Expression of Tissue Inhibitor of Metalloproteinases 1 and 2 Is Increased in Fibrotic Human Liver

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**Background & Aims:** Tissue inhibitors of metalloproteinases may contribute to liver fibrosis by preventing remodeling of fibrillar collagens by interstitial collagenase. This hypothesis was investigated by comparing the relative expression of messenger RNA for interstitial collagenase, gelatinase A, and tissue inhibitor of metalloproteinases 1 and 2 in fibrotic and normal human liver. **Methods:** Hepatic expression of metalloproteinases and their inhibitors was examined using ribonuclease protection assay, immunocytochemistry, and immunoassay. Lipocyte expression of tissue inhibitors of metalloproteinases was examined using Northern blotting and reverse zymography. **Results:** Messenger RNA levels for tissue inhibitors of metalloproteinase 1 and 2 were elevated to 260%–526% of levels in normal liver in biliary atresia, primary biliary cirrhosis, and primary sclerosing cholangitis. In fibrotic livers, tissue inhibitor of metalloproteinase 1 protein was 367%–724% of normal. Gelatinase A messenger RNA level increased to 324%–430% of normal values in fibrotic liver, but interstitial collagenase messenger RNA level was not significantly altered. Normal human liver lipocytes activated by culture on plastic expressed messenger RNA and protein for tissue inhibitor of metalloproteinase 1 and 2. **Conclusions:** Increased expression of tissue inhibitors of metalloproteinases relative to interstitial collagenase may promote deposition of interstitial collagens in liver fibrosis. Our studies further suggest that lipocytes are an important source of tissue inhibitors of metalloproteinases in progressive liver fibrosis.

Liver fibrosis is characterized by a net increase in the hepatic content of collagen I, III, and IV and other extracellular matrix proteins that disrupts normal hepatic architecture and impairs liver function. The extracellular matrix seems to be an active determinant of normal hepatocellular function, maintaining the phenotype of hepatocytes, sinusoidal endothelial cells, and lipocytes. Isolated hepatocytes, for example, maintain specific gene functions such as albumin or cytochrome P450 expression when maintained on basement membrane–like matrices but not on collagen I. The deposition of fibrotic matrix in the perisinusoidal space of Disse may therefore induce abnormal function in adjoining parenchymal and nonparenchymal liver cells.

Hepatic lipocytes (fat-storing or Ito cells) are liver-specific pericytes considered to be major contributors to liver fibrosis. In areas of liver injury, lipocytes proliferate and transform to a myofibroblastic phenotype (activated lipocytes) highly active in the synthesis of fibrillar collagens. Isolated lipocytes remain in a quiescent state when maintained on a matrix similar to basement membrane, whereas cells cultured on plastic or collagen I are activated to produce extracellular matrix proteins. Such studies provide insights into the mechanism of the development of fibrosis in injured liver based on altered lipocyte/matrix interactions.

Alterations in extracellular matrix may result not only from changes in deposition but also in degradation. A family of Zn²⁺-dependent matrix metalloproteinases (MMPs) plays an important role in the latter process. Interstitial collagenase, MMP-1 (EC 3.4.24.7), digests native fibrillar collagens I, II, and III. Gelatinase A, MMP-2 (EC 3.4.24.24), digests denatured collagens I and III and native collagen IV, an important component of basement membranes. Activity of MMPs can be regulated at the level of transcription, proenzyme activation, or binding of proenzyme or active enzyme to specific inhibitors such as the tissue inhibitor of metalloproteinase (TIMP) 1 and 2.

Activated lipocytes have the potential to remodel extracellular matrix as they produce both gelatinase A and stromelysin, MMP-3 (EC 3.4.24.17). Interstitial
Collagenase expression in liver has been ascribed to Kupffer cells and hepatocytes. Lipocytes may also produce this enzyme: passaged cell outgrowths of human liver, possibly of lipocyte origin, express interstitial collagenase constitutively; this is up-regulated by interleukin 1 and tumor necrosis factor α. Rat cultured hepatic lipocytes also express interstitial collagenase in response to polyunsaturated lecithin.

