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Inhibition of Apoptosis of Activated Hepatic Stellate Cells by Tissue Inhibitor of Metalloproteinase-1 Is Mediated via Effects on Matrix Metalloproteinase Inhibition

IMPLICATIONS FOR REVERSIBILITY OF LIVER FIBROSIS*

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The activated hepatic stellate cell (HSC) is central to liver fibrosis as the major source of collagens I and III and the tissue inhibitors of metalloproteinase-1 (TIMP-1). During spontaneous recovery from liver fibrosis, there is a decrease of TIMP expression, an increase in collagenase activity, and increased apoptosis of HSC, highlighting a potential role for TIMP-1 in HSC survival. In this report, we use tissue culture and *in vivo* models to demonstrate that TIMP-1 directly inhibits HSC apoptosis. TIMP-1 demonstrated a consistent, significant, and dose-dependent antiapoptotic effect for HSC activated in tissue culture and stimulated to undergo apoptosis by serum deprivation, cycloheximide exposure, and nerve growth factor stimulation. A nonfunctional mutated TIMP-1 (T2G mutant) in which all other domains are conserved did not inhibit apoptosis, indicating that inhibition of apoptosis was mediated through MMP inhibition. Synthetic MMP inhibitors also inhibited HSC apoptosis. Studies of experimental liver cirrhosis demonstrated that persistent expression of TIMP-1 mRNA determined by PCR correlated with persistence of activated HSC quantified by α smooth muscle actin staining, while in fibrosis, loss of activated HSC correlated with a reduction in TIMP-1 mRNA. We conclude that TIMP-1 inhibits apoptosis of activated HSC via MMP inhibition.

Liver fibrosis represents the final common pathological outcome for the majority of chronic liver insults (*e.g.* alcohol, autoimmune, or viral injury) (1). Current evidence indicates that the central mediator of liver fibrosis is the hepatic stellate cell (HSC)[1] (2). During fibrotic injury, these retinoid-rich perisinusoidal cells proliferate and undergo a phenotypic transformation to myofibroblast-like cells, a process termed activation (3, 4). Previous work has demonstrated that in addition to collagen-I (5), activated HSC also express tissue inhibitors of metalloproteinases 1 and 2 (TIMP-1 and -2), leading to the hypothesis that matrix degradation is inhibited during progressive fibrosis (6–12). This hypothesis is supported by findings that overexpression of TIMP-1 enhances experimental fibrosis (13) and that spontaneous recovery from liver fibrosis is associated with a diminution of TIMP expression and an increase in collagenase activity with consequent matrix degradation (14). A further finding in this study was that apoptosis was responsible for mediating HSC loss during recovery from fibrosis (14–17). This has highlighted the control of HSC apoptosis as a key process regulating fibrosis *in toto*. Indeed, we have recently demonstrated that induction of HSC apoptosis has an antifibrotic effect (18).

While previous work has emphasized the potential importance of TIMPs to fibrosis via inhibition of matrix degradation, individual TIMPs may regulate cell division and apoptosis independently of this activity. TIMP-1 has been shown *in vitro* to suppress apoptosis of Burkitt's lymphoma cell lines (19) and human breast epithelial cells (20). In these studies the antiapoptotic effect of TIMP-1 was reported to be independent of its ability to inhibit MMP activity. Moreover, several neoplasms demonstrate a positive correlation between TIMP-1 and TIMP-2 expression and metastatic spread. This observation is counterintuitive to the previously accepted hypothesis that TIMP-1-mediated inhibition of matrix degradation would retard tumor dissemination (21). TIMP-1 was originally identified as a growth factor for myeloid elements and has also been demonstrated to promote fibroblast proliferation (22, 23). These data suggest that TIMPs may be important regulators of cell growth and apoptosis.

The TIMPs appear to have divergent effects on proliferation and apoptosis in different cell types. For example, TIMP-2 acts as a growth factor for mesenchymal cells in rat kidney development (24), whereas it is a proapoptotic stimulus for human T lymphocytes (25). Adenoviral overexpression of TIMP-1, -2, and -3 has been studied in rat aorta smooth muscle cells. Within the same cell type, the TIMPs had divergent effects; TIMP-1 overexpression had no effect on cell proliferation, whereas TIMP-2 produced a dose-dependent reduction in proliferation. This effect was not mimicked by a synthetic matrix metalloproteinase inhibitor. TIMP-3 overexpression induced DNA synthesis and promoted apoptosis in myofibroblasts (26). In contrast, TIMP-2

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[1] The abbreviations used are: HSC, hepatic stellate cell; TIMP, tissue inhibitors of metalloproteinase; TBS, Tris-buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; BSA, bovine serum albumin; NGF, nerve growth factor; MMP, matrix metalloproteinase; MMPI, MMP inhibitor; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling.

overexpression in B16F10 melanoma cells protects these cells from apoptosis but had no effect on proliferation (27). The close correlation between the reduction of TIMP-1 expression and apoptosis of HSC observed *in vivo* during recovery from liver fibrosis highlights a possible role for TIMP-1 in regulating HSC survival.

Recently, a mutated N-TIMP-1 (threonine 2 to glycine, or T2G, mutant) has been developed, which has virtually no MMP inhibitory activity compared with the wild type TIMP-1, while its other domains are conserved. Furthermore, the secondary structure of this mutated protein is not significantly different from the wild type protein (28). This mutated TIMP-1 now allows the dissection of any anti- or proapoptotic effect in stellate cells to be examined. In particular, this facilitates experiments to determine whether any TIMP effects on apoptosis are due to MMP inhibition or via alternative and novel mechanisms. We have therefore studied the role of TIMP-1 in regulating the survival of HSC in primary culture and rat models of fibrosis *in vivo*. We have gone on to demonstrate that TIMP-1 is antiapoptotic for HSC, and the antiapoptotic effect of TIMP-1 for HSC is dependent on MMP inhibition. We have also demonstrated that persistent TIMP-1 expression in a 12-week model of carbon tetrachloride-induced cirrhosis is associated with persistence of HSC and decreased recovery of liver fibrosis.

