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Tissue Inhibitor of Metalloproteinase-1 and -2 RNA Expression in Rat and Human Liver Fibrosis

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The remodeling of extracellular matrix during chronic liver disease may partially be attributed to altered activity of matrix metalloproteinases and their tissue inhibitors (TIMPs). Expression of TIMP-1 and -2 was studied by* in situ *hybridization combined with immunohistochemistry in rat (acute and chronic carbon tetrachloride intoxication and secondary biliary fibrosis) and human livers and on isolated rat hepatic stellate cells. TIMP-1 and -2 transcripts appeared in rat livers within 1 to 3 hours after intoxication, pointing to a role in the protection against accidental activation of matrix metalloproteinases, and were present at high levels in all fibrotic rat and human livers predominantly in stellate cells. TIMP-2 RNA distribution largely matched with previously reported patterns of matrix metalloproteinase-2 (72-kd gelatinase) expression, suggesting generation of a TIMP-2/matrix metalloproteinase-2 complex (large inhibitor of metalloproteinases). Isolated stellate cells expressed TIMP-1 and -2 RNA. Addition of transforming growth factor-β1 enhanced TIMP-1 and matrix metalloproteinase-2 RNA levels* in vitro, *whereas TIMP-2-specific signals were reduced, likely to result in a stoichiometric excess of matrix-metalloproteinase-2 over TIMP-2. In the context of previous demonstrations of transforming growth factor-β1 and matrix metalloproteinase-2* in vivo, *these patterns suggest an intrahepatic environment permitting only limited matrix degradation, ultimately resulting in redistribution of extracellular matrix with relative accumulation of collagen type I. (Am J Pathol 1997, 150:1647–1659)

Liver fibrosis and cirrhosis are associated with excessive deposition of extracellular matrix (ECM) in a distribution and composition different from the nonfibrotic organ.[1,2] These alterations relate particularly to an absolute and relative accumulation of collagen type I in fibrotic and cirrhotic livers.[3] Whereas the quantitative aspect may be conceivable in the light of increased *de novo* synthesis of almost all ECM components, the understanding of the qualitative changes within the ECM requires evaluation of additional mechanisms such as fibrolytic activities of matrix metalloproteinases (MMPs).[1–4] MMPs represent a family of neutral proteinases with variable substrate spectra.[5,6] Their differential expression was suggested to influence the turnover of ECM components resulting in preferential accumulation of interstitial collagens, in particular, collagen type I, in fibrotic liver.[7]

By *in situ* hybridization, MMP-1 (interstitial collagenase) transcripts were undetectable in liver with active fibrogenesis although the potential of mesenchymal liver cells such as hepatic stellate cells (HSCs) to express this gene was evident in primary cell culture.[7] In addition, MMP-3 (transin/stromelysin-1) was transiently expressed in a distinct time frame by hepatocytes and mesenchymal cells in response to a single injection of carbon tetrachloride.[8] MMP-2

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(72-kd gelatinase/type IV collagenase), on the other hand, was expressed by mesenchymal cells at elevated levels in all fibrotic livers.[7,9]

Because of their destructive potential, MMPs are tightly regulated at the levels of transcription, secretion, and proteolytic activation.[5,6,10] Furthermore, MMP activities are restricted by plasma- and tissue-derived inhibitors. Tissue inhibitors of metalloproteinases such as TIMP-1 and TIMP-2 are low molecular weight proteins sharing structural similarity. Individual members of this family of proteins display selective affinities for different members of the MMP family of enzymes.[5,6] TIMP-1 controls most MMP, in particular, MMP-1, activity, whereas TIMP-2 is the major inhibitor of MMP-2 not only by virtue of its inhibiting activity but also by preventing the formation of active enzyme from zymogen by autocatalytic activation.[11–13] Moreover, large inhibitor of metalloproteinases (LIMP), the noncovalently associated, stoichiometric MMP-2/TIMP-2 complex, functions as an additional inhibitor of MMP-1.[14,15] MMP-2 may be liberated from the complex with TIMP-2 by action of matrilysin (MMP-7)[16] and membrane-bound MMP (MMP-14).[17] A recently discovered additional member of the TIMP family of proteins, TIMP-3, was not found in liver.[18] TIMP transcription is activated by serum responsive elements, various growth factors, phorbol esters, steroids, and glucocorticoids,[19–21] in part through c-*fos*/c-*jun*-dependent pathways.[22] Depending on the cell type tested, cytokines such as transforming growth factor (TGF)-β1, platelet-derived growth factor (PDGF), and interleukin (IL)-6 were reported to modulate TIMP expression *in vitro*.[23,24]

TIMP expression was previously studied in conditions such as wound healing, arthritis, and embryogenesis.[25–28] Little is known, however, about hepatic TIMP expression *in vivo*. *In vitro*, TIMP-1 expression was found to be up-regulated in the human hepatoma cell line HepG2 under the influence of TGF-β1 and IL-6.[23] The finding of increased TIMP-1 and -2 expression in extracts of fibrotic human liver[29] as well as the finding of TIMP-1 expression by HSCs in primary culture[30] suggested a contribution of this cell type to progression of liver fibrosis. In normal liver, HSCs (also known as Ito cells, lipocytes, fat-storing cells, and perisinusoidal cells) are situated in the space of Disse and are the major storage site of vitamin A. In response to liver injury and under the influence of TGF-β, HSCs proliferate, lose their lipid droplets, acquire a myofibroblastic phenotype with expression of smooth muscle α-actin and PDGF receptors, and synthesize numerous ECM components in large amounts.[31–33]

To address questions as to the cellular localization, steady-state levels, and kinetics of TIMP-1 and -2 transcripts in normal and fibrotic rat and human liver as well as in cultured HSCs, *in situ* hybridization was applied using [^{35}S]-labeled single-stranded RNA probes. A combination of immunohistochemistry followed by *in situ* hybridization was employed to characterize the phenotype of TIMP-expressing cells. Rat livers at different time points after a single injection or repeated injections of CCl_4 or subsequent to common bile duct ligation and scission were used to focus on kinetic aspects of TIMP expression. Human fibrotic liver lesions comprised cirrhotic liver disease of different etiology, cases of focal nodular hyperplasia, and hepatic carcinoma metastases with a prominent desmoplastic stromal reaction. Furthermore, HSC cultures were prepared to analyze TIMP RNA expression over periods of several days in the presence or absence of exogenous TGF-β1.