We have previously shown that TIMP-1 is increasingly expressed by lipocytes as they become activated in vitro. If overproduced in injured liver in vivo, TIMP-1 might promote the development of fibrosis. Progressive liver fibrosis is indeed associated with inhibition of interstitial collagenase activity. Collagenolytic activity in whole-liver homogenates decreases as liver fibrosis develops both in carbon tetrachloride–induced liver fibrosis in rats and in alcoholic liver disease in humans and baboons. It has been suggested that reduced collagenase activity in cirrhosis may prevent remodeling of fibrous lesions. How inhibition of collagenolysis occurs is uncertain, but the involvement of metalloproteinase inhibitors is suggested by studies of murine schistosomiasis showing decreased synthesis of interstitial collagenase and its increased binding to α2-macroglobulin as fibrosis develops.

In the present study, we compared the expression of messenger RNA (mRNA) for TIMP-1, TIMP-2, interstitial collagenase, and gelatinase A in liver biopsy specimens from normal individuals and patients with end-stage cirrhosis. The results indicate that increased expression of MMP inhibitors is a feature of advanced liver fibrosis and suggest that activated lipocytes contribute to increased hepatic expression of these inhibitors.

Materials and Methods

Liver Samples

Samples of snap-frozen fibrotic explant human liver (removed at orthotopic liver transplantation) from patients with diagnoses of biliary atresia, primary biliary cirrhosis (PBC), and primary sclerosing cholangitis (PSC) and samples of normal pediatric and adult human liver (not used for liver transplantation) were obtained from the Queen Elizabeth Hospital Liver Unit, Edgbaston, Birmingham, England.

RNA Isolation

Approximately 50–100-mg samples of human liver were homogenized in 1 mL of guanidinium isothiocyanate, pH 5.0, and RNA was purified using phenol-chloroform extraction. RNA isolated from each sample was analyzed by electrophoresis on 1% denaturing agarose gels; RNA was judged to be intact and suitable for further analysis when the 28S and 18S ribosomal RNAs were visualized as clearly defined bands after staining with ethidium bromide. RNA concentrations were assessed by absorbance at 260 nm using UV spectrophotometry.

Ribonuclease Protection Assays

Radiolabeled antisense riboprobes were prepared by in vitro transcription from relevant complementary DNA (cDNA) subcloned into pGEM 7 Zf-plasmid. The templates used were cDNA for gelatinase A (0.21 kilobase pairs [kbp]), TIMP-1 (0.9 kbp), TIMP-2 (0.9 kbp), and interstitial collagenase (1.4 kbp) provided by Dr. Gillian Murphy (Strangeways Research Laboratories, Cambridge, England). Identity of each cDNA was confirmed by sequencing approximately 200 bases of each cDNA. In vitro transcription was performed using 1 μg of linearized template cDNA in the presence of [α-32P]uridine 5′-triphosphate and either SP6 RNA polymerase (interstitial collagenase, TIMP-2) or T7 RNA polymerase (gelatinase A, TIMP-1). Riboprobes were purified using phenol-chloroform extraction followed by ethanol precipitation and confirmed to be of the expected molecular weight by denaturing agarose gel electrophoresis with autoradiography.

Aliquots of total liver RNA were subjected to ribonuclease protection assay for TIMPs, gelatinase A, and interstitial collagenase using a commercially available kit (RPA II kit; Ambion Bioscience, Whitney, Oxon, England). Briefly, sample RNA was hybridized at 45°C for 16 hours with 105 cpm of relevant 32P-labeled antisense riboprobes. The amount of RNA subjected to ribonuclease protection assay was standardized to 10 μg for TIMP-1 and TIMP-2 and 40 μg for interstitial collagenase and gelatinase A as assessed by optical density at 260-nm wavelength. However, because UV absorbance is mainly caused by the presence of ribosomal RNA (with mRNA contributing only 1%–2%), a more accurate estimate of the mRNA content of each sample was determined by performing, in parallel, ribonuclease protection assay for ribosomal protein S14, a constitutive “housekeeping” protein (antisense riboprobe prepared from a 0.59-kbp cDNA, a gift of Dr. J. Maher, Liver Center Laboratories, University of California, San Francisco, CA).

After overnight hybridization, RNA complexes were digested with ribonucleases A/T1, after which undigested protected fragments were subjected to electrophoresis on a 5% denaturing polyacrylamide-urea gel. After drying, gels were exposed to preflashed Fuji AR x-ray film (Fuji, Tokyo, Japan) for 1–10 days at ~70°C. Densitometry readings were taken of the major (largest) protected fragment of riboprobe in each lane of the gel. For each sample, the density of gelatinase A, TIMP-1, TIMP-2, and interstitial collagenase major protected fragments were normalized for sample mRNA content by expressing as a ratio of the major S14 protected fragment density for the same sample.