MATERIALS AND METHODS

Isolation of Human and Rat Hepatic Stellate Cells—Human HSC were extracted from the margins of normal human liver resected for colonic metastatic disease as previously described (29). Rat HSC were extracted from normal rat liver by Pronase and collagenase digestion and purified by centrifugal elutriation as described (10). Extracted HSC were cultured on plastic until they were activated to a myofibroblastic phenotype after 7–10 days. Human and rat HSC were used for experiments after activation in primary culture or before fourth passage. Cells were cultured in Dulbecco's modified Eagle's medium in the presence of 16% fetal calf serum and antibiotics.

Effect of TIMP-1 on HSC Proliferation—HSC were cultured in 24-well tissue culture plates. These were washed with serum-free medium for 24 h, and then the cells were exposed to TIMP-1 at a concentration range of 1–100 ng/ml for 24 h and then pulsed with tritiated thymidine (0.5 μCi/well) for 18 h before scintillation counting as previously described (30).

Stimulation of HSC Apoptosis and Examination of Nuclear Morphology by Acridine Orange—Apoptosis of HSC was induced by absolute serum deprivation, cycloheximide treatment (15), or exposure to nerve growth factor as previously described (31). HSC were cultured in 24-well tissue culture plates. Rat and human HSC were exposed to proapoptotic stimuli with and without recombinant TIMP-1 (Biogenesis, Poole, UK) and other manipulations as detailed below. Following a 4-h incubation at 37 °C, nuclear morphology was assessed by adding acridine orange to each well (final concentration 1 μg/ml) and observing the cells under blue fluorescence. The total number of apoptotic bodies was counted, and any apoptotic bodies floating in the supernatant were included by racking up the objective lens. The total number of cells per field was counted, and an apoptotic index was calculated. Each condition was performed in duplicate, and three high power fields were counted for each well. Experiments were repeated in parallel following an 18-h incubation in serum-free conditions. To examine for autocrine effects, HSC were incubated for 18 h with azide-free polyclonal neutralizing antibodies to TIMP-1 and a nonimmune IgG control (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and responses were assessed by acridine orange staining and counting. Parallel experiments using the nonfunctional T2G mutant N-TIMP-1 and wild type TIMP-1 proteins were performed in which apoptosis was induced by cycloheximide and assessed by the acridine orange technique.

TUNEL Staining—HSC were cultured on glass chamber slides and then exposed to 50 μM cycloheximide for 18 h with and without TIMP-1 (100 ng/ml). Slides were then stained for DNA fragmentation characteristic of apoptosis by the TUNEL reaction as previously described (14) with the modifications recently described to reduce false positivity (32). Each slide was then analyzed by a blinded observer who counted the number of TUNEL-positive apoptotic figures and the TUNEL-negative cells over 10 high power fields for each condition.

Determination of Caspase-3 Activity—To support the data from acridine orange counting and TUNEL staining, experiments with recombinant TIMP-1, the inactive T2G mutant N-TIMP-1, the wild type TIMP-1 proteins, and the broad spectrum caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone were repeated. Apoptosis was quantified by a colorimetric assay for caspase-3 activity (Promega) according to the manufacturer's instructions. To determine whether TIMP-1 directly inhibited apoptosis of caspase-3, each recombinant protein was incubated with recombinant caspase-3 for 1 h before adding the caspase-3 substrate, and then caspase-3 activity was measured as described above.

Measurement of DNA Concentration by PicoGreen Fluorescence—Cultured HSC were harvested with a sterile cell scraper, pelleted by centrifugation, and then resuspended in 500 μl of TE buffer (10 mmol/Tris-HCl, 1 mmol/liter EDTA, pH 8.0) before sonication for 15 min. 100 μl of PicoGreen (Molecular Probes, Inc., Eugene, OR) at 1:200 dilution was added to 100 μl of sample and incubated in the dark at room temperature for 5 min. Standards were made from herring sperm DNA. Fluorescence was measured using a Cytofluor II Microwell Fluorescence reader (Perceptive Biosystems, Framingham, MA) at standard wavelengths (excitation 485 nm, emission 530 nm). Concentrations of double-stranded DNA in the samples were calculated from the standard curve.

Western Blotting for α Smooth Muscle Actin and Bcl-2—Western blot analysis of rat liver tissue and HSC was undertaken using a monoclonal anti-α smooth muscle action antibody (Sigma) and a monoclonal antibody to Bcl-2 (Santa Cruz Biotechnology) to detect protein expression. The extracted proteins were subjected to electrophoresis on 12% SDS-PAGE gel after normalization for protein content. After resolution, the protein samples were electrotransferred onto polyvinylidene difluoride. The membrane was blocked for 1 h in 5% nonfat dry milk in TBS. Membranes were incubated overnight at room temperature with the primary antibody (1:500) or with nonimmune IgG (as negative control) in TBS. Membranes were washed three times for 15 min in 0.1% Tween TBS (TTBS) before the addition of the secondary antibody (rabbit anti-mouse IgG horseradish peroxidase in a 1:2000 dilution) in TBS containing 0.5% nonfat dry milk for 1 h. The membranes were then washed in TTBS twice for 10 min, followed by distilled water for 10 min. Reactive bands were identified using ECL (Amersham Biosciences) and autoradiography according to the manufacturer's instructions.

