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Synthesis of tissue inhibitor of metalloproteinase-1 (TIMP-1) in human hepatoma cells (HepG2)

Up-regulation by interleukin-6 and transforming growth factor β1

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Metalloproteinases and their specific inhibitors, believed to play a role in extracellular matrix metabolism, are regulated by inflammatory cytokines. Here we have addressed the question of whether liver, the major site of synthesis of plasma proteinase inhibitors, is also capable of synthesizing the tissue inhibitor of metalloproteinase-1 (TIMP-1). We show at mRNA and protein levels that TIMP-1 is expressed in differentiated human hepatoma cells (HepG2) and that its synthesis is up-regulated by interleukin-6 (IL-6), transforming growth factor β1 and phorbol 12-myristate 13-acetate. The physiological role of this phenomenon is underlined by the fact that lipopolysaccharide administration into rats in vivo, as well as IL-6-stimulation of rat hepatocytes in primary culture, also leads to an increase of TIMP-1 mRNA in liver cells.

Tissue inhibitor of metalloproteinase-1 (TIMP-1); Metalloproteinase; Interleukin-6 (IL-6); Transforming growth factor β1 (TGFβ1); Hepatoma HepG2 cell; Hepatocyte

1. INTRODUCTION

Recently much attention has been paid to proteinases and their inhibitors involved in tissue remodelling. Particularly, several metalloproteinases have been shown to play a role in the degradation and turnover of the extracellular matrix composed of collagens, proteoglycans, and glycoproteins like fibronectin and laminin. The metalloproteinases are synthesized and secreted as inactive precursors and their activity in the extracellular milieu is regulated by inhibitors. The major inhibitors of metalloproteinases are α_2-macroglobulin and tissue inhibitors of metalloproteinases (TIMP-1 and TIMP-2) (reviewed in [1–4]). TIMP-1 is a glycoprotein with a molecular mass of about 29 kDa mainly produced by cells of mesodermal origin [3].

Thus far, TIMP-1 synthesis has been described as being regulated by growth factors and cytokines such as epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), transforming growth factor-β1 (TGFβ1), interleukin-1 (IL-1) and tumor necrosis factor-α TNFα [5–9]. Recently, IL-6 has been shown to stimulate TIMP-1 expression in human chondrocytes, fibroblasts and synoviocytes [10,11]. It has also been reported that TIMP-1 is present in serum of normal individuals [12,13].

Since liver is the major site of synthesis of plasma proteinase inhibitors, we asked whether hepatocytes also synthesize TIMP-1 and whether this expression is regulated by cytokines. As a model system for human hepatocytes, we used the human hepatoma cell line, HepG2, which has been shown to be capable of synthesizing and secreting essentially the whole spectrum of human acute-phase proteins [14]. Here, we describe for the first time that HepG2 cells synthesize and secrete TIMP-1. We also present data on the up-regulation of TIMP-1 by IL-6, TGFβ1 and phorbol 12 myristate 13-acetate (PMA). Furthermore, we show that TIMP-1 mRNA increases in livers of lipopolysaccharide (LPS)-treated rats, as well as in rat hepatocytes in primary culture after stimulation with IL-6.

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Abbreviations: α_1ACT, α_1-antichymotrypsin; bFGF, basic fibroblast growth factor; BSF, B-cell stimulatory factor; DMEM, Dulbecco's minimal essential medium; EGF, epidermal growth factor; FCS, fetal calf serum; IL, interleukin; LPS, lipopolysaccharide, PMA, phorbol 12-myristate 13-acetate; TIMP, tissue inhibitor of metalloproteinases; TGF, transforming growth factor; TNF, tumor necrosis factor.

