

Tissue inhibitor of metalloproteinases-2 (TIMP-2) in rat liver cells is increased by lipopolysaccharide and prostaglandin E₂

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Abstract To explore the functional role of TIMP-2 in liver, we determined TIMP-2 mRNA levels in primary rat hepatocytes and in total rat liver. Rat hepatocytes constitutively express TIMP-2 mRNA at a low level. Incubation with dexamethasone, prostaglandin E₂ and a combination of inflammatory cytokines leads to an up-regulation of TIMP-2 mRNA. In rats in vivo we found a dramatic increase of TIMP-2 expression after intraperitoneal injection of lipopolysaccharide. Compared to our previous findings on TIMP-1 we conclude that TIMP-2 mRNA expression is regulated in a distinct and partially opposite manner. Over-production of TIMP-2 could inhibit the activity of metalloproteinases and thus lead to matrix accumulation. Dysregulation of TIMP-2 synthesis might be involved in the development of liver fibrosis.

Key words: Inflammatory mediator; Tissue inhibitor of metalloproteinase; Extracellular matrix; Fibrosis; Liver; Rat hepatocyte

1. Introduction

Tissue inhibitor of metalloproteinases-2 (TIMP-2) is a 21 kDa protein which forms a non-covalent complex with a 1:1 stoichiometry with the latent and active matrix metalloproteinase type IV collagenase and the 92 kDa gelatinase [1–4]. The N-terminal amino acid sequence of the protein is more than 60% identical to a consensus sequence of mammalian TIMP-1 [5]. TIMP-2 shares several properties with TIMP-1, including conservation of the positions of the 12 cysteine residues and resistance to cleavage by trypsin.

TIMP-2 is secreted by melanoma cells [1], fibrosarcoma cells [6], lung fibroblasts [7], human alveolar macrophages [8], human osteoblast-like cells [9], human vein and microvascular endothelial cells [10] and found in human plasma [11] as well as in the majority of 26 human tumor cell lines [12]. TIMP-2 was purified from human rheumatoid synovial fluid and serum-free medium of a human hepatoma cell line HLE [2,13]. Interestingly TIMP-2 levels in the sera of patients with rheumatoid arthritis and hepatocellular carcinomas were significantly higher than those of normal subjects [14]. Northern blot analysis of RNA from human melanoma or fibrosarcoma cell lines,

normal and tumor tissue samples demonstrated two distinct transcripts of 1.0 and 3.5 kb for TIMP-2 mRNA. Both transcripts are down-regulated in response to transforming growth factor- β and remain unchanged in response to PMA [6]. In most other human cell lines, TIMP-2 expression was refractory to IL-1, PMA and TNF- α treatment [15]. In human alveolar macrophages treatment with LPS, denatured type I collagen and zymosan resulted in a dose-dependent and profound decrease of TIMP-2 production [8]. In rabbit uterine cervical fibroblasts, progesterone increased the production of TIMP-2 [16]. In contrast the expression of TIMP-1 mRNA is induced by serum, PMA, fibroblast growth factor and platelet-derived growth factor [17–19]. Oncostatin M, interleukin-6 (IL-6), leukemia inhibitory factor, and IL-1 α elevate TIMP-1 expression at the mRNA level in human lung fibroblasts [20]. Thus, TIMP-1 and TIMP-2 transcription is differentially regulated in vivo and in vitro in cell culture, indicating a different physiologic role for the two TIMPs [21,22].

In a previous report we have demonstrated that TIMP-1 is up-regulated by inflammatory cytokines in rat hepatocyte primary cultures and we discussed a possible involvement of TIMP-1 in the pathogenesis of liver fibrosis [23,24]. Here we show for the first time that TIMP-2 expression is up-regulated by cytokines and prostaglandin E₂ in primary rat hepatocytes and by LPS in rat liver. Furthermore we demonstrate that TIMP-1 and TIMP-2 are differently regulated genes in liver cells.

