimulation of liver cells with inflammatory cytokines. After addition of recombinant murine TIMP-1 as competitor (Fig. 3, lane 2) immunoprecipitation of rat TIMP-1 could partly be prevented. Besides TIMP-1 a protein of 100–150 kDa is detected by the antiserum A. These proteins might be matrix metalloproteinases tightly associated with newly synthesized TIMP-1. Taken together with the finding that TIMP-1 is elevated in serum of patients with chronic liver diseases [27] TIMP-1 could possibly be involved in the pathogenesis of liver fibrosis.

TIMP-1 has been identified in the culture medium of many connective tissue cells, explants, and in synovial fluids [28]. Its occurence has been implicated with tissue sclerosis and fibrosis [1,2,27]. So far, for the mouse or rat system no antisera have been described to detect the protein. With the antiserum described in this study it will be possible to detect local changes in TIMP-1 expression in murine and rat models for tumor metastasis, fibrosis or pathological tissue breakdown like arthritis. Progress in this area should help to elucidate the role of TIMP-1 in the pathogenesis of these diseases and should indicate a route for rational strategies for the design of therapeutic compounds.

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