2. MATERIALS AND METHODS

2.1 *Chemicals*

Restriction enzymes and random primed DNA labelling kit were

purchased from Boehringer-Mannheim (Mannheim, Germany) [α-^{32}P]dATP (110 TBq/mmol) was obtained from Amersham International (Amersham, UK). DMEM and DMEM/F12 was from Gibco (Eggenstein, Germany) *Escherichia coli* LPS was from Sigma (Munich, Germany) Human TGFβ1 was purchased from Biermann (Bad Nauheim, Germany). Plasmid pTrPCI9 containing human TIMP-1 cDNA was kindly provided by Dr. M Naruto (Toray Industries Inc., Japan). A 1 kb *Eco*RI fragment was used for labeling. Plasmid pBSmTIMP-1 containing mouse TIMP-1 cDNA was a gift from Dr D.R. Edwards (University of Calgary, Canada) For hybridization a 825 bp *Hin*dIII–*Bam*HI fragment was used. The plasmid pACT containing human α_1-antichymotrypsin (α_1ACT) cDNA was a gift from Dr E Berger (University of Zurich, Switzerland). For hybridization a 1.5 kb *Pst*I fragment was used. Recombinant human IL-6 (rhIL-6) was prepared by J Müllberg using a T7 cDNA expression system.

2.2. *Animals and cell culture*

Male Sprague–Dawley rats of 200–300 g body weight were injected intraperitoneally with 10 mg LPS per kg body weight After the times indicated the animals were killed by asphyxiation and the livers were removed immediately.

HepG2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured in DMEM/DMEM-F12 medium supplemented with 10% FCS, streptomycin (100 mg/l) and penicillin (61 mg/l)

Primary rat hepatocytes were prepared by perfusion of the liver as described [15]. Hepatocytes were cultured in M 199 medium supplemented with 4% FCS, streptomycin (100 mg/l), penicillin (61 mg/l), dexamethasone (10^{-7} M) and insulin ($5\ 10^{-10}$).

2.3 *RNA preparation and Northern blot analysis*

Total RNA was prepared using the phenol extraction method as described in [16,17] 5 μg of RNA were heated to 65°C for 10 min in 50% formamide, 20 mM morpholinopropane sulphonic acid (MOPS), 5 mM sodium acetate, 1 mM EDTA, 2.2 M formaldehyde prior to gel electrophoresis in 1% agarose containing 2.2 M formaldehyde, 20 mM MOPS, 5 mM sodium acetate and 1 mM 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–DeNemours, Dreieich, Germany) according to the supplier's instructions. The filters were prehybridized at 68°C for 3 h in 10% dextran sulfate, 1 M sodium chloride, 1% SDS, and hybridized in the same solution with cDNA fragments labelled by random priming [18]. After hybridization, non-specifically bound radioactivity was removed by washing in 2× standard saline solution (SSC) at room temperature, followed by two subsequent washes in 2 × SSC/1% SDS at 68°C for 30 min each. The filters were then subjected to autoradiography using intensifying screens.

2.4. *Metabolic labelling of HepG2 cells and immunoprecipitation*

HepG2 cells were grown to confluency on 35-mm plastic dishes and stimulated for 18 h with IL-6 (100 U/ml), TGFβ1 (5 ng/ml) or PMA (10^{-7} M) Cells were washed with phosphate-buffered saline and incubated for 3 h with 150 μl [^{35}S]methionine/cysteine (Tran^{35}S-label, ICN, Meckenheim, Germany) in 500 μl of methionine/cysteine-free DMEM containing 0.2% bovine serum albumin and 10 mM Hepes, pH 7.3. After addition of 10 × the normal concentration of methionine/cysteine, cells were further incubated for 2 h at 37°C.

Medium was removed and centrifuged for 5 min in a microfuge The supernatant was adjusted to 1% Triton X-100 and 0.1% SDS and preincubated with Pansorbin (Calbiochem, Frankfurt, Germany) for 1 h at 4°C. After centrifugation for 5 min, the supernatant was removed and immunoprecipitated with a polyclonal rabbit antiserum raised against human TIMP-1 followed by protein A–Sepharose (Pharmacia, Freiburg, Germany) absorption. Immunoprecipitates were eluted by boiling in SDS-PAGE sample buffer and subjected to 12% SDS-PAGE according to Laemmli [19]. Gels were fixed and impregnated with 1 M sodium salicylate [20] and exposed to a Cronex-4 (DuPont-DeNemours, Dreieich) film at −80°C.