Materials and Methods

Animals

Female Sprague-Dawley rats (weight, 200 to 250 g) from the Zentralinstitut für Versuchstiere (Hannover, Germany) were treated by intraperitoneal injection of CCl_4 (1.25 ml/kg of body weight), mixed with an equal volume of vegetable oil. For the study of the acute response to CCl_4, animals were sacrificed 0.5, 1, 3, 6, 12, 24, 48, or 72 hours after injection. For the chronic CCl_4 intoxication model, animals were injected with a reduced dose of 1 ml/kg of body weight in 4-day intervals for up to 3 weeks and killed 12, 24, or 48 hours after the last injection. Rats treated with vegetable oil without CCl_4 served as controls. To induce biliary fibrosis in rats, the common bile duct was interrupted by ligation and subsequent scission under mild ether anesthesia. Animals were killed 2, 3, and 4 weeks after surgery, with sham-operated rats serving as controls. The morphology of the changes induced by CCl_4 intoxication and by bile duct ligation and division has been described in detail previously.[34–36]

Human Tissues

Twenty tissue samples were obtained from patients undergoing orthotopic liver transplantation and were snap-frozen immediately after explantation. Three livers were transplants, and retransplantation was required because of chronic rejection, recurrent hepatitis B virus graft hepatitis, or ischemic biliary

Table 1. *Human Liver Samples*

Case	Age Sex (years)	Material	Clinical Diagnosis/ aetiology	Histology
1	47 M	E	Chronic rejection	Portal fibrosis, vanishing bile ducts
2	49 M	E	Cirrhosis, HBV-related	Micronodular cirrhosis, moderate inflammatory activity
3	49 F	E	Recurrent graft HBV hepatitis	Moderate fibrosis, moderate inflammatory activity, severe cholestasis
4	52 F	E	Cirrhosis, HCV-related	Micronodular cirrhosis, moderate inflammatory activity, severe cholestasis
5	35 M	E	Cirrhosis, autoimmune	Micronodular cirrhosis, moderate inflammatory activity
6	37 M	E	Cirrhosis, HBV/HDV-related	Micronodular cirrhosis, moderate inflammatory activity
7	41 F	E	Alcoholic cirrhosis	Cirrhosis, moderate inflammatory activity, prominent bile duct proliferatons
8	58 F	E	Biliary cirrhosis secondary to bile duct injury	Severe portal fibrosis, moderate inflammatory activity, severe cholestasis
9	21 M	E	Cirrhosis, autoimmune	Micronodular cirrhosis, high inflammatory activity
10	54 M	E	Cirrhosis, HBV-related	Micronodular cirrhosis, high inflammatory activity
11	49 M	E	Alcoholic cirrhosis	Micronodular cirrhosis, moderate inflammatory activity
12	37 M	E	Cirrhosis, HBV-related	Micronodular cirrhosis, moderate inflammatory activity
13	44 M	E	Cirrhosis, HBV-related	Micronodular cirrhosis, moderate inflammatory activity
14	53 M	E	Alcoholic cirrhosis	Micronodular cirrhosis, moderate inflammatory activity, prominent bile duct proliferations
15	63 F	E	Cirrhosis, HCV-related	Micronodular cirrhosis, moderate to high inflammatory activity
16	48 M	E	Ischemic-type biliary lesions, transplantation 22 months previously	Severe biliary fibrosis, portoportal septa, cholestasis, moderate inflammatory activity
17	45 M	E	HBV post-hepatitic cirrhosis	Micronodular cirrhosis, moderate inflammatory activity
18	59 F	E	Cirrhosis, HCV-related	Micronodular cirrhosis, high inflammatory activity, prominent bile duct proliferation
19	47 F	E	Primary biliary cirrhosis	Primary biliary cirrhosis stage IV, high inflammatory activity, cholestasis
20	25 F	E	Primary sclerosing cholangitis	Sclerosing cholangitis, secondary biliary cirrhosis, moderate inflammatory activity
21	40 F	R	Focal lesion left lobe	Focal nodular hyperplasia, normal histology distant to lesion
22	68 M	R	Metastatic colon carcinoma	Moderately differentiated adenocarcinoma, normal histologh distant to lesion
23	57 F	R	Metastatic colon carcinoma	Moderately differentiated adenocarcinoma, normal histology distant to lesion

M, male; F, female; E, liver explant, R, resectate; HBV, HCV, HDV, hepatitis viruses, B, C, and D, respectively.

lesions. The other livers showed cirrhosis clinically related to alcoholic liver disease (n = 3), micronodular cirrhosis with moderate to high activity related to infection with hepatitis B virus (n = 5), hepatitis C virus (n = 3), and hepatitis B and D viruses (n = 1) or due to autoimmune chronic active hepatitis (n = 2), primary biliary cirrhosis (n = 1), biliary cirrhosis following primary sclerosing cholangitis (n = 1), and secondary biliary cirrhosis following accidental bile duct injury during cholecystectomy (n = 1). The clinical data and histological changes are summarized in Table 1. Additional samples were obtained from resectates with focal nodular hyperplasia (n = 1) or metastases of colon adenocarcinomas (n = 2). Normal liver samples were obtained from tissue at the margins of the latter three specimens 2 to 3 cm distant from the focal lesions, showing minimal portal tract fibrosis and scant lymphoid infiltration. Informed consent was obtained before surgery.

Tissue Sectioning and Fixation

Liver samples (5 to 7 mm^3) were immediately snap-frozen in liquid nitrogen. Frozen sections (4 μm) were collected onto slides coated with 3-aminopropyl triethoxysilane, dried briefly on a hot plate at 80°C, and fixed in 4% paraformaldehyde/phosphate-buffered saline (PBS), pH 7.4, for 20 minutes. After two washes in PBS, dehydration in graded ethanols, and short air drying, sections were immediately used for *in situ* hybridization.[36] For immunohistochemical studies, frozen sections were air dried overnight and stored at −80°C.

Isolation, Characterization, and Culture of Human HSCs

Human HSCs were obtained from wedge sections (10 to 20 g) of normal liver unsuitable for transplan-

Table 2. *Summary of* in Situ *Hybridization Results*

Tissue		TIMP-1, 15 days exposure				TIMP 2, 25 days exposure			
		NEC	HC	BDC	CA	NEC	HC	BDC	CA
	0 hour	−	−	−		−	−	−	
	0.5 hour	−	−	−		−	−	−	
Acute	1 hour	(+)	−	−		−	−	−	
CCl_4	3 hours	(+)	−	−		+	−	−	
Intoxication,	6 hours	+	−	−		+	−	−	
rat liver	12 hours	+	(+)	−		+	−	−	
	24 hours	+	−	−		+	−	−	
	48 hours	++	−	−		++	−	−	
	72 hours	+++	−	−		+++	−	−	
Chronic	12 hours	++	(+)	−		++	−	−	
CCl_4	24 hours	++	−	−		++	−	−	
intoxication,	48 hours	++	−	−		++	−	−	
rat liver	72 hours	++	−	−		++	−	−	
Bile duct	Sham	−	−	−		−	−	−	
ligation,	2 weeks	++	−	(+)		++	−	+	
rat liver	3 weeks	++	−	(+)		++	−	+	
	Normal	(+)	−	−		(+)	−	−	
Human	Cirrhotic	++++	+	+		+++	−	+	
liver	FNH	+	−	−		+	−	−	
	Metastasis	+++	(+)	(+)	++	+++	−	(+)	++