In each ribonuclease protection assay, specificity of the riboprobe for their target mRNA was shown by the absence of protected fragments after hybridization of riboprobes with yeast transfer RNA (10 or 40 μg as relevant).

Hydroxyproline Assay

Samples of 100–200 mg of human liver were dried at 80°C for 6 hours and accurately weighed. Dried samples were
Table 1. Immunocytochemical Detection of TIMP-1 and Interstitial Collagenase in Normal and Fibrotic Human Liver Biopsy Specimens

<table>
<thead>
<tr>
<th>Disease group</th>
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<th>Collagenase</th>
<th>TIMP-1/collagenase colocalization</th>
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<tbody>
<tr>
<td>Normal (n = 5)</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>PSC (n = 2)</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Fibrosis and cirrhosis (n = 4)</td>
<td>3</td>
<td>2</td>
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NOTE. Biopsy specimens of human liver were incubated with 5 μmol/L monensin for 6 hours before snap-freezing in liquid N2. Cryostat sections were used for immunodetection of TIMP-1 and interstitial collagenase as described.

aTwo alcoholic, 1 PBC, and 1 cryptogenic.

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hydrolyzed in 3 mL of 6 mol/L HCl for 16 hours at 105°C. Total hydroxyproline content of the hydrolysates was determined in duplicate by spectrophotometry using a dye-binding assay as described. Hydroxyproline content of samples was calculated by reference to hydroxyproline standards (2.6–9.8 μg assayed in parallel) and expressed as micrograms of hydroxyproline per gram of dry weight.

Human TIMP-1 Enzyme-Linked Immunosorbent Assay

Samples of human livers (~200 mg) were homogenized with an Ultra Turrax homogenizer (Janke and Kunkel Labortechnik) in 5 mL of ice-cold 50 mmol/L phosphate buffer (pH 7) containing 1 mol/L NaCl, 0.2% Tween 20, and 5 mmol/L CaCl2. Samples were clarified by centrifugation (14,000g for 20 minutes at 4°C), and 30-μL aliquots were assayed for total protein content using the bicinchoninic acid kit (Amersham International, Amersham, Buckinghamshire, England), pH 7.6. Activated gels—containing preflashed Fuji AR film for 48–72 hours at −70°C.

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Immunolocalization of TIMP-1 and Collagenase

Ethical committee permission was obtained to use a 3–5-mm core of routine Menghini needle liver biopsy specimens for immunostaining. Specimens were obtained from 6 patients with fibrotic liver disease. The histological diagnosis, determined using H&E stain for each biopsy, is listed in Table 1. In addition, five samples of normal liver were obtained from an area close to the resection margin from patients undergoing segmental liver resection for metastatic liver cancer. To promote intracellular accumulation of TIMP-1 and interstitial collagenase, fresh liver specimens were incubated for 6 hours in Dulbecco’s minimal essential medium (DMEM; Gibco, Paisley, England) containing 5 μmol/L monensin (Sigma Chemical Co.) at 37°C before snap-freezing in liquid nitrogen.

Immunostaining was performed using 3-μm cryostat sec-

tions air-fixed for 30 minutes. Sheep antibodies against human TIMP-1 and interstitial collagenase (gifts of Dr. R. Hembrey, Strangeways Laboratory) were applied at a concentration of 60 μg/mL for 1 hour in phosphate-buffered saline containing 5% bovine serum albumin. Nonimmune sheep immunoglobulin G (60 μg/mL) was used in negative control sections. Secondary antibody was a rabbit anti-sheep antibody labeled with fluorescein isothiocyanate (Southern Biotech, Europath, England) diluted 1:50 in phosphate-buffered saline plus 5% bovine serum albumin. Specimens were mounted in Citifluor (City University, London, England), viewed, and photographed using a Zeiss photomicroscope (Carl Zeiss Ltd., Welwyn Garden City, Herts, England).

Isolation and Culture of Human Hepatic Lipocytes

Human hepatic lipocytes were obtained from Wisconsin solution—perfused normal donor human liver as previously described and from the margin of hepatic resection specimens by a modification of the method of Pinzani et al. Cell purity, determined by endogenous vitamin A fluorescence and uptake of killed bakers yeast, consistently exceeded 90% with Kupffer cells as the only significant contaminant. Lipocytes were plated into plastic tissue culture flasks and maintained for 14 days in DMEM with 10% fetal calf serum.