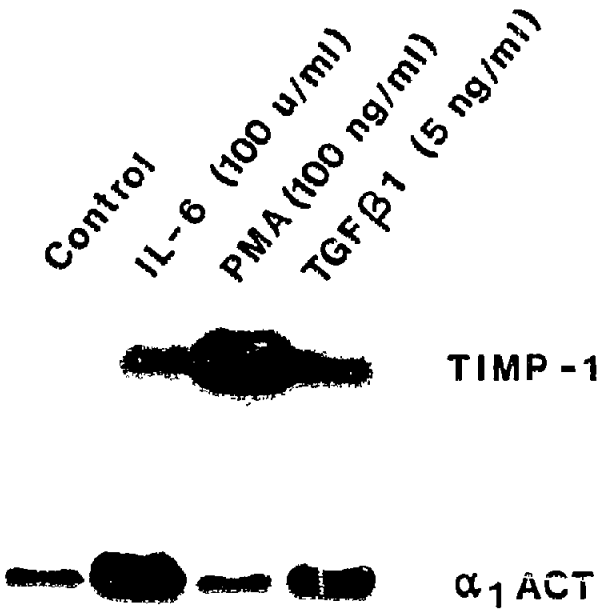

Fig. 1. Effect of IL-6, PMA and TGFβ1 on mRNA levels of TIMP-1 and α1-ACT. Confluent HepG2 cells in medium containing 10% FCS were stimulated with 100 BSF-2 U of IL-6 per ml, 100 ng of PMA per ml and 4 ng of TGFβ1 per ml medium for 18 h. Total RNA was isolated and 5 μg were used for Northern blot analysis of TIMP-1 and α_1ACT-mRNA.

3. RESULTS

HepG2 cells were incubated with rhIL-6 and the known stimulators of TIMP-1 synthesis, TGFβ1 and PMA. mRNA levels for TIMP-1 and α_1ACT were determined by Northern blot analysis. Fig. 1 (upper panel) shows that untreated HepG2 cells constitutively express TIMP-1 mRNA. Upon incubation with IL-6 or TGFβ1, TIMP-1 mRNA levels increased 2–3-fold. A dramatic stimulation of TIMP-1 mRNA was observed after treatment of HepG2 cells with PMA. For comparison we have included the Northern blot for the well-known human acute-phase protein, α_1ACT (Fig. 1, lower panel). As expected, α_1ACT mRNA levels increased >5-fold and 2–3-fold upon treatment with IL-6 or TGFβ1, respectively. In contrast to TIMP-1, concentrations of α_1ACT mRNA were slightly reduced after PMA. The dose- and time-dependence of TIMP-1 mRNA induction by IL-6 is shown in Fig. 2. A slight induction was already observed at an IL-6 concentration of 10 U/ml, maximal TIMP-1 mRNA levels were achieved at 50 U/ml of IL-6 (Fig. 2A). TIMP-1 mRNA was fully induced 4 h after incubation with IL-6 (Fig. 2B).

In order to demonstrate TIMP-1 synthesis and regulation by IL-6, TGFβ1 and PMA also at the protein level, we have metabolically labelled HepG2 cells with [^{35}S]methionine/[^{35}S]cysteine for 3 h and immunoprecipitated the newly synthesized and secreted TIMP-1 from

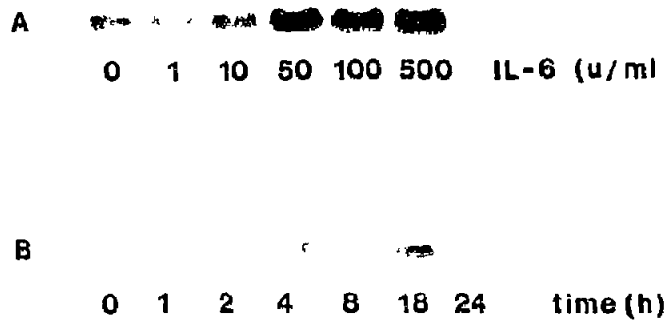

Fig. 2 Dose- and time-dependence of TIMP-1 mRNA induction by IL-6. Confluent HepG2 cells in medium containing 10% FCS were stimulated with increasing amounts of IL-6 (A) or with 100 BSF-2 U of human recombinant IL-6 per ml for the times indicated in B. As described in the legend to Fig. 1, total RNA was isolated and used for Northern blot analysis.