Because of the longer exposure time requird for TIMP-2, the scale is characteristic for each probe and cannot be used to directly compare absolute transcript copy numbers for TIMP-1 and TIMP-2. Results are expressed as the relative abundance of grains over individual cells after hybridization with TIMP-1 and TIMP-2 probes after 15 and 25 days of autoradiographic exposure, respectively: −, background signal; (+), 2 to 5 times; +, 5 to 10 times; ++, 10 to 15 times; +++, >15 times the background signal as defined by hybridizations with sense (control) probe. NEC, non-epithelial cells; HC, hepatocytes; BDC, bile duct epithelial cells, CA, carcinoma cells. Time points refer to the interval between CCl_4 intoxication and hepatectomy after a single (acute intoxication model), the last one of two to four CCl_4 of 4 days (chronic intoxication model), or the interval between common bile duct ligation/scission and hepatectomy (biliary fibrosis model).

tation and cirrhotic liver explants from patients with chronic hepatitis C virus infection. After combined 0.5% pronase/0.05% collagenase tissue digestion, HSCs were isolated by ultracentrifugation over four gradients (1/1.080/1.058/1.053) of stractan (Larex-LO) and characterized as previously described.[32] After isolation, cells were plated onto 100-mm plastic petri dishes (Falcon Primaria, Becton Dickinson, Oxnard, CA) at an approximate density of 1×10^6 cells in Iscove's modified Dulbecco's medium (BRL-Gibco, Gaithersburg, MD) supplemented with 20% fetal calf serum (FCS), 0.6 IU/ml insulin, 2 mmol/L glutamine, 0.1 mmol/L nonessential amino acids, 1 mmol/L sodium pyruvate, and antibiotic/antifungal solution (Sigma Chemical Co., St. Louis, MO). Primary cultures were allowed to grow to confluency and then subcultured after digestion with 0.025% trypsin/0.5 mmol/L EDTA (BRL-Gibco). Confluent HSCs were then incubated in serum-free/insulin-free medium for 48 hours. In some experiments, medium containing 20% FCS was added and the cells incubated for another 24 hours at 37°C.

Isolation, Culture and Characterization of Rat HSCs

Nonparenchymal rat liver cells were prepared by the pronase-collagenase method in a sequence of non-circulating and recirculating perfusions with minor modifications as described elsewhere.[37,38] From this cell suspension, HSCs were purified by a single-step density gradient centrifugation with Nycodenz (analytical grade, Nyegaard C., Oslo, Norway). Cells recovered at this level (15 to 43×10^6 cells/liver) were 98% viable as verified by trypan blue exclusion and 97% pure as determined by phase-contrast microscopy and immunocytochemistry with antibodies specific for intermediate filaments (cytokeratin, vimentin, and desmin; see below). Kupffer cell and endothelial cell contaminations were excluded by their inability to phagocytose latex beads, to express Fc receptors, and to stain for peroxidase.[35] Cells were cultured in Dulbecco's modified Eagle's medium (Boehringer Mannheim, Mannheim, Germany) supplemented with 4 mmol/L L-glutamine, penicillin (100 IU/ml), streptomycin (0.1 mg/ml), and 10% FCS (Boehringer Mannheim) at a seeding density of 1.8×10^6 cells per 75 cm^2. The first change of medium was made 24 hours after seeding, after which the purity of HSCs was >97%. Cells were subsequently recovered by incubation with 0.05% trypsin/10 mmol/L EDTA in PBS, pH 7.4, washed with PBS, applied to glass slides with a cytocentrifuge at 500 rpm, dried for 20 minutes at room temperature, and fixed as described above.

To test for the influence of TGF-β1 on the expression of TIMP-1 and -2 and MMP-2, rat HSCs were cultured in the presence of FCS for 24 hours and kept under serum-free conditions for another 24 hours. Cultivation was continued for an additional 48 hours in serum-free medium with or without TGF-β1 polypeptide (Serva, Heidelberg, Germany) at concentrations of 1, 5, and 20 ng/ml. Statistical evaluation of cell culture results was performed by the Wilcoxon-Mann-Whitney *U* test for independent samples.

Preparation and Labeling of Probes

TIMP-1 and TIMP-2 gene probes were generated by oligo(dT)-primed reverse transcription of total cell RNA prepared from human placenta and HT144 melanoma cells (HTB 63, American Type Culture Collection, Rockville, MD), respectively, with subsequent amplification using specific oligodeoxyribonucleotide primers. Primers for TIMP-1 corresponded to nucleotides 96 to 116 and 645 to 665 and for TIMP-2 to nucleotides 490 to 514 and 981 to 1001 of the published sequences.[39,40] The products were then cloned into the run-off transcription vector pAMP1 (BRL-Gibco). Authenticity of the 0.56- and 0.51-kb amplification products was verified by restriction endonuclease digestion and partial sequence analysis. For detection of MMP-2 transcripts, pGEM1-based transcription vectors were constructed by subcloning of the 1300-bp *Eco*RI-*Bgl*II and 750-bp *Bgl*II-*Eco*RI fragments of pK121, kindly provided by Dr. K. Tryggvason.[41] A 110-bp cDNA fragment of the glyceraldehyde 3-phosphatase dehydrogenase (GAPDH) gene, subcloned in the *Hin*dIII site of pT7.2,[42] was used as a control for RNase protection assays. All constructs were subjected to nucleic acid sequence analysis and proved to fully conform to published sequences. After linearization of the plasmids with either *Hin*dIII or *Eco*RI restriction endonuclease, T7 or SP6 RNA polymerase (BRL-Gibco), respectively, was employed to obtain run-off transcripts of either the antisense (complementary to mRNA) or sense (anti-complementary, negative control) strands. Transcription and [^{35}S] or [^{32}P] labeling of RNA probes were performed as described previously.[31] The specific activity routinely obtained was 1.2 to 1.4 $\times$ 10^9 cpm/μg.

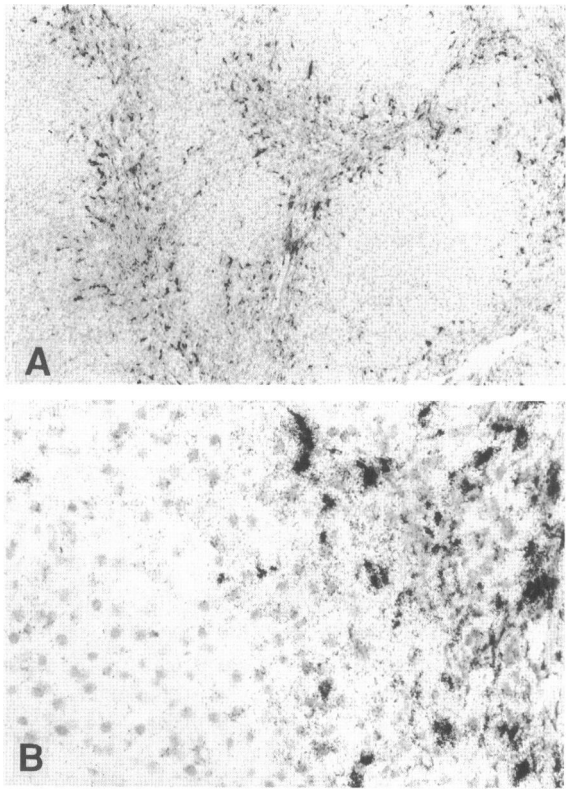

Figure 1. In situ *hybridization with [^{35}S]-labeled single-stranded TIMP-1 RNA probe on a human liver with alcohol-induced micronodular cirrhosis (case 9).* **A:** *Intensely labeled spindle-shaped mesenchymal cells accumulate at the interface between regeneration nodules and fibrotic stroma.* **B:** *Hepatocytes at the periphery but not in the center of regeneration nodules display weak autoradiographic signals. Cryostat sections; exposure time 15 days; magnification, ×75 (A) and ×380 (B).*