2. Materials and methods

2.1. Materials

The random primed DNA labeling kit was purchased from Boehringer-Mannheim (Mannheim, Germany). [α -³²P]dATP (110 TBq/mmol) was obtained from Amersham International (Amersham, UK). M199 and FCS were from Gibco (Eggenstein, Germany); *Escherichia coli* LPS, PGE₂ were from Sigma (Munich, Germany). Recombinant human (rh) IL-1 β with a specific activity of 2×10^7 units/mg protein was a generous gift of Dr. A.R. Shaw (Glaxo Institute for Molecular Biology, Geneva, Switzerland). rh IL-6 with a specific activity of 1.5×10^6 B-cell stimulatory factor 2 units/mg protein was generously supplied by Drs. T. Hirano and T. Kishimoto (Osaka, Japan). rh IL-11 with a specific activity of 2.5×10^6 units/mg protein in a T10 cell bioassay was obtained from Genetics Institute (Boston, MA). The cloning of murine TIMP-1 [25] and TIMP-2 [21] has recently been described.

2.2. Preparation of hepatocyte primary cultures

After perfusion of rat livers with collagenase, rat hepatocytes were prepared as described in [23,26]. Hepatocytes were cultured serum-free for 48 h prior to the experiments.

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Abbreviations: TIMP, tissue inhibitor of metalloproteinases; IL, interleukin; LPS, lipopolysaccharide; PGE₂, prostaglandin E₂; PMA, 4 β -phorbol-12-myristate-13-acetate.

2.3. Animal experiments

Male Sprague–Dawley rats weighing 220–250 g were injected intraperitoneally with 2.3 mg of LPS. 0, 2 and 24 h after injection, rats were sacrificed, the livers were excised, immediately frozen in liquid nitrogen and stored at -80°C for subsequent analysis. For Northern blotting at each time point tissues were analyzed individually. Animal studies were performed in accordance with required standards of the local animal welfare committee.

2.4. RNA isolation and Northern analysis

Total RNA was isolated from liver tissues and from rat hepatocytes in primary culture as described in [27]. 5 μg of RNA were heated to 65°C for 10 min in 50% formamide, 20 mmol/l 3-[N-morpholino]propane sulfonic acid (MOPS), 5 mmol/l sodium acetate, 1 mmol/l EDTA and 2.2 mol/l formaldehyde before gel-electrophoresis in 1% agarose containing 2.2 mol/l formaldehyde, 20 mmol/l MOPS, 5 mmol/l sodium acetate and 1 mmol/l EDTA. Equal loading of the RNA gel was checked by ethidium bromide staining of 18 S and 28 S ribosomal RNA. The separated RNA was transferred to GeneScreen Plus membranes (DuPont-De-Nemours, Dreieich, Germany) according to the supplier's instructions. The filters were prehybridized at 68°C for 2 h in 10% dextran sulfate, 1 mol/l sodium chloride and 1% SDS and hybridized in the same solution with cDNA fragments labeled by random priming [28]. After hybridization, unspecifically bound radioactivity was removed by washing in $2 \times$ standard saline citrate solution (SSC) at room temperature, followed by two consecutive washes in $2 \times$ SSC, 1% SDS at 68°C for 30 min each. The filters were then subjected to autoradiography with intensifying screens at -80°C . Autoradiograms from at least three separate experiments were analyzed.

3. Results

Total RNA isolated from the murine cell lines B78 and NIH 3T3, total rat liver, primary rat hepatocytes and the rat hepatoma cell line H35 was analyzed for TIMP-2 mRNA expression (Fig. 1). In comparison to a high expression in the murine melanoma cells B78, rat liver cells displayed low TIMP-2 mRNA levels. When rat hepatocytes were incubated with conditioned medium from LPS-stimulated human monocytes, known to contain many different inflammatory mediators, a dramatic increase of TIMP-2 mRNA was observed (Fig. 2). Among different agents tested, dexamethasone, prostaglandin E_2 (in low concentrations) and also conditioned medium from unstimulated human monocytes enhanced the expression of TIMP-2 mRNA, whereas fetal calf serum had no effect. When rat hepatocytes were stimulated with conditioned medium of LPS-treated human monocytes, the lower TIMP-2 transcript showed a slightly higher mobility (Fig. 2). Inflammatory cytokines alone had no marked effect on the expression of TIMP-2 mRNA, but the combinations of IL-1 β + IL-6 or IL-1 β + IL-6 + IL-11 led to a synergistic up-regulation of TIMP-2 mRNA expression (Fig. 3). Surprisingly only the 1.0 kb TIMP-2 transcript increased upon incubation with the combination of cytokines.