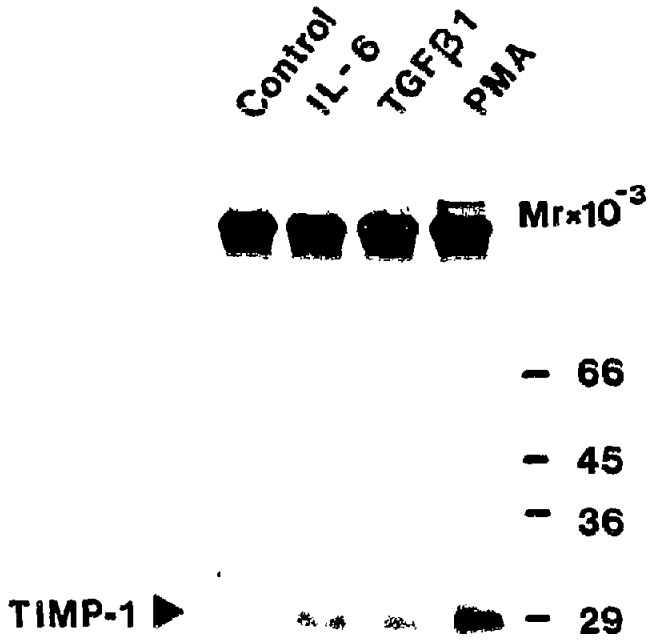

Fig. 3. Stimulation of TIMP-1 synthesis by IL-6, TGFβ1 and PMA. Confluent HepG2 cells in medium containing 10% FCS were stimulated with 100 BSF-2 U of human IL-6 per ml, with 2 ng of human TGFβ1 per ml, or 100 ng of PMA per ml of medium for 18 h. Subsequently, cells were labelled with [^{35}S]methionine/[^{35}S]cysteine as described in section 2. Radioactively labelled TIMP-1 was immunoprecipitated from the supernatants with a polyclonal antiserum to human TIMP-1. The immunoprecipitated TIMP-1/antibody complexes were subjected to SDS-PAGE and fluororadiography.

the media. Fig. 3 shows that TIMP-1 protein is constitutively produced by HepG2 cells and up-regulated by IL-6, TGFβ1 and PMA. Specificity of the immunoprecipitated protein at a mobility corresponding to an apparent molecular mass of 29 kDa was confirmed by competition studies with purified unlabelled TIMP-1 (not shown). The band on top of the gel represents high molecular weight material (non-specifically bound to the antibody upon immunoprecipitation since it could not be competed out with an excess of unlabelled TIMP-1. We also examined TIMP-1 expression in rat liver in vivo and in rat hepatocytes in vitro. LPS was injected intraperitoneally into male rats. Such an experimental approach has been widely used to mimic inflammatory conditions because it leads to the secretion of inflammatory cytokines such as IL-1, TNFα and IL-6 by monocytes [21]. Total RNA was isolated from livers after 24 h and subjected to Northern blot analysis. It is evident from Fig. 4A that LPS administration strongly induced TIMP-1 mRNA expression in rat liver. An induction of TIMP-1 mRNA was also found in rat hepatocytes in primary culture after stimulation with IL-6 (Fig. 4B).

4. DISCUSSION

We have shown in the present study that TIMP-1 is constitutively synthesized and secreted by human hepatoma cells (HepG2). Our major finding is the stimulation of TIMP-1 synthesis by IL-6 in hepatic cells. IL-6 is characterized by a pleiotropic spectrum of action [22,23]. Besides its function in the immune system and during hematopoiesis, IL-6 has been shown to be a major regulator of acute-phase protein synthesis in liver cells [24–29]. Most of the acute-phase proteins which are induced by IL-6 in hepatic cells are proteinase inhibitors such as α_1-antitrypsin, α_1-ACT, α_2-macroglobulin and C1-inhibitor [21,30,31]. Except for α_2-macroglobulin [1], none of these inhibitors is capable of inhibiting metalloproteinases. Metalloproteinases released during tissue injury or inflammatory processes from monocytes, macrophages, neutrophils, fibroblasts or endothelial cells need to be controlled by specific inhibitors to prevent damage of extracellular matrix of healthy tissue [1]. TIMP-1 and TIMP-2 have been found and characterized as highly specific inhibitors of metalloproteinases [12,32]. Thus far, IL-6 stimulation of TIMP-1 expression has only been shown in fibroblasts, synoviocytes and chondrocytes [10,11]. In all three cases, the stimulation was performed under low serum (<1%) conditions. In the presence of 10% FCS, we could not induce TIMP-1 synthesis in human chondrocytes by IL-6 (Graeve et al., unpublished work). However, in HepG2 cells TIMP-1 up-regulation by IL-6 was observed in the presence of 10% FCS. The increase in TIMP-1 mRNA and secreted protein after IL-6 stimulation was about 2–3-fold. Comparable moderate stimulatory effects were observed in various other cell lines and primary cultures after induction of TIMP-1 synthesis by IL-1β, TGFβ1, bFGF, EGF, TNFα and LPS [5–9].