Immunohistology Combined with in Situ *Hybridization*

Prehybridization, hybridization, washing procedures including removal of nonspecifically bound probe by RNase A digestion were performed for both antisense and sense strand [^{35}S]-labeled RNA probes as described in detail.[36] Sections from all time points after CCl_4 administration and bile duct ligation were always processed simultaneously using the same batches of probes and reagents. Hybridization of tissues pretreated with *Micrococcus* nuclease verified that cellular RNA was the target of hybridization. In double-labeling procedures, immunohistology was performed immediately before prehybridization using ribonuclease-free buffers and glassware. Sections were stained with monoclonal antibodies specific for cytokeratin (lu5, Boehringer Mannheim), vimentin (V9, DAKO, Glostrup, Denmark), desmin (D33, DAKO), smooth muscle α-actin (1A4, Sigma), the endothelial cell marker CD31 (JC70A, DAKO), and the monocyte/macrophage antigen CD68 (PG-M1, DAKO) using the immunoalkaline phosphatase (APAAP) method with affinity-purified rabbit anti-mouse immunoglobulin antibodies (DAKO), APAAP complex (1:20 dilution, DAKO), and new fuchsin.[41] All reagents were made up to 5000 IU/ml with heparin sodium salt (Sigma) and 0.6 mg/ml

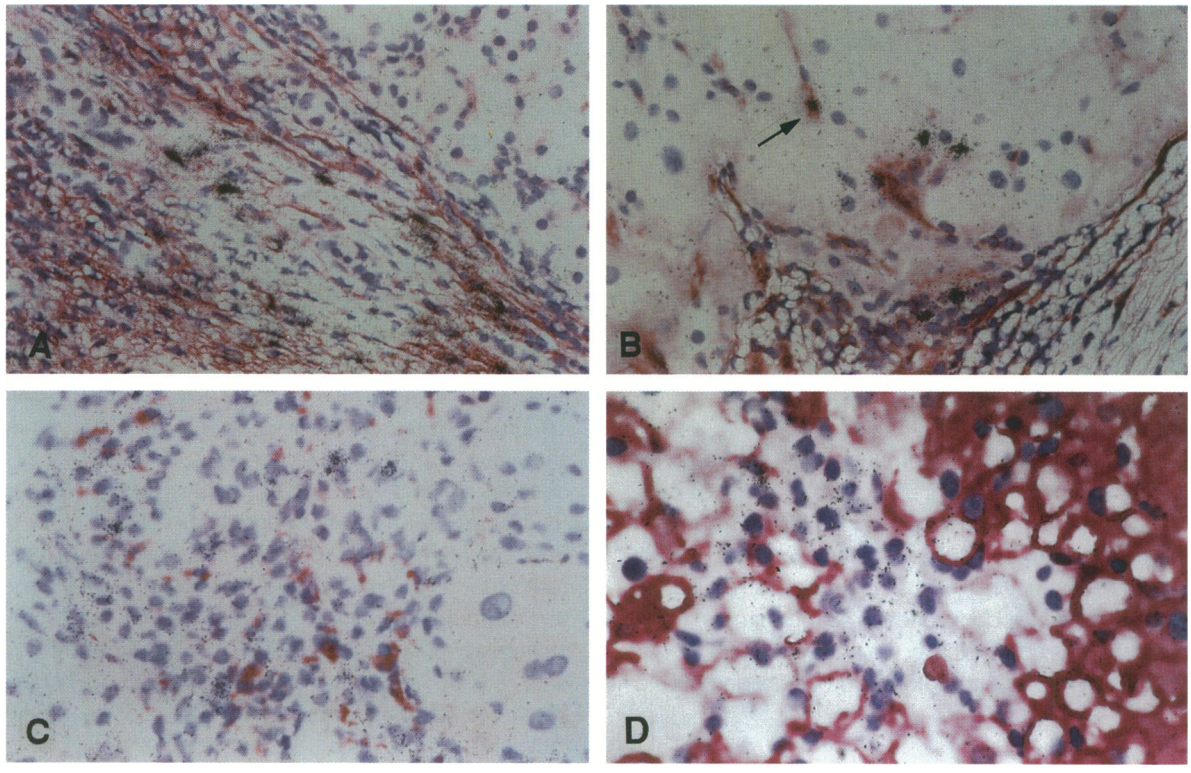

Figure 2. *Immunostaining for smooth muscle α-actin* (**A**), *the endothelial cell antigen CD31* (**B**), *the macrophage-specific antigen CD68* (**C**), *and cytokeratin* (**D**) *followed by* in situ *hybridization with [^{35}S]-labeled TIMP-1 antisense RNA probes on a human liver with micronodular cirrhosis related to HBV infection (case 3,* **A** *to* **C**) *or in rat liver 24 hours after the last one of three CCl_4 injections in 4-day intervals* (**D**). *In human liver, grains are localized over smooth muscle α-actin-positive cells* (**A**) *and some endothelial cells* (**arrow**) *characterized by CD31 positivity* (**B**) *but not over macrophages/Kupffer cells* (**C**). *Many of the strongly α-actin-positive, TIMP-1-negative cells represent vascular and periductular smooth muscle cells. In the rat liver specimen, grains are localized over cytokeratin-negative mesenchymal cells that have replaced necrotic hepatocytes. Cryostat sections; APAAP procedure; autoradiographic exposure time 14 days; magnification, ×280* (**A** *to* **C**), *and ×480* (**D**).

yeast tRNA (BRL-Gibco) to inhibit ribonuclease activity. The alkaline phosphatase substrate was developed with new fuchsin as described. Immunohistology without subsequent *in situ* hybridization required no addition of heparin and tRNA. For autoradiographic detection of bound activity, dehydrated slides were dipped into nuclear emulsion (LM1, Amersham, Braunschweig, Germany) that had been melted at 42°C and diluted with an equal volume of 0.6 mol/L ammonium acetate. After 2 hours of drying, the slides were stored in light-proof boxes containing dessicant, exposed at 4°C for 3 to 21 days, developed in Kodak D19 solution (Kodak, Hemel Hempstead, UK) for 2.5 minutes, rinsed in 1% acetic acid, and fixed in Kodak fixer for 3 minutes. After extensive washing in tap water, slides were then counterstained in hematoxylin and coverslipped.

RNA Extraction

Human HSCs were washed twice in cold PBS, pH 7.4, scraped with a rubber policeman in a 4 mol/L guanidinium isothiocyanate solution, vortexed, and immediately stored at −70°C. Liver tissue was ground to fine powder using a mortar and liquid nitrogen. Total RNA was then isolated by the guanidinium isothiocyanate-phenol-chloroform method[44] using RNAzol (Biotecx Laboratories, Houston, TX).