Analysis of Lipocyte TIMP Expression by Northern Blotting and Reverse Zymography

Lipocyte RNA was isolated as described and subjected to electrophoresis on 1% agarose gels containing formaldehyde as denaturant. RNA was transferred to Hybond N nylon filters (Amersham International) by overnight capillary elution and fixed by baking at 80°C for 2 hours. Filters were probed with cDNA for human TIMP-1 and TIMP-2 random-primed using [α-32P]adenosine triphosphate as described. Before overnight hybridization at 42°C, filters were washed sequentially at 42°C (20 minutes) and 60°C (20 minutes) in 0.1× sodium chloride/sodium citrate (1× SSC is 0.15 mol/L NaCl plus 15 mmol/L sodium citrate) containing 0.1% sodium dodecyl sulfate. Filters were subjected to autoradiography using preflashed Fuji AR film for 48–72 hours at −70°C.

TIMP-1 and TIMP-2 secretion into lipocyte culture media was assessed using reverse zymography as described. Before harvest of media, cells were washed threefold in DMEM, preincubated in serum-free DMEM for 4 hours (which was discarded), and then incubated in serum-free DMEM for 24 hours. Aliquots of 10 μL of supernatant from lipocytes cultured on plastic for 14 days were subjected to electrophoresis at 4°C in 12% polyacrylamide gels containing 0.1% sodium dodecyl sulfate and 0.5 mg/mL gelatin. After electrophoresis, gels were washed twice for 20 minutes in 2.5% Triton X-100 and incubated for 16 hours at 37°C in a solution of 50 mmol/L Tris, 5 mmol/L CaCl2, and 0.04% Brij 35 (Imperial Chemical Industries, Leatherhead, Surrey, England), pH 7.6. Activated gelatinase A (7 U/mL), derived from rabbit epithelial cells (a gift
from Dr. Gillian Murphy, Strangeways Research Laboratory), was then added to this buffer to degrade gelatin within the polyacrylamide gel. Gels were then stained with Coomassie blue, after which stained bands manifest the presence of gelatinase inhibitory activity corresponding to TIMPs in the polyacrylamide gels.

Data Presentation and Statistical Analysis

Data are expressed as mean ± SEM. Differences between means were tested for significance using the Mann–Whitney U test. Mean values were considered to be significant at $P$ values of $<0.05$.

Results

Detection of Gelatinase A, Interstitial Collagenase, and TIMP mRNA in Normal and Fibrotic Liver

Figure 1 shows representative autoradiograms of ribonuclease protection assays measuring the expression of MMPs and TIMPs in RNA isolated from liver specimens from 4 normal children or 5 individuals with biliary atresia. Measurements were performed on three independent occasions for TIMP-1, interstitial collagenase, and gelatinase A and on two occasions for TIMP-2. The appropriate $^{32}$P-labeled antisense probes for TIMP-1, TIMP-2, interstitial collagenase, gelatinase A, and ribosomal protein S14 each detected their relevant mRNA. As usually found in this assay, the major protected fragments were of slightly lower molecular weight than undigested probes (position shown by arrow in Figure 1). For each individual sample used for the above assays, total mRNA content was determined in parallel using ribonuclease protection assay for a "housekeeping" protein, ribosomal protein S14, as described above. As expected, no significant changes were apparent in S14 expression between normal and diseased tissue. None of the antisense probes was protected after hybridization with an equivalent amount of yeast transfer RNA used as a negative control.

Expression of mRNA for TIMPs and MMPs was also determined in adult fibrotic liver. Figure 2 shows autoradiograms of ribonuclease protection assay gels from one of two independent experiments quantifying these mRNAs in the liver of normal individuals or those with end-stage PBC or PSC.

Figure 3 summarizes the changes in the expression of MMPs and TIMPs in liver specimens with biliary atresia, PBC, and PSC after correction for S14 mRNA loading into the assay. In each fibrotic liver disease, expression of TIMP-1, TIMP-2, and gelatinase A was significantly increased above normal. When the content of each mRNA species in normal liver was arbitrarily expressed as 100%, it was found that the mRNA level of TIMP-1 was elevated to 237%–526% ($P < 0.02$; $n = 5$), the level of TIMP-2 to 260%–458% ($P < 0.02$; $n = 5$), and the level of gelatinase A to 324%–430% ($P < 0.01$; $n = 5$). In contrast, interstitial collagenase mRNA expression was increased only in PBC livers, although this change was not significant overall.