Experimental Models of Progressive Fibrosis and Fibrosis Recovery—Experimental models of reversible fibrosis and cirrhosis were established by injecting cohorts of 12 Sprague-Dawley rats with carbon tetrachloride twice weekly intraperitoneally for 6 and 12 weeks, respectively. For each model, livers were harvested at peak fibrosis (immediately after the final injection of carbon tetrachloride) and at 5 and 15 days of spontaneous recovery ($n = 4$ at each time point in each model). Harvested livers were split and fixed for hematoxylin and eosin and Sirius Red staining, and a portion was snap frozen for biochemical and molecular analysis. Histological analysis of each liver was undertaken, and in addition samples of frozen liver at peak fibrosis and 15 days of recovery were analyzed for hydroxyproline and total collagenase activity as previously described (14). The MMPs that would be expected to show activity in this assay are the interstitial collagenases (MMP-1 and MMP-13), gelatinase A (MMP-2), and membrane type 1 MMP (MMP-14). Further sections were cut from each liver, deparaffinized, and subjected to microwave antigen retrieval before being immunostained for α smooth muscle actin exactly as previously described (14). Three normal untreated rat livers were also harvested for use as controls in individual experiments. The number of α smooth muscle actin positive cells was counted by a blinded observer exactly as described previously (14).

Determination of Messenger RNA for TIMP-1 and GAPDH Using Taqman Real Time Quantitative PCR—Total RNA was extracted from snap frozen 6- and 12-week carbon tetrachloride-treated rat livers at day 0 (peak fibrosis) and after 15 days of spontaneous recovery (Qiagen). The first strain cDNA synthesis was undertaken using random primers and the Moloney murine leukemia virus reverse transcriptase system (Promega). All primers and probes were designed using the Taqman Primer Express program, and real time Taqman PCR mRNA quantitation using the PerkinElmer Applied Biosystems 7700 Sequence Detection System. Primers and probe sequences of rat GAPDH used were as follows: sense, 5′-ggcctacatggcctccaa-3′; antisense, 5′-tctctcttgctctcagtatccttgc-3′; and probe, 5′-agaaaccctggaccacccagccc-3′. Rat TIMP-1 primers and probe sequences used were as follows: sense, 5′-agcctgtagctgtgccccaa-3′; antisense, 5′-aactcctcgctgcggttctg-3′; probe, 5′-agaggctctccatggctggggtgta-3′.

1 μl of first strand cDNA (10 ng of RNA), 0.3 μM primers, and 0.3 μM probe were used per 25-μl real time Taqman PCR. Taqman 2× Univer-