RNase Protection Assay

A 10-μg aliquot of total RNA was hybridized to 1 × 10^6 cpm of [^{32}P]-labeled antisense probes specific for TIMP-1 and TIMP-2 transcripts (specific activity, 1.5 × 10^9 cpm/μg) in 80% formamide, 10 mmol/L Pipes, pH 6.4, 400 mmol/L NaCl, 1 mmol/L EDTA for 16 hours at 50°C. After hybridization, the reaction mixture was treated with RNase A (50 mg/ml) and RNase T1 (2 mg/ml) and then with proteinase K (0.16 mg/ml). The protected fragments were analyzed by electrophoresis on a 7 mol/L urea/6% acrylamide gel, followed by autoradiography.

Northern Blot Hybridization

A 50-μg aliquot of total RNA was separated on a 1% agarose/3% formaldehyde gel. RNA was transferred

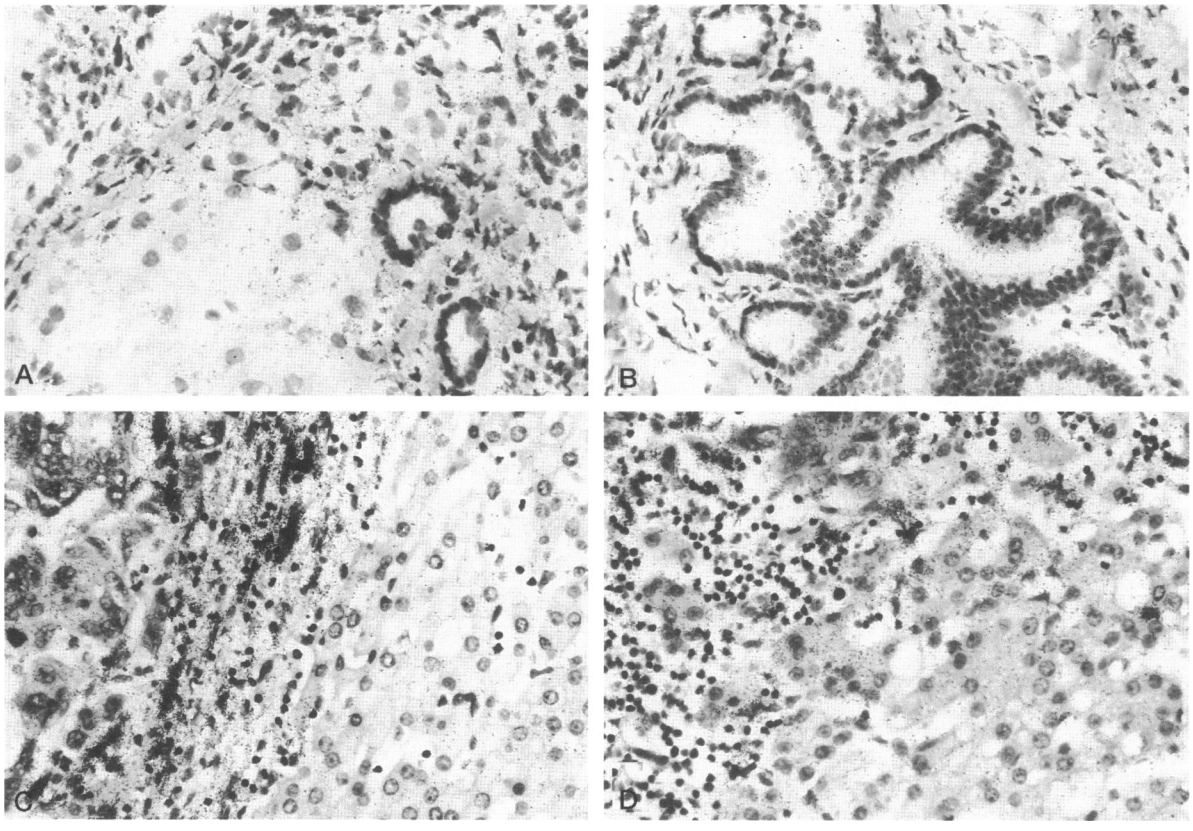

Figure 3. In situ *hybridization with [^{35}S]-labeled single-stranded TIMP-1 RNA probe on a human liver with alcohol-induced micronodular cirrhosis (case 14) and metastatic colon carcinoma (case 22).* **A** *and* **B**: *Epithelial cells of small proliferating bile ductules* (**A**) *display weaker levels of TIMP-1 transcripts as compared with ducts of intermediate size* (**B**). *Cells of a colon carcinoma metastasis display TIMP-1 signals of intermediate intensity. Higher intensities are found in inflammatory and other mesenchymal cells at the border of the metastasis; hepatocytes do not label with the TIMP-1 probe* (**C**) *except for a few hepatocytes surrounded by carcinoma and inflammatory cells* (**D**). *Ca, carcinoma cells. Cryostat* (**A** *and* **B**) *and paraffin sections* (**C** *and* **D**); *exposure time 8 days; magnification, ×380.*

to nitrocellulose filters (Genescreen, New England Nuclear, Dreieich, Germany) by capillary blotting overnight in 10X standard saline citrate (SSC) and immobilized by baking for 2 hours at 80°C. Prehybridization was carried out for 4 hours at 42°C in a buffer containing 50% formamide, 5X SSC, 5X Denhardt's solution, 0.1% sodium dodecyl sulfate (SDS), 50 mmol/L $NaPO_4$, pH 6.4, and 0.2 mg/ml denatured salmon sperm DNA. Hybridization was performed at 42°C for 18 hours in the buffer described above containing 0.2 volumes of 50% dextran sulfate and 10^6 cpm/ml [^{32}P]cDNA probes labeled by random priming. The filters were washed with three changes of 15 minutes each in 2X SSC, 0.1% SDS, 1X SSC, 0.1% SDS, and 0.1X SSC, 0.1% SDS, respectively, at 65°C, and then exposed to Kodak XAR-5 film at −70°C, developed, and quantitated by scanning laser densitometry.