Although showing variation between individual samples, ribosomal S14 protein mRNA expression was not significantly altered in the fibrotic livers compared with
normal livers. When results from three separate ribonuclease protection assays were pooled, expression of S14 as percentage of normal was 89% ± 9% (n = 15) in biliary atresia specimens, 107% ± 9% in PBC specimens (n = 18), and 112% ± 12% (n = 15) in PSC specimens.

**Correlation of TIMP mRNA Expression With Liver Hydroxyproline Content**

To assess whether TIMP-1 and TIMP-2 expression in the liver was related to the extent of fibrosis, TIMP mRNA content, determined by densitometry of ribonuclease protection assay autoradiograms, was compared with the tissue content of hydroxyproline, used as an indicator of collagen deposition, in each sample. As expected for samples derived from individuals with end-stage cirrhosis, hydroxyproline content of fibrotic livers was significantly greater than that of normal liver samples. These values (micrograms of hydroxyproline per gram) were 164 ± 13 for normal liver specimens (n = 11), 441 ± 62 for PBC specimens (P < 0.002; n = 6), 482 ± 40 for PSC specimens (P < 0.003; n = 6), and 509 ± 38 for biliary atresia specimens (P < 0.003; n = 5). As shown in Figure 4, there was a significant correlation between expression of mRNA for TIMP-1 and TIMP-2 in liver samples and their content of hydroxyproline. For technical reasons, it was not possible to pool ribonuclease protection assay data from adult liver samples (adult normal, PBC, and PSC specimens) with pediatric samples (child normal and biliary atresia specimens); correlation coefficients were therefore calculated separately. Calculated correlation coefficients were as follows: TIMP-1 vs. hydroxyproline (adult), r = 0.652 (P < 0.05; n = 12); TIMP-1 vs. hydroxyproline (child), r = 0.857 (P < 0.01; n = 9); TIMP-2 vs. hydroxyproline (adult), r = 0.798 (P < 0.01; n = 16); and TIMP-2 vs. hydroxyproline (child), r = 0.774 (P < 0.02; n = 9).

**TIMP-1 Protein Content of Liver Samples**

To complement the above studies of TIMP-1 mRNA expression, levels of TIMP-1 protein in liver homogenates were determined using a commercial sandwich ELISA. An equal quantity (50 μg of total protein) of each liver homogenate was assayed. The results of the ELISA showed that there were significant increases in TIMP-1 concentrations in liver extracts of each type of fibrotic disorder compared with that of normal samples. TIMP-1 concentrations (nanograms of TIMP-1 per mi-

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**Figure 2.** Quantitation of TIMP and MMP mRNA in normal and fibrotic adult human liver. Total RNA isolated from the liver of normal adults or from individuals with PBC or PSC was subjected to ribonuclease protection assay for mRNA of TIMP-1, TIMP-2, interstitial collagenase (MMP-1), gelatinase A (MMP-2), or ribosomal protein S14. Results are a representative autoradiogram from one of two independent experiments. Arrows mark position and molecular weight of antisense riboprobes used in each experiment.

**Figure 3.** Changes in TIMP and MMP mRNA expression in fibrotic vs. normal liver. Scanning densitometry was used to quantitate the amount of the major protected fragment (i.e., the highest band) in each lane of ribonuclease protection assay gels. The density of the fragment protected in assays for TIMP-1, TIMP-2, interstitial collagenase (MMP-1), and gelatinase A (MMP-2) was expressed as a ratio of S14 mRNA content of each sample. Abundance of each mRNA in normal liver specimens was assigned a value of 100%. □, Biliary atresia; ■, PBC; □, PSC.
Immunolocalization of TIMP-1 and Interstitial Collagenase in Human Liver Biopsy Specimens

TIMP-1 was detected in diseased liver samples only (4 of 6) and could not be found in normal human liver. The protein was predominantly perisinusoidal with an intracellular distribution in cells with small nuclei and cytoplasmic extensions (Figure 5A). Labeling was not evident in hepatocytes. Without preincubation with monensin, TIMP-1 could not be detected, even in diseased samples, indicating that TIMP-1 accumulates within the perisinusoidal cells during a 6-hour period of monensin exposure. The overall results for TIMP-1 immunostaining are shown in Table 1. The histological diagnoses of the livers positive for TIMP-1 were as follows: PSC, 1 of 2; alcoholic cirrhosis, 1 of 2; PBC, 1 of 1; and cryptogenic fibrosis, 1 of 1.