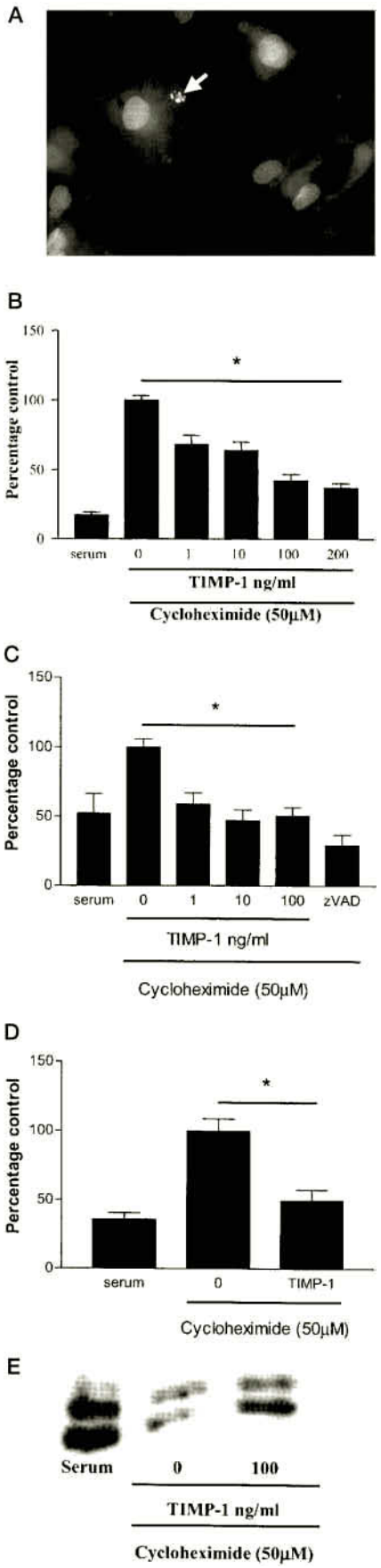

FIG. 1. *A*, example of an apoptotic HSC (*arrow*) induced by cycloheximide exposure for 4 h and identified *in situ* by acridine orange staining. A normal cell lies adjacent to the apoptotic body. *B*, TIMP-1 significantly reduces apoptosis of activated hepatic stellate cells induced by 4-h cycloheximide exposure in a dose-dependent manner over the concentration range 1–200 ng/ml (0.035–7 nM). Apoptosis was determined by acridine orange staining and counting. Data presented are mean ± S.E. expressed as percentage of control given the arbitrary value of 100%. *, $p < 0.001$ for cycloheximide *versus* cycloheximide with 200 ng/ml TIMP-1 by Student's t test, $n = 5$. *C*, caspase-3 activity of cell extracts from HSC treated with cycloheximide with and without TIMP-1 were compared with HSC incubated in serum and benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone controls. TIMP-1 showed a dose-dependent reduction in caspase-3 activity over the concentration range 1–100 ng/ml. Data are expressed as mean ± S.E. and are presented as percentage of control given the arbitrary value of 100%. *, $p < 0.001$; $n = 3$. *D*, activated hepatic stellate cells were induced to undergo apoptosis by cycloheximide treatment in the presence and absence of TIMP-1. Treatment with TIMP-1 (100 ng/ml) resulted in significantly reduced numbers of apoptotic cells defined by the presence of DNA fragmentation detected by the TUNEL technique. Data are expressed as mean ± S.E. and presented as percentage of control, which has been given the arbitrary value of 100%. *, $p < 0.001$; $n = 2$. *E*, Western blotting for Bcl-2 of equal quantities (determined by protein concentration) of protein extracts from HSC exposed to serum alone, cycloheximide alone, and cycloheximide with TIMP-1 protein (100 ng/ml). Treatment with TIMP-1 resulted in an increase in Bcl-2 expression relative to HSC treated with cycloheximide alone.

sal PCR Master Mix and 0.2 ml of optical reaction tube (PerkinElmer Applied Biosystems) were employed. The conditions of the reaction were as follows. Initial steps were 50 °C for 2 min and 95 °C for 10 min, followed with a denaturing step for 15 s at 95 °C and an annealing extension step at 60 °C for 1 min. Determination of the expression of the housekeeping gene, GAPDH, was employed, and all reactions were undertaken in triplicate. After detection of the threshold cycle for each mRNA in each sample, relative concentrations were calculated and normalized to GAPDH analyzed in parallel.

Enzyme-linked Immunosorbent Assay for Fas and Fas Ligand—Human HSC were grown to confluence and exposed to BSA with and without TIMP-1. Cells and supernatants were harvested, and protein extracts were assayed for Fas and Fas ligand by commercial enzyme-linked immunosorbent assay following the manufacturer's instructions (Calbiochem). The quantities of Fas and Fas ligand were normalized to cell number by DNA quantification using the PicoGreen technique.

RESULTS

TIMP-1 Inhibits Apoptosis Induced by Cycloheximide, Serum Deprivation, and Nerve Growth Factor—We and others have previously demonstrated that the major mechanism mediating the reduction in HSC numbers during spontaneous recovery from liver fibrosis is apoptosis (14–17). Because our previous data suggested a possible correlation between TIMP-1 expression and survival of the activated HSC population, we went on to study the potential antiapoptotic effect of TIMP-1 in tissue culture. Assessment of nuclear morphology following acridine orange staining (Fig. 1*A*) showed that 4-h incubation with TIMP-1 significantly reduced apoptosis of human and rat HSC induced by cycloheximide in a dose-dependent manner at a concentration range of 1–200 ng/ml (Fig. 1*B*). An identical effect with TIMP-1 was observed after a 24-h incubation in serum-free conditions (data not shown). Bovine serum albumin, used as a carrier for the TIMP-1 had no antiapoptotic effect.

Furthermore, parallel experiments with human hepatic stellate cells treated with cycloheximide for 4 h or serum deprivation for 18 h demonstrated identical antiapoptotic effects for TIMP-1 (data not shown; $n = 4$).

TIMP-1-treated HSC Have Reduced Caspase-3 Activity following Induction of Apoptosis by Cycloheximide—Caspase-3 is a central caspase in the proapoptotic cascade (33) and can be used as an alternative assay to assess apoptosis. HSC cultured in 50 μM cycloheximide with TIMP-1 (1–100 ng/ml) demonstrated a dose-dependent reduction in caspase-3 activity compared with cycloheximide alone (Fig. 1*C*). Although the mean caspase-3 activity of the cells treated with 10 ng/ml TIMP-1 was slightly lower than that treated with 100 ng/ml of TIMP-1,

this was not statistically significant ($p = 0.67$ by Student's t test). Therefore, this did not represent a reversal of the dose trend observed by acridine orange staining and counting.

The caspase-3 data and acridine orange morphological data did not correlate exactly with each other. For example, TIMP-1 at a concentration of 10 ng/ml caused a 50% reduction in caspase-3 activity but only a 30% reduction in apoptotic morphology by acridine orange staining and counting. At the higher dose of 100 ng/ml, TIMP-1 appeared to reduce apoptosis by 50% measured by both techniques. These observed differences in dose response may be due to two factors. First, the precision of each assay is unlikely to be the same. Second, while the caspase-3 activity assay is accepted as a measure of apoptosis, it is at best only a measure of one out of the 16 known caspase enzymes in what is clearly a complicated enzymatic cascade, which ends in the morphological changes that are characteristic of apoptosis.

To exclude a direct effect of TIMP-1 on caspase-3 activity, recombinant human caspase-3 (Calbiochem) was incubated with TIMP-1 in varying concentrations (285–2850 ng/ml) for 1 h before caspase-3 substrate was added to the reaction. TIMP-1 did not reduce caspase-3 activity directly (data not shown).

TIMP-1-treated HSC Have Reduced DNA Fragmentation Assessed by the TUNEL Technique following Induction of Apoptosis by Cycloheximide—A further pathognomonic feature of apoptosis is the fragmentation of DNA into oligonucleosomal lengths (34). Fragmented DNA can be identified by the TUNEL technique, which can therefore be used to further quantify the apoptotic response of HSC in the presence and absence of cycloheximide. Activated HSC cultured on glass chamber slides and exposed to cycloheximide for 18 h with TIMP-1 demonstrated significantly reduced numbers of cells containing fragmented DNA assessed by the TUNEL technique compared with controls treated without TIMP-1 (Fig. 1*D*).