Results

TIMP-1 RNA was detectable in all human and fibrotic rat livers (Table 2). Human livers with normal architecture showed a weak signal over few scattered perisinusoidal, perivenular, and portal tract stromal cells. Slightly larger numbers of mesenchymal cells with increased TIMP-1 steady-state RNA levels were present in the focal nodular hyperplasia specimen within the lesion and in a moderately fibrotic liver with vanishing bile duct syndrome (case 1). Dramatically enhanced signals, however, were observed in cirrhotic human livers in the neighborhood to and within areas of active fibrogenesis (Figure 1A). In addition to mesenchymal cells, hepatocytes at the periphery of regenerative nodules displayed moderate levels of TIMP-1 RNA in human livers (Figure 1B), whereas only vimentin-positive mesenchymal liver cells were labeled in noncirrhotic specimens. In agreement with the morphological characteristics, the vast majority of TIMP-1-positive cells were vimentin positive, and further analysis revealed that most TIMP-1 RNA-positive cells within and in the vicinity of fibrotic septa expressed the activation marker smooth muscle α-actin (Figure 2A). Few of the TIMP-1-positive cells were labeled with the endothelial cell marker CD31 (Figure 2B), whereas TIMP-1 RNA and CD68 anti-

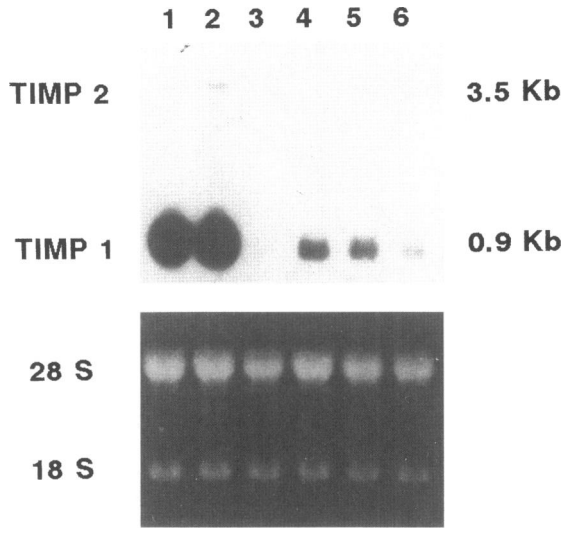

Figure 4. *Northern blot hybridization of total RNA (50 μg per lane) extracted from cultured human HSCs* (lanes 1 *and* 2) *as well as normal* (lane 3) *and cirrhotic* (lanes 4 *to* 6) *human liver tissues. Large amounts of transcripts for TIMP-1 are visible in cultured HSCs and in cirrhotic liver samples. A weak expression of TIMP-2 mRNA is detected only in isolated HSCs but not in liver tissues. Normal liver does not show relevant amounts of transcripts for both TIMPs. Autoradiographic exposure time was 5 days. The ethidium bromide staining intensities of 28 and 18 S RNA indicate that similar amounts of total RNA were applied to each lane* (lower panel).

gen, characteristic for monocytes/macrophages and Kupffer cells, did not co-localize (Figure 2C).

Bile duct epithelial cells displayed moderate TIMP-1-specific signals. However, steady-state levels over epithelial cells of small proliferating bile ducts were reduced as compared with bile ducts of intermediate size (Figure 3, A and B). Mesenchymal cells in the vicinity as well as in the stroma of colonic adenocarcinoma metastases expressed

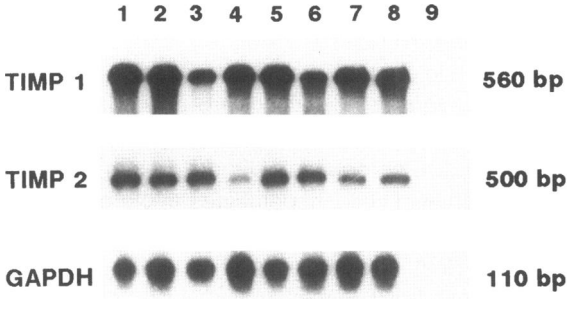

Figure 5. *RNAse protection assay showing TIMP-1 and TIMP-2 mRNA expression in human HSCs isolated from four individual normal livers* (lanes 1 *and* 2, 3 *and* 4, 5 *and* 6, *and* 7 *and* 8, *respectively). Single protected fragments of 560 bp for TIMP-1 and approximately 500 bp for TIMP-2 mRNA are present in all cell isolates. HSCs were cultured for 48 hours in serum-free/insulin-free medium* (lanes 1, 3, 5, *and* 7) *or kept for 48 hours in serum-free/insulin-free medium and subsequently in medium supplemented with 20% FCS for an additional 24 hours* (lanes 2, 4, 6, *and* 8). *A glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe protecting a 110-bp RNA fragment served as a control. Reaction mixtures representing each 10 μg of total cellular RNA were applied to* lanes 1 *to* 8, *and 10 μg of yeast tRNA was applied to* lane 9 *for control.*

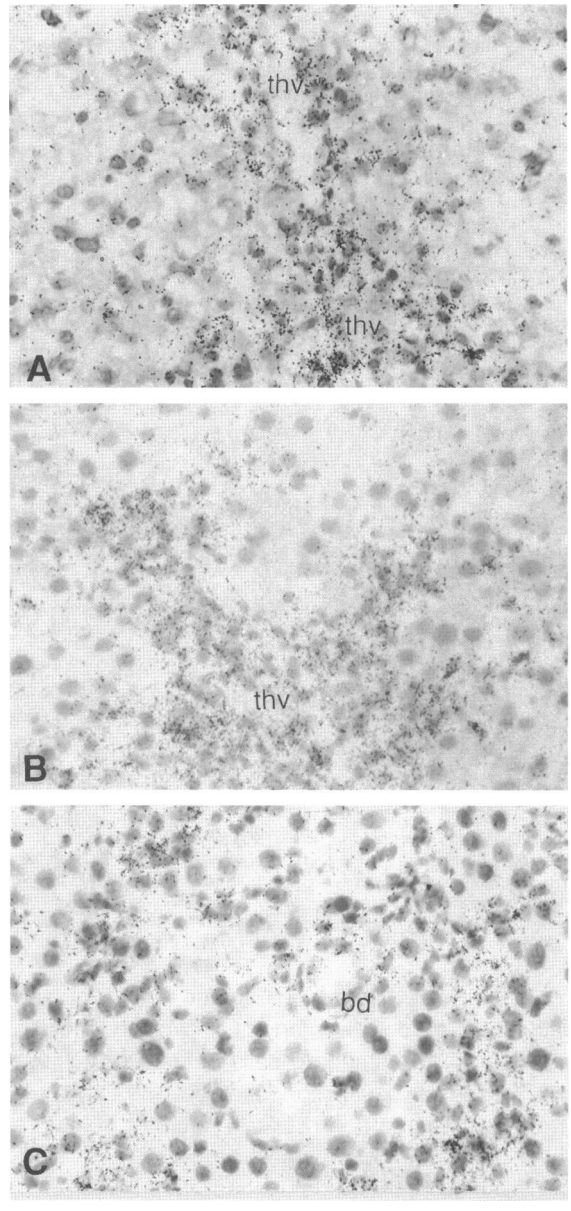

Figure 6. In situ *hybridization with [^{35}S]-labeled single-stranded TIMP-1 RNA probe in rat liver 72 hours after a single* (A) *12 hours after the last one of four CCl_4 administrations* (B) *and 3 weeks after bile duct ligation* (C). *Highest signal intensity is displayed by mesenchymal cells in the area of toxic damage* (A *and* B) *and in the neighborhood of proliferating biliary ductules also displaying low TIMP-1-specific signal* (C). *bd, bile ductule; thv, terminal hepatic venule. Cryostat sections; exposure time 7 days; magnification, ×380.*

TIMP-1 RNA at high steady-state levels similar to septal mesenchymal cells in cirrhotic livers (Figure 3C); additionally, tumor cells displayed high autoradiographic signals (Figure 3D).