Interstitial collagenase was detected in 1 normal and 3 diseased livers (Table 1). Interstitial collagenase alone was detected in 1 normal liver, but in the 3 further interstitial collagenase–positive livers (PSC, alcoholic cirrhosis, and cryptogenic fibrosis), TIMP-1 was also detected by immunostaining. Immunocytochemical staining of liver sections showed that interstitial collagenase resembled TIMP-1 in being localized to cells with a perisinusoidal distribution (Figure 5B). No specific fluorescence was detected in the nonimmune immunoglobulin G controls (Figure 5C).

Analysis of TIMP-1 and TIMP-2 Production by Human Cultured Lipocytes

Northern blots of RNA isolated from lipocytes activated by culture on plastic for 14 days in the presence of serum were probed for the presence of TIMP-1 and TIMP-2 mRNA as described (Figure 6). Both TIMP-1 and TIMP-2 mRNA were detected, each with a size of approximately 0.9 kilobases. To prevent the possibility of cross-hybridization of the riboprobes with heterologous mRNAs (human TIMP-1 and TIMP-2 show limited sequence homology of 40%18), hybridizations were performed under stringent conditions (0.1× SSC, 60°C).

Lipocyte production of TIMP-1 and TIMP-2 protein was further shown using reverse zymography (Figure 7). In supernatants from two separate lipocyte cultures, undegraded areas of gelatin due to presence of MMP inhibitors were detected at approximately 29 and 20 kilodaltons (Figure 7, lanes A and B). These bands were found to correspond exactly with the position of authentic TIMP-1 and TIMP-2, respectively, when these were run in parallel (lane C).

**Discussion**

The results of this study show that there is a significant increase in the expression of mRNA for the...
TIMP inhibitors TIMP-1 and TIMP-2 in both pediatric fibrotic liver disease (biliary atresia) and adult fibrotic liver disease (PBC and PSC) in the absence of any significant change in interstitial collagenase mRNA expression. There was a significant correlation between expression of mRNA for the TIMPs and the liver content of hydroxyproline, which supports the hypothesis that these inhibitors play an important role in promoting collagen deposition. The studies of mRNA expression were supported by ELISA measurements of TIMP-1 protein levels in liver homogenates in which fibrotic livers were shown to contain 367%–724% of normal liver TIMP-1 content.

The molecular investigations were complemented by immunohistochemical analysis of TIMP-1 and interstitial collagenase protein in various liver diseases. The studies required incubation of the liver samples in monensin (5 μmol/L) to promote intracellular accumulation of proteins; its necessity was shown by the total lack of immunoreactivity for either protein in the absence of monensin pretreatment. The present study showed results that are similar to our studies of mRNA levels; TIMP-1 was readily detectable in diseased and particularly fibrotic

**Figure 5.** Immunostaining for TIMP-1 and interstitial collagenase in a monensin-pretreated human liver biopsy from a patient with cryptogenic fibrosis. Human liver biopsy specimens were immunostained for (A) TIMP-1 or (B) interstitial collagenase as described. TIMP-1 and interstitial collagenase were localized in cells with a perisinusoidal distribution. (C) No specific staining was observed in the nonimmune immunoglobulin G control.

**Figure 6.** Northern blot detection of TIMP-1 and TIMP-2 mRNA in human activated lipocytes. RNA isolated from lipocytes cultured on plastic for 14 days was subjected to agarose gel electrophoresis and transfer to nylon filters. Messenger RNA for TIMP-1 or TIMP-2 was detected after hybridization with relevant 32P-labeled cDNA probes and autoradiography as described.
We have also shown that gelatinase A released from activated human lipocytes is predominantly complexed with TIMPs, with <5% being in the form of free enzyme. 

Accumulation of TIMPs would be expected to promote extracellular matrix deposition in fibrosis through their inhibition of several MMPs responsible for matrix degradation, including interstitial collagenase, stromelysins, and gelatinases. This hypothesis is supported by previous findings showing metalloproteinase inhibition associated with fibrosis in kidney, lung, and liver. TIMP-1 expression is markedly increased in the kidneys of a mouse model of lupus nephritis, whereas both TIMP-1 and TIMP-2 expression is increased in glomeruli of humans with glomerulonephritic renal disease. 