TIMP-1 Enhances Expression of Bcl-2 Protein—The protein Bcl-2 regulates the properties of cells to undergo apoptosis by interpolating into the mitochondria membrane (33). Bcl-2 increases the resistance of cells to apoptosis. To define changes in the protein level of Bcl-2, extracts from HSC treated with cycloheximide, in the presence and absence of 200 ng/ml TIMP-1 for 18 h, were analyzed by Western blotting. Relative to cells treated with cycloheximide alone, cells treated with TIMP-1 and cycloheximide demonstrated enhanced levels of Bcl-2 protein expression (Fig. 1*E*), which approached the levels observed in HSC maintained in serum alone.

TIMP-1 Inhibits Apoptosis Induced by Nerve Growth Factor—We have previously demonstrated that HSC express low affinity nerve growth factor receptor (p75) and undergo apoptosis in response to nerve growth factor (NGF) stimulation (31). To determine whether TIMP-1 reduced NGF-induced apoptosis, NGF-activated HSC were exposed to NGF (100 ng/ml) in conditions of absolute serum deprivation with and without TIMP-1 (142.5 ng/ml). As expected, NGF induced significantly more apoptosis in HSC than cells treated with BSA carrier alone (data not shown). TIMP-1 significantly reduced the apoptosis induced by nerve growth factor (Fig. 2).

TIMP-1 Is an Autocrine Survival Factor for HSC—We and others have previously demonstrated that TIMP-1 is major synthetic product of activated HSC. Therefore, TIMP-1 is potentially an autocrine survival factor for HSC. To determine the effect of neutralizing HSC-derived TIMP-1, HSC were incubated with azide free polyclonal neutralizing antibodies to TIMP-1 for 18 h in 5% bovine serum albumin. This demonstrated significantly increased apoptosis of HSC treated with TIMP-neutralizing antibodies compared with a nonimmune IgG control, suggesting that TIMP-1 acts as a survival factor in an autocrine manner for activated HSC (Fig. 3).

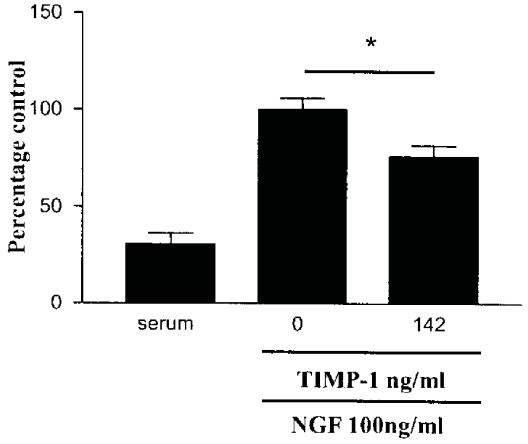

FIG. 2. **To determine whether TIMP-1 could reduce apoptosis induced by nerve growth factor, activated HSC were incubated in serum and serum-free conditions.** Apoptosis induced by exposure to NGF (100 ng/ml) in serum-free conditions, was significantly inhibited by TIMP-1 (142.5 ng/ml). Data are expressed as mean ± S.E. and presented relative to control given the arbitrary value of 100%; *, $p < 0.02$ for NGF treated alone *versus* NGF with TIMP-1 treatment by Student's t test; $n = 3$.

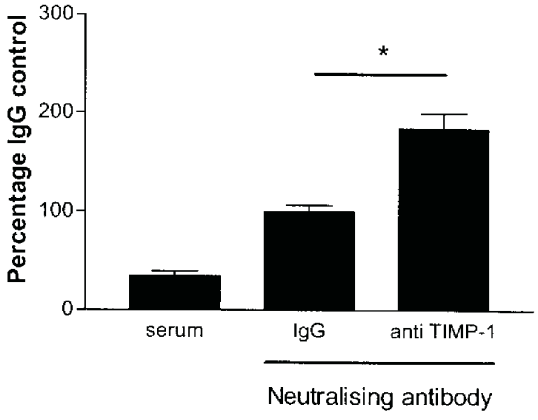

FIG. 3. **Activated hepatic stellate cells were incubated in serum-free conditions with 5% BSA for 18 h in the presence and absence of neutralizing antibodies to TIMP-1 and compared with a nonimmune IgG control antibody as described under "Materials and Methods."** Neutralizing TIMP-1 significantly increased apoptosis of activated HSC compared with exposure to the nonimmune IgG control. All antibodies were in azide-free buffer. Apoptosis was quantified by the acridine orange technique. Data are expressed as mean ± S.E. and presented relative to control, which has been given the arbitrary value of 100%. *, $p < 0.0001$ by Student's t test for HSC treated with neutralizing antibodies for TIMP-1 relative to nonimmune IgG control; $n = 3$.