Northern blot analysis of RNA extracted from cultured human HSCs and total liver revealed TIMP-1 transcripts of 0.9 kb in large amounts in cultured HSCs, moderate amounts in cirrhotic liver tissue extracts, and no signal in an extract from liver with

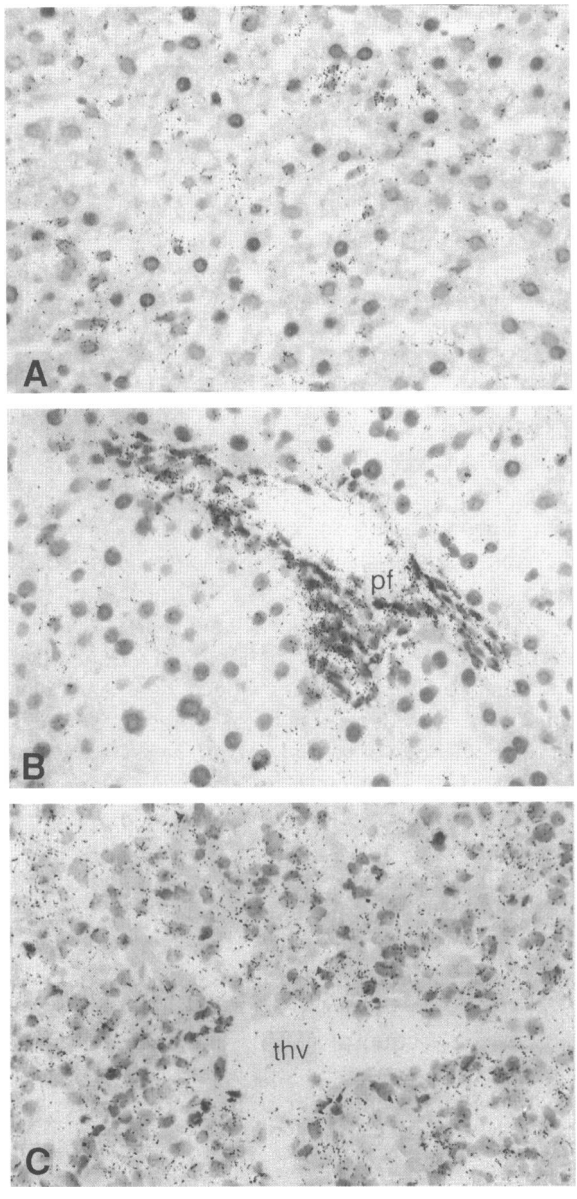

Figure 7. In situ *hybridization with [^{35}S]-labeled single-stranded TIMP-2 RNA probe in rat liver 3 hours* (**A** *and* **B**) *and 48 hours* (**C**) *after a single injection of* CCl_4. *Perisinusoidal* (**A**) *and most portal tract cells* (**B**) *display specific autoradiographic signals. The signal increases in pericentral mesenchymal cells along with the appearance of hepatocellular necroses* (**C**). *pf, portal field; thv, terminal hepatic venule. Cryostat sections; exposure time 12 days; magnification,* ×520.

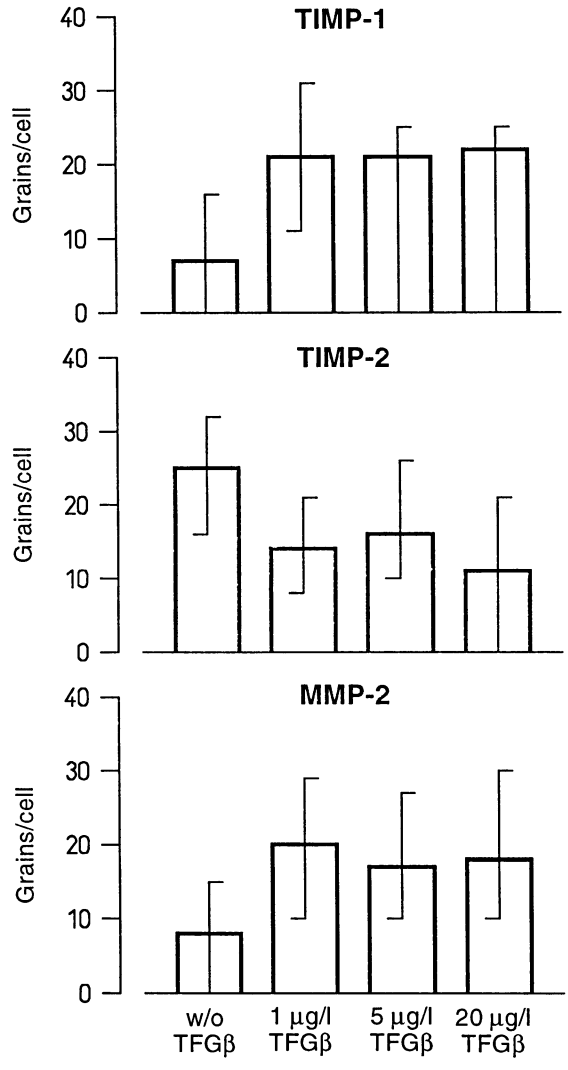

Figure 8. *Evaluation of autoradiographic signal intensities of rat HSCs hybridized to TIMP-1 and -2 and MMP-2 probes after 4 days of culture, with or without the presence of exogenous TGF-β1 at different concentrations during the last 48 hours of culture. Columns indicate the median of grain numbers attributable to individual cells in three independent experiments, and bars indicate the 25th and 75th percentiles. Differences between TGF-β1-treated cells in comparison with untreated controls were statistically significant* ($P < 0.005$). *All sense controls displayed an average of three grains per cell. Autoradiographic exposure time was 5 days.*

largely normal histology (Figure 4). RNase protection assays showed a protected TIMP-1 fragment of 560 bp in HSC samples cultivated with or without FCS (Figure 5). Addition of FCS to HSC cultures resulted in apparently slightly enhanced (up to 1.5-fold) transcript levels.

In rat liver, TIMP-1 RNA expression was noticeable in perisinusoidal, perivenular, and portal tract stromal cells 1 hour after injection of CCl_4. Over the next 11 hours, these signals increased in relative intensity but without changes in the number and localization of TIMP-expressing cells. Beginning 12 to 24 hours after intoxication, coinciding with morphological manifestations of hepatocellular damage, the numbers of TIMP-1-positive perisinusoidal cells and the signal intensity increased in the centrolobular area. The highest levels were noted 72 hours after CCl_4 injection (Figure 6A), and autoradiographic signals of similar intensity were found in centrolobular areas at all time points analyzed after repeated CCl_4 injection (Figures 2D and 6B; Table 2). Only few scattered TIMP-1-positive hepatocytes were found in cen-

trolobular areas 12 hours after a single injection or repeated CCl_4 injections. In bile-duct-ligated rats, TIMP-1 expression predominated in periductular mesenchymal cells but was also detectable in epithelial cells of small proliferating biliary ductules. Phenotypic analysis of TIMP-1-positive rat liver cells revealed virtually exclusive or predominant co-localization with vimentin and desmin, respectively.