To analyze the expression of TIMP-2 *in vivo* we injected rats intraperitoneally with LPS. 2 and 24 h after LPS administration total hepatic RNA was prepared and subjected to Northern blot analysis. Fig. 4A illustrates the induction of TIMP-2 mRNA expression by LPS in comparison to control animals injected with NaCl. Similar to our observation in cell culture when rat hepatocytes were incubated with conditioned medium of LPS-stimulated human monocytes, we found an additional smaller transcript of TIMP-2 after 24 h. Interestingly, we also observed a TIMP-1 transcript of higher mobility after LPS treatment *in vivo* (Fig. 4B).

4. Discussion

The present study shows for the first time that rat hepatocytes in primary culture and total rat liver *in vivo* express TIMP-2 mRNA. Furthermore, we could demonstrate an up-regulation of TIMP-2 mRNA by conditioned medium of LPS-stimulated human monocytes, by a combination of inflammatory cytokines, by prostaglandin E_2 or by dexamethasone. In primary rat hepatocytes TIMP-2 expression is unaffected by fetal calf serum (Fig. 2), ciliary neurotrophic factor and leukemia inhibitory factor (data not shown) or by a stimulation with IL-1 β , IL-6 or IL-11 alone (Fig. 3).

In a previous report we demonstrated that rat hepatocytes in primary culture constitutively express TIMP-1 mRNA at a low level. Incubation with conditioned medium from LPS-stimulated human monocytes led to a dramatic increase of TIMP-1 mRNA. The inflammatory cytokines IL-1 β , IL-6, IL-11 and CNTF were also capable of stimulating TIMP-1 mRNA in a dose- and time-dependent manner. Among these cytokines IL-6 was the most potent stimulator. A combination of IL-1 β , IL-6 and IL-11 synergistically increased mRNA levels of TIMP-1. On the other hand the synthetic glucocorticoid dexamethasone inhibited basal and IL-6-induced expression of TIMP-1 dose-dependently.

The regulation of TIMP-2 expression in rat hepatocytes by dexamethasone and inflammatory cytokines, however, differs from that of TIMP-1. Like others [22] we propose different (referring to the dexamethasone effect and the effect of single inflammatory cytokines) but also similar (referring to the effect of conditioned medium and a combination of cytokines) mechanisms regulating TIMP-1 and TIMP-2 expression, indicating both different and also partly similar physiological roles for the two TIMPs. The closely related regulation of TIMP-1 and TIMP-2 might be of importance for the fine tuning of the homeostasis of the extracellular matrix.

The balance between activated metalloproteinases and available inhibitors is thought to be a critical determinant of matrix protein turnover associated with a variety of pathologic processes, including tumor cell invasion and fibrosis [29–32]. TIMP-2 is considered as a potential tumor terminator [33]. Addition of endogenous TIMP-2 protein blocks tumor invasion of human fibrosarcoma HT-1080 cells [34]. Transfection of invasive metastatic rat cells with TIMP-2 resulted in reduced local invasion and partially suppressed hematogenous metastasis

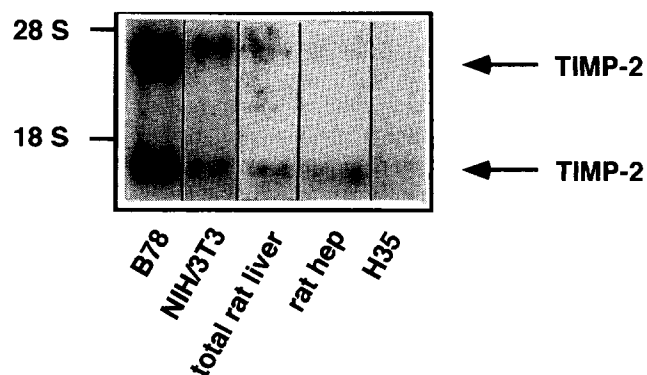


Fig. 1. Expression of TIMP-2 in different murine and rat cells. Total RNA was isolated and 5 μg were used for Northern blot analysis of TIMP-2 mRNA.