The Antiapoptotic Effect of TIMP-1 for HSC Is Mediated via MMP Inhibition—Previous studies have suggested that the observed antiapoptotic effect of TIMP-1 in other cell systems was independent of MMP inhibition. Our data showed an effective antiapoptotic activity for TIMP-1. To determine whether this effect might be mediated via MMP inhibition, we undertook further experiments using a mutated nonfunctional TIMP-1 (T2G) in which all other domains were conserved (28). The T2G mutant N-TIMP-1 had no inhibitory effect on rat or human hepatic stellate cell apoptosis induced by cycloheximide, whereas the wild type N-TIMP-1 protein at an identical concentration (142.5 ng/ml) significantly inhibited apoptosis (Fig. 4*A*) determined by *in situ* counting after acridine orange staining. Moreover, while the wild type TIMP-1 reduced caspase-3 activity in HSC treated with cycloheximide, no effect

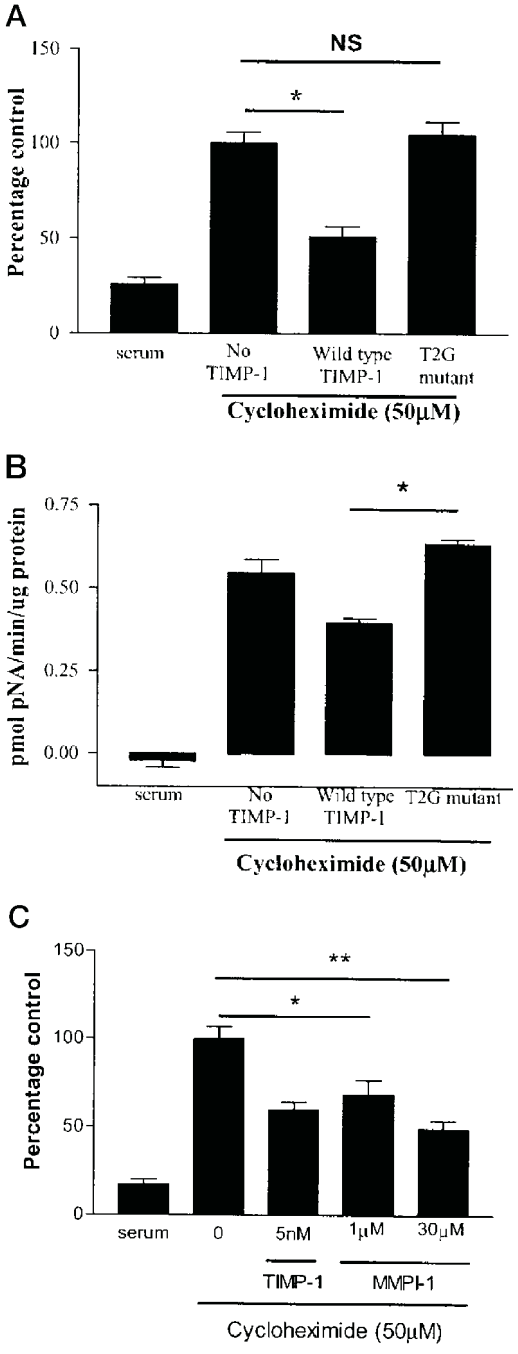

FIG. 4. *A*, acridine orange staining and counting of HSC exposure to cycloheximide with and without wild type and T2G mutant TIMP-1 demonstrated that while the wild type TIMP-1 significantly inhibited apoptosis, the T2G mutant N-TIMP-1 (with no MMP inhibitory activity) had no effect on apoptosis at equal concentration (142.5 ng/ml). Data are expressed as mean ± S.E. and presented as a percentage of control, which has been given the arbitrary value of 100%. *, $p < 0.01$ by Student's *t* test. *NS*, not significant by Student's *t* test; $n = 3$. *B*, graph demonstrating that the caspase-3 activity of cell extracts from HSC treated with cycloheximide in the presence of wild type (active) TIMP-1 and the T2G mutant (which has no MMP inhibitory activity). The wild type TIMP-1 significantly reduced caspase-3 activity relative to the T2G mutant. Data are presented as mean ± S.E.; $p < 0.01$; $n = 3$. *C*, studies comparing the anti-apoptotic effect of TIMP-1 (142.5 ng/ml; 5 nM) and the synthetic MMP inhibitor MMPI-1 were undertaken as described under "Materials and Methods." As previously described, TIMP-1 inhibited apoptosis induced by cycloheximide exposure. At a concentration of 1–30 μM, MMPI-1 significantly inhibited HSC apoptosis induced by cycloheximide determined by acridine orange staining and cell counting. Data are expressed as mean ± S.E. and presented relative to control, which has been given the arbitrary value of 100%. *, $p < 0.001$; **, $p < 0.0001$ by Student's *t* test; $n = 3$.

was observed with the T2G nonfunctional mutant (Fig. 4*B*). These data suggest that the inhibition of apoptosis by TIMP-1 was MMP-dependent. We therefore undertook further studies using a synthetic MMP inhibitor (MMPI-1; Calbiochem). The concentration of inhibitor used was calculated to provide a level of MMP inhibition comparable with 142.5 ng/ml recombinant TIMP-1 on the basis of the published K_i for the inhibitor and the recombinant TIMP-1. The synthetic matrix metalloproteinase inhibitor MMPI-1 also demonstrated a dose-dependent protective effect at a concentration of 1–30 μM. This suggested that the antiapoptotic effect in HSC could be brought about by matrix metalloproteinase inhibition alone (Fig. 4*C*).

Effect of TIMP-1 on FAS/APO-1/CD95 and Fas Ligand—The cleavage and release of the proapoptotic ligand Fas ligand has been demonstrated to be MMP-dependent (35, 36). Moreover, it has been suggested that Fas/Fas ligand may regulate HSC apoptosis (16, 17, 37). We therefore went on to determine whether TIMP-1 regulated Fas ligand cleavage in human HSC. HSC were incubated for 18 h in conditions of absolute serum deprivation with BSA or BSA with TIMP-1 (142.5 ng/ml). HSC were extracted, and supernatants were collected. After normalizing for cell number (by DNA concentration using the PicoGreen technique), these extracts were analyzed by enzyme-linked immunosorbent assay for Fas and Fas ligand as described under "Materials and Methods." TIMP-1 treatment of human HSC had no effect on cellular Fas or Fas ligand protein levels compared with control cells treated with BSA alone. Supernatant Fas and Fas ligand protein levels were undetectable in all experimental conditions (data not shown, $n = 3$).