Increased amounts of a collagenase inhibitor activity has also been detected in homogenates of fibrotic vs. normal human lung. In murine schistosomiasis, there is increased binding of α₂-macroglobulin to interstitial collagenase as fibrosis develops, whereas liver fibrosis after CCl₄ administration to rats is associated with reduced collagenolytic activity of liver homogenates. 

Increased amounts of an interstitial collagenase and gelatinase A inhibitor of 40 kilodaltons have also been detected in human livers with cirrhosis of diverse etiology, including viral hepatitis and biliary cirrhosis. 

Our results provide further evidence associating increased expression of MMP inhibitors with fibrosis in human liver disease.

In fibrotic liver, we have found that expression of gelatinase A mRNA differs from that of interstitial collagenase in being increased 3–4 times the levels in normal liver, a change similar to that observed with the TIMPs. These studies confirm recent findings in which Northern blotting and in situ hybridization were used to show increased expression of gelatinase A in liver fibrosis of viral etiology. Gelatinase A was localized to perisinusoidal cells expressing desmin and α-actin that were presumably lipocytes. This is in accord with our previous studies showing increased gelatinase A expression by human lipocytes after in vitro activation by culturing on plastic. It is possible that production of gelatinase A by lipocytes, and possibly other cells, after liver injury disrupts the type IV collagen framework of the subendothelial (perisinusoidal) basement membrane normally required for maintenance of hepatic function and lipocyte quiescence. Gelatinase A activity may therefore give rise to two of the key features of liver fibrosis: lipocyte proliferation and hepatocyte dysfunction. However, our findings of increased gelatinase A mRNA expression in advanced fibrosis seem inconsistent with the accumulation of type IV collagen that actually occurs in human cir-
TIMPs as important profibrogenic mediators in the liver. Nevertheless, our data provide a basis for considering ways of manipulating TIMP levels in liver in vivo.

Normal rat liver release a neutral metalloproteinase that degrades interstitial collagenase and other metalloproteinases in localized areas of fibrotic liver together with transforming growth factor \( \beta_1 \), showing that this is an autocrine stimulator of these cells. An alternative suggestion is provided by the observation that lipocyte activation is accompanied by hydrolysis and release of intracellular retinoids. This mechanism may be relevant to lipocyte activation in cell culture or in vivo because retinoids are known to up-regulate TIMP-1 and gelatinase A expression in fibroblasts, a pattern of change that we now describe in whole liver. Activated lipocytes express receptors for and produce transforming growth factor \( \beta_1 \), suggesting that this is an autocrine stimulator of these cells.

Differential expression of gelatinase A and TIMP-1 relative to interstitial collagenase in fibrotic liver may be mediated by transforming growth factor \( \beta_1 \), whose expression is increased in the liver during progression of fibrosis. This cytokine is known to increase TIMP-1 and gelatinase A synthesis in fibroblasts, a pattern of change that we now describe in whole liver. Activated lipocytes express receptors for and produce transforming growth factor \( \beta_1 \), suggesting that this is an autocrine stimulator of these cells. An alternative suggestion is provided by the observation that lipocyte activation is accompanied by hydrolysis and release of intracellular retinoids. This mechanism may be relevant to lipocyte activation in cell culture or in vivo because retinoids are known to up-regulate TIMP-1 and gelatinase A in other cell types. However, the signal for TIMP-2 up-regulation that we describe in liver fibrosis remains uncertain: TIMP-2 expression is regulated differently from TIMP-1 and is actually decreased by transforming growth factor \( \beta_1 \).

Whatever the underlying signal mechanisms, our studies indicate that there is an excess expression of TIMPs relative to interstitial collagenase in the human fibrotic liver, supporting the concept that TIMPs play a role in the pathogenesis of liver fibrosis. We recognize that our studies are largely descriptive at this stage, concentrating on total liver mRNA expression and protein detection by immunocytochemistry. More definitive evidence must await development of methods to measure net activity of interstitial collagenase and other metalloproteinases in localized areas of fibrotic liver together with ways of manipulating TIMP levels in liver in vivo. Nevertheless, our data provide a basis for considering TIMPs as important profibrogenic mediators in the liver.

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