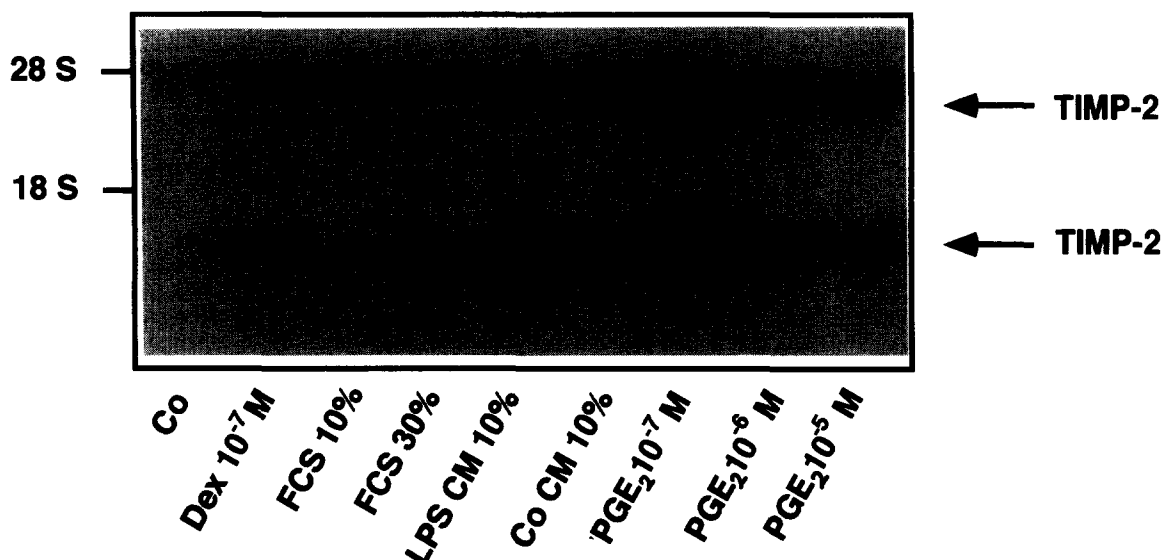


Fig. 2. Effect of dexamethasone, fetal calf serum, prostaglandin E_2 and conditioned medium from LPS-stimulated human monocytes on TIMP-2 levels in rat hepatocyte primary cultures. Rat hepatocytes (4×10^6 cells per dish) were incubated in the presence of 10^{-7} mol/l dexamethasone, 10% and 30% fetal calf serum (FCS), 1 ml of conditioned medium (CM) from LPS-stimulated human monocytes ($1 \mu\text{g/ml}$ for 18 h) and prostaglandin E_2 (PGE_2) in different concentrations (10^{-7} , 10^{-6} and 10^{-5} mol/l). Total RNA was isolated and $5 \mu\text{g}$ were used for Northern blot analysis of TIMP-2 mRNA. When the TIMP-2 cDNA probe was used an unspecific cross hybridization to 28 S ribosomal RNA was observed, which served as an internal standard.

[35]. Independent of its proteinase inhibitory activity TIMP-2 is able to inhibit cultured endothelial cell proliferation, which suggests additional actions limiting neovascularization associated with solid tumor growth and metastasis [36]. Expression of TIMP-1 and TIMP-2 correlates with the development of glomerulosclerosis. Both TIMP genes are expressed in normal glomeruli, and their expression is increased in glomerulosclerosis associated with renal carcinoma [29]. Furthermore, endogenous TIMPs play a central role in regulating both physiological and pathological bone resorption [37].

Fibrosis is characterized by an accumulation of extracellular proteins, which might be the result of an increase in their synthesis or a decrease in degradation or a combination of both. Our results provide evidence that in hepatocytes, besides TIMP-1, TIMP-2 may also be involved in the regulation of extracellular matrix protein metabolism. A high TIMP-2 activity would reduce metalloproteinase activity and thus result in matrix accumulation by an impairment of matrix degradation. Our data also suggest that TIMP-2, like TIMP-1 [23], might be involved in liver fibrosis. Additional support for this conclusion