TIMP-1 Has No Effect on HSC Proliferation—Because previous studies have demonstrated a potential proproliferative effect for TIMP-1, this was analyzed in activated HSC. TIMP-1 at concentrations of 1–100 ng/ml had no effect on proliferation of rat HSC ($n = 4$) over a 24-h incubation period compared with bovine serum albumin carrier used as a negative control (data not shown).

Persistence of TIMP-1 Expression Is Accompanied by Persistence of Activated HSC and Decreased Resolution of Liver Fibrosis—We have previously demonstrated that TIMP-1 falls during spontaneous recovery of experimental fibrosis following 4 weeks of carbon tetrachloride intoxication (14). To determine whether TIMP-1 mRNA remained elevated in liver cirrhosis, we undertook a further model of experimental fibrosis. Rats injured with carbon tetrachloride as described under "Materials and Methods" were harvested after 12 and 6 weeks of intoxication and after a further 5 and 15 days of spontaneous recovery for each model. Taqman quantification of TIMP-1 mRNA was undertaken in the same livers. Over the 15 days of spontaneous recovery, there was only a 2-fold decrease in expression of TIMP-1 mRNA after 12 weeks of carbon tetrachloride, whereas during recovery, there was a 13-fold decrease in TIMP-1 mRNA expression after 6 weeks of carbon tetrachloride (Fig. 5*A*).

To determine whether the persistence of TIMP-1 expression after 12 weeks of carbon tetrachloride correlated with persistence of activated HSC and a failure of matrix degradation, immunohistochemistry for α smooth muscle actin and histological analysis were undertaken on the same livers. Immunostaining of sections for α smooth muscle actin with cell counting and Western analysis of liver homogenates for α smooth muscle actin demonstrated that there was only a slight decrease in α smooth muscle actin-positive activated HSC during recovery (Fig. 5, *B* and *C*). Indeed, significant numbers of α smooth muscle actin-positive activated HSC were present in the 15-day recovery livers after 12 weeks of carbon tetrachlo-