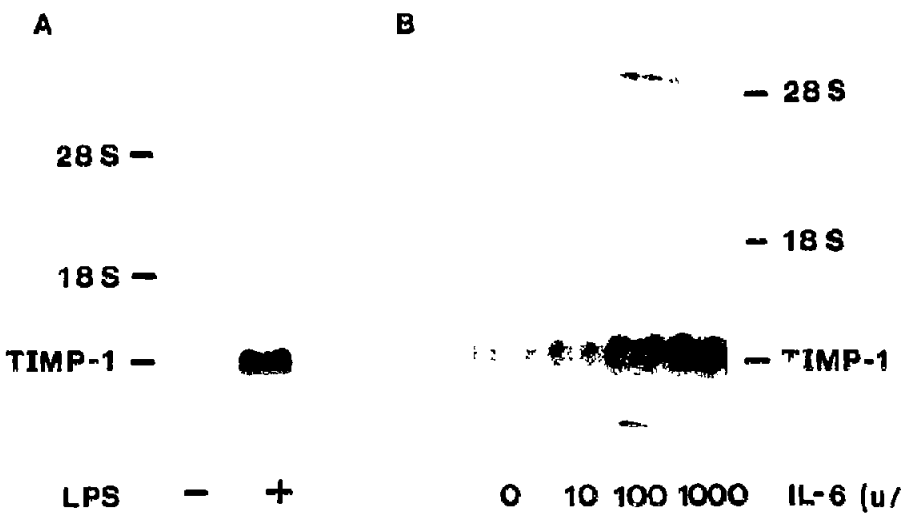

Fig. 4. Effect of LPS and IL-6 on TIMP-1 mRNA expression in rat liver and in rat hepatocytes in primary culture. (A) Rats were injected intraperitoneally with 10 mg LPS per kg body weight. After 24 h RNA was prepared from rat livers and subjected to Northern blot analysis using a cDNA probe for murine TIMP-1. (B) Primary rat hepatocytes were stimulated with different amounts of IL-6 for 18 h. Isolated RNA was analyzed by Northern blotting.

Among the various stimulators tested in HepG2 cells, PMA exhibited by far the most dramatic induction of TIMP-1 mRNA. This observation has also been made in other systems [5,8]. The TIMP-1 gene has recently been cloned and its promoter characterized [33,34]. A PMA-responsive element was identified. It should be noted that the huge increase in TIMP-1 mRNA after PMA stimulation was not followed by a comparable increase in newly synthesized TIMP-1 protein (see Fig. 3). A similar observation has been made by other investigators in human fibroblasts [7] and synovial cells [8]. Presently the nature of the physiological mediator whose action is mimicked by PMA is unknown.

In addition to the results obtained with human hepatoma cells we performed in vivo and in vitro experiments with rat hepatocytes. A strong increase in TIMP-1 mRNA concentrations was observed in rat liver after intraperitoneal LPS injection. This clearly shows that TIMP-1 expression is induced in liver under inflammatory conditions. At present we cannot rule out the possibility that besides hepatocytes endothelial cells, Kupffer and Ito cells which are minor cell populations in the liver, also contribute to this effect. Furthermore, additional mediators may play a role in TIMP-1 induction in vivo, particularly because LPS triggers the release of a whole spectrum of cytokines from monocytes/macrophages. This question is currently under investigation. However, in preliminary experiments we have found that IL-6 stimulated TIMP-1 expression in rat hepatocytes (see Fig. 4B). In conclusion we propose TIMP-1 to be an acute-phase reactant inducible by IL-6 in vitro and by LPS in vivo.

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