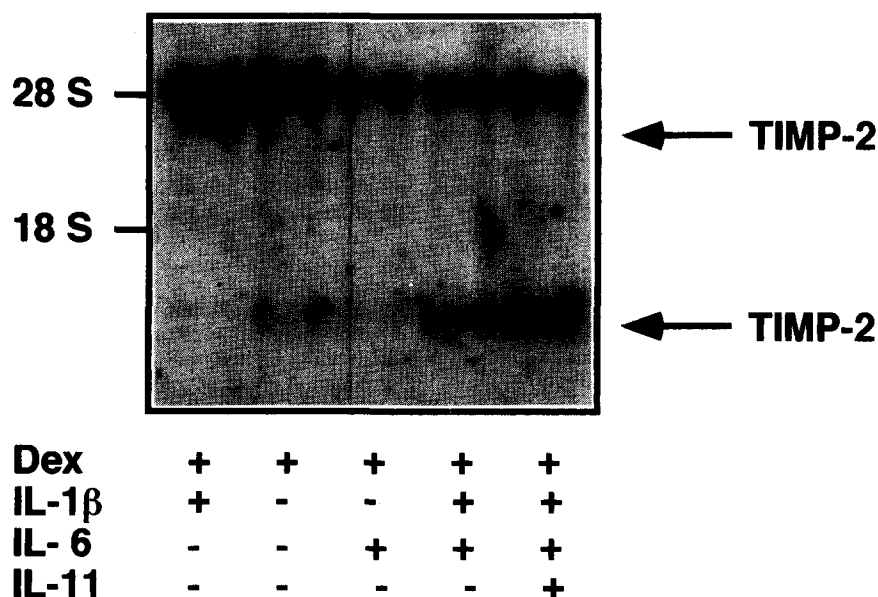


Fig. 3. TIMP-2 mRNA stimulation by a combination of cytokines. Rat hepatocytes in primary culture were incubated for 8 h in the presence of 10^{-7} mol/l dexamethasone and combinations of IL-6 (100 U/ml), IL-1 β (100 U/ml) and IL-11 (100 U/ml) as indicated in the figure. Total RNA was extracted and $5 \mu\text{g}$ were analyzed by Northern blotting. When the TIMP-2 cDNA probe was used, an unspecific cross-hybridization to 28 S ribosomal RNA was observed, which served as an internal standard.

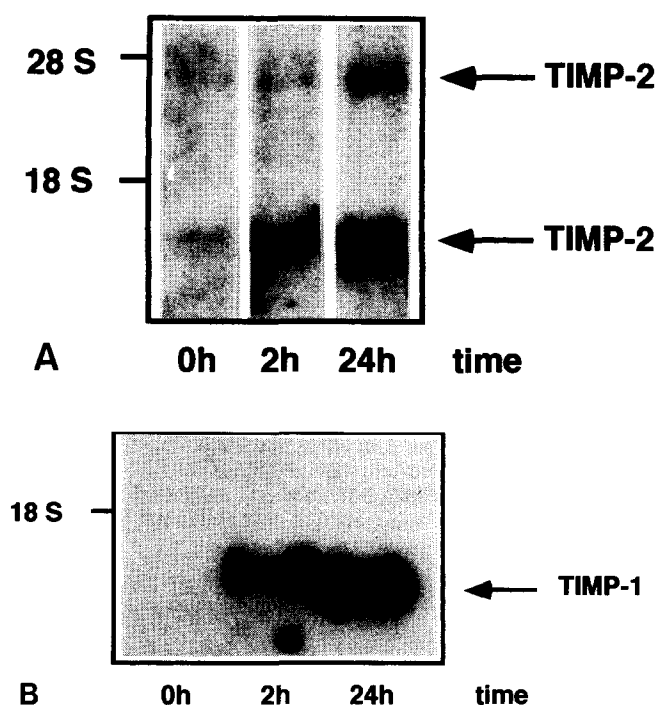


Fig. 4. Expression of TIMP-2 in vivo. Rats were injected intraperitoneally with LPS. 2 and 24 h after LPS administration, RNA from total rat liver was analyzed by Northern blotting. (A) Hybridization with a murine TIMP-2 cDNA. (B) Hybridization with a murine TIMP-1 probe.

is derived from some recent clinical studies. TIMP-2 levels in sera of patients with hepatocellular carcinoma were significantly higher than those of normal subjects [14]. The level of TIMP-2 expression was increased in glomerulosclerosis associated with renal carcinoma [29]. Serum TIMP-1 levels were closely correlated with the histological degree of liver fibrosis in chronic liver diseases, such as chronic persistent and chronic active hepatitis, liver cirrhosis, primary biliary cirrhosis and hepatocellular carcinoma [38]. Further studies with experimental models of liver fibrosis and biopsy samples from patients with chronic liver disease are required to establish a functional relationship between the observed changes in TIMP expression and hepatic fibrosis. To define the role of the different TIMP proteins in liver fibrosis, studies with transgenic or knock-out animals for TIMP-1/TIMP-2 have to be performed.

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