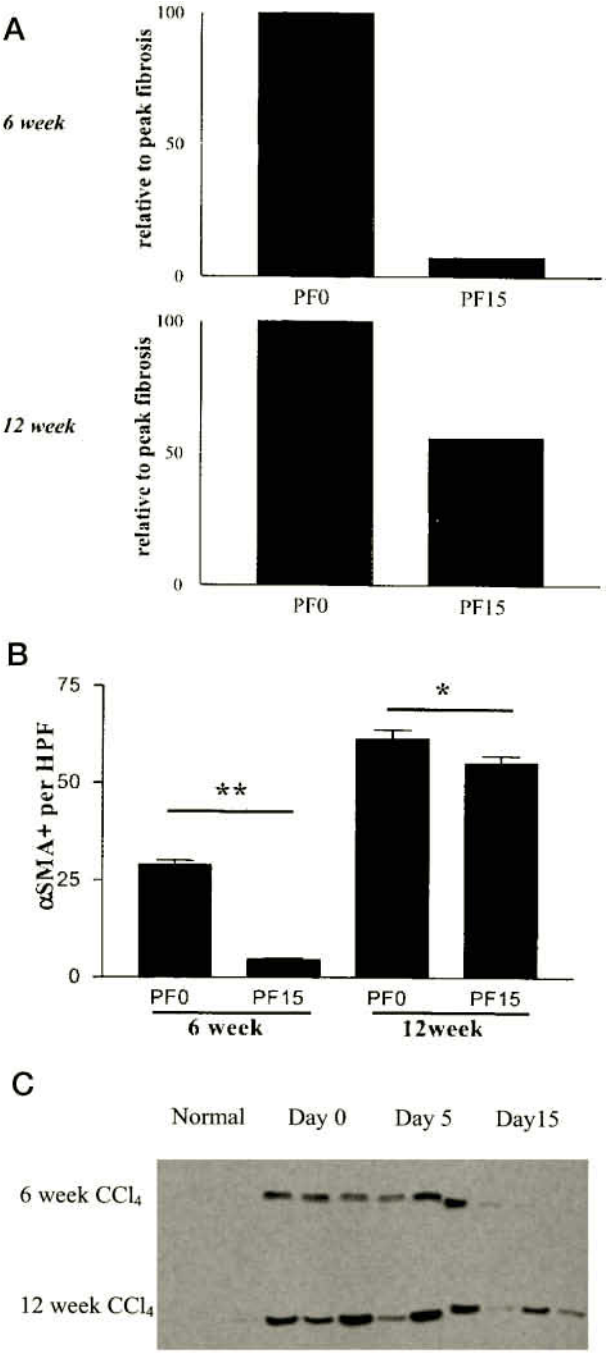

FIG. 5. *A*, TIMP-1 mRNA expression was determined by Taqman quantitative PCR in total liver RNA. After 6-week treatment with carbon tetrachloride, a 13-fold decrease in TIMP-1 expression occurs during the first 2 weeks of spontaneous recovery (compare *PF0* and *PF15*; 6 weeks of CCl_4). In contrast, there is only a 2-fold fall in TIMP-1 mRNA during the first 15 days of recovery in the 12-week injured rat liver (compare *PF0* and *PF15*; 12 weeks of CCl_4). Data are presented as mean change relative to peak fibrosis, which has been given the arbitrary value of 100 for each data set. All values have been normalized for GAPDH expression determined in parallel. *PF0*, peak fibrosis, immediately after the final injection of carbon tetrachloride; *PF15*, after 15 days of spontaneous recovery. $n = 3$ for each experiment group at each time point. *B*, the numbers of α smooth muscle actin (αSMA)-positive HSC were quantified in section form after 6 and 12 weeks of carbon tetrachloride intoxication and after 15 days of spontaneous recovery as described under "Materials and Methods." During the first 2 weeks of spontaneous recovery from rat liver fibrosis, there is minimal change in the number of α smooth muscle actin-positive HSC in the 12-week injured liver (compare *PF0* and *PF15*; 12 weeks), whereas in the liver injured for 6 weeks, there is a dramatic decrease in the number of α smooth muscle actin-positive staining cells (compare *PF0* and *PF15*; 6 weeks). Data presented are mean ± S.E. *PF0*, peak fibrosis, immediately after the final injection of carbon tetrachloride; *PF15*, after 15 days of spontaneous recovery; $n = 4$ for each experimental group at each time point; **, $p < 0.0001$; *, $p < 0.03$. *C*, Western blotting of whole liver homogenate for α smooth muscle actin demonstrates reduction in levels of liver α smooth muscle actin protein over the first 15 days of recovery after 6 weeks of carbon tetrachloride intoxication (compare *Day 0* and *Day 15*; 6 weeks of CCl_4). In contrast, levels of α smooth muscle actin protein remain elevated in whole liver extracts from the animals injured with carbon tetrachloride for 12 weeks even after 15 days of spontaneous recovery (compare *Day 0* and *Day 15*; 12 weeks of CCl_4). In both models, the liver α smooth muscle actin protein level is increased at peak fibrosis (*Day 0*) relative to normal livers (*Normal*). *Normal*, untreated liver control; *Day 0*, immediately after the final injection of carbon tetrachloride; *Day 5* and *Day 15*, after 5 and 15 days of spontaneous recovery, respectively; $n = 3$ for each time point.

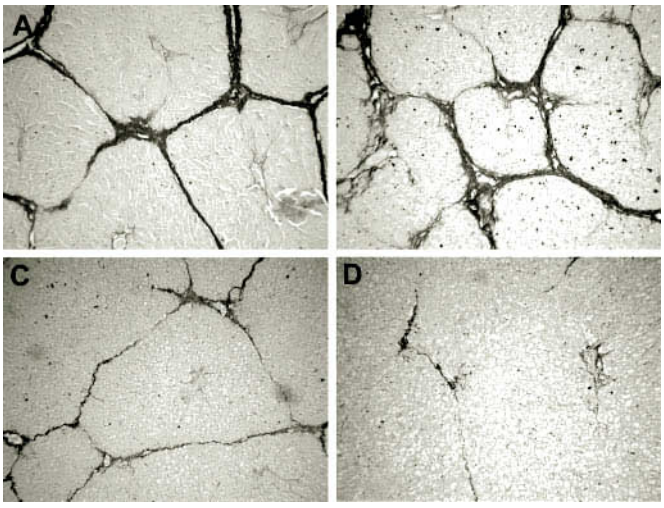

FIG. 6. *A–D*, histological analysis (Sirius Red stain) of rat livers harvested after 6 and 12 weeks of carbon tetrachloride intoxication twice weekly as described under "Materials and Methods." Livers were harvested at peak fibrosis (PF0) following 12 (*A*) and 6 (*C*) weeks of treatment and after a further 15 days of spontaneous recovery (*B* and *D*, respectively). In the 12-week model, there is more substantial fibrosis (indeed, cirrhosis is present) compared with the 6 weeks of injury (*A* and *C*, respectively). Furthermore, there is evidence of only modest matrix remodeling during the 15 days of spontaneous recovery in the 12-week model (*A* and *B*). In the 6-week model, there is an established septal fibrosis present (*C*), which demonstrates evidence of remodeling over 15 days (*C* and *D*).

ride. By Sirius Red staining, the 12-week carbon tetrachloride model had histological evidence of an established cirrhosis at peak fibrosis (Fig. 6*A*) and demonstrated only modest remodeling over the 15-day spontaneous recovery period (Fig. 6*B*). In contrast, after 6 weeks of carbon tetrachloride, the numbers of α smooth muscle actin-positive HSC demonstrated a highly significant decrease (Fig. 5, *B* and *C*). Indeed, α smooth muscle actin expression was not detectable by Western analysis in the 6-week treated livers after 15 days of recovery (Fig. 5*C*). Histological analysis of the 6-week model peak fibrosis samples demonstrated an established septal fibrosis (Fig. 6*C*). Over the 15 days of spontaneous recovery, there was evidence of significant matrix remodeling (Fig. 6*D*). The remodeling of fibrosis in the 6-week model during 15 days of recovery was accompanied with a 50% drop in liver hydroxyproline content to a level identical to that seen in untreated control liver. In contrast, the 12-week model showed increased levels of hydroxyproline of 150% of normal liver at peak fibrosis, which did not significantly change over the 15 days of spontaneous recovery.

To determine whether the observed changes in TIMP-1 mRNA expression were associated with MMP inhibition, collagenase activity in whole liver homogenate was undertaken. This demonstrated that after 12 weeks of carbon tetrachloride, at no time point (days 0 or 5 or 15 days of recovery) was activity

above that seen in normal untreated liver (collagenase activities expressed as percentage of normal liver ± S.E. were as follows: day 0, 70 ± 1.9%; day 5, 60 ± 3.3%; day 15, 55 ± 3.7%). In contrast, after 6