TIMP-2 RNA-specific signals were largely superimposable to the previously reported patterns of MMP-2 expression in rat and human liver (Table 2). In comparison with TIMP-1, TIMP-2 transcript levels were in all instances lower, requiring extended autoradiographic exposure times. Similar to TIMP-1, TIMP-2 RNA was detectable as early as 3 hours after CCl_4 intoxication (Figure 7, A and B). TIMP-2 RNA was also expressed at low levels in bile duct epithelial cells and tumor cells of adenocarcinoma metastases but not in hepatocytes of normal or cirrhotic livers. TIMP-2 was not detectable in CD68-positive cells of the monocyte/macrophage lineage, which were previously shown to secrete the 92-kd gelatinase (MMP-9).[45]

Northern blot analysis of RNA extracted from cultured human HSCs and total liver revealed TIMP-2 transcripts of 3.5 kb in minimal amounts in only one of the HSC cultures (Figure 4). With the more sensitive RNase protection assay, a single protected TIMP-2 fragment of approximately 500 bp became apparent in all of the HSC isolates cultivated with or without addition of FCS (Figure 5). TIMP-1 and -2 transcripts were clearly detectable by *in situ* hybridization in rat HSCs over 7 days of primary culture. Addition of TGF-β1 at various doses resulted in significant changes of the signal intensity and the number of specifically labeled cells (Figure 8). Overall, a twofold enhancement of TIMP-1- and MMP-2-specific signals was induced by TGF-β1, whereas TIMP-2 RNA-specific signals were decreased by approximately 50%.

Discussion

Expression of the major matrix-degrading enzymes, MMPs, is tightly regulated at the levels of gene expression, transcript stabilization, secretion, and activation, because of the destructive potential inherent to MMP activity.[4–6] In addition to these control mechanisms, MMPs are usually secreted in the presence of specific inhibitors, TIMP-1 and -2, which prevent accidental activation and limit the range of enzymatic activity.[5,6] Evaluation of the biological significance of MMP expression patterns therefore requires detailed knowledge of TIMP expression *in vivo*. Here we report on TIMP-1 and -2 RNA expression resolved by semiquantitative *in situ* hybridization at the single-cell level.

Application of TIMP-1 and -2 probes to rat liver tissue at various time points after CCl_4 administration revealed activation of TIMP expression in mesenchymal cells as early as 1 hour after intoxication, suggesting a role for TIMP in protection against accidental MMP activation, similar to other acute-phase proteins such as α_2-macroglobulin. Notably, expression of the immediate-early genes c-*jun* and c-*fos*, which also regulate basal TIMP expression,[22] precedes TIMP expression in CCl_4-treated livers.[36] *In situ* hybridization revealed dramatically elevated levels of TIMP-1 and TIMP-2 RNA transcripts in livers with active fibrogenesis. Whereas TIMP-2 proved to be expressed largely by those cells previously identified as sources of MMP-2 RNA in rat and human liver, TIMP-1 was found in a variety of cell types, even in hepatocytes at the periphery of regenerative nodules in cirrhosis. Hepatocytes in these particular sites were previously found to express TGF-β1,[43] further pointing to participation of some parenchymal cells in the control of fibrogenesis and fibrolysis. These observations are in good agreement with previous demonstrations of TIMP-1 expression in HepG2 hepatoma cells,[23] in primary rat hepatocytes stimulated with IL-1β and IL-6,[46] and in keratinocytes in the context of wound healing,[28] indicating that the expression of TIMP may occur dissociated from that of MMP in an epithelial-mesenchymal interplay. Although the spectrum of TIMP-1-expressing cell types appears to be broad, the highest TIMP-1 transcript levels were found in a subpopulation of α-actin-positive cells of human liver and desmin-positive cells in the rat, morphologically and phenotypically compatible with activated HSCs. The finding that expression of TIMP-2 and MMP-2 was mainly co-localized points to the generation of a MMP-2/TIMP-2 complex (LIMP) *in situ*, which acts as an additional MMP-1 inhibitor synergizing with TIMP-1.[14,15] The patterns of TIMP expression underline the notion that liver fibrosis may be perceived as a condition of ECM production (fibrogenesis) that is not met by adequate ECM degradation (fibrolysis), in particular, with respect to the predominating collagen type I.

Although TIMP RNA was clearly detectable in carcinoma cells, the relative RNA steady-state levels within the tumor were below those observed in the neighborhood to normal parenchyme. Therefore, at variance with cirrhotic liver, the balance of MMP and TIMP gene expression in central parts of carcinoma metastases seems to be tilted in favor of enzymatic

activities for rapid turnover of ECM facilitating infiltrative tumor growth. The observations in metastatic tumors are similar to those noticed in the corresponding primary malignancies.[47,48] Interestingly, the TIMP expression patterns observed in carcinoma metastases were similar to those of proliferating bile ductules.

Well in agreement with previous demonstrations of MMP-2 and TIMP-1 secretion by cultured human HSCs,[30,49] isolated rat HSCs expressed TIMP-1 and -2 RNA over 7 days of primary culture. As previously demonstrated for cell lines,[50] exogenous TGF-β1 increased TIMP-1 and MMP-2 RNA steady-state levels in our rat HSC cultures, whereas the TIMP-2-specific signal was reduced. The differences in TIMP transcript levels in human HSCs with or without addition of FCS are also suggestive of the action of factors such as TGF-β. Thus, TIMP-1 and TIMP-2 are independently regulated, and TGF-β seems to shift the equilibrium between MMP-2 and TIMP-2 expression by HSCs in favor of MMP-2, likely to result in an imbalance of enzyme over inhibitor. This may ultimately permit generation of active enzyme and result in local destruction of ECM, specifically that of basement membrane.

Enhanced TGF-β1 expression has previously been observed in rat livers with acute and chronic CCl_4-induced liver damage[51] or biliary fibrosis after bile duct ligation.[43] TGF-β1 expression at high steady-state levels was a constant finding in human chronic liver disease of different etiology[43] and was also found in all human liver samples analyzed for TIMP expression in this study (data not shown). With the demonstration of TGF-β1-mediated down-regulation of MMP-1 and MMP-3 gene transcript levels,[21] our data further support the pivotal role for TGF-β in the stimulation of fibrogenesis and the regulation of fibrolysis *in vivo*. Accordingly, enzymes required for the degradation of native interstitial collagen are either not synthesized or kept inactive in the parenchyme of livers with active fibrogenesis or cirrhosis.

In conclusion, chronic rat and human liver disease is characterized by a down-regulation of MMP-1 and TIMP-2 expression and a stimulation of MMP-2 and TIMP-1 production. Correlating with TGF-β1 expression, this suggests the virtual absence of MMP-1 activities in the presence of MMP-2 in fibrotic liver, resulting in patterns of enzyme activity that favor the degradation of most ECM constituents except for native collagen type I, which is the target of MMP-1.[10] In view of the enhanced *de novo* production of virtually all ECM components,[1,2] this may explain the quantitative and qualitative derangements and the histological redistribution of ECM constituents in cirrhotic as compared with normal liver, with particular reference to the well documented relative increase in collagen type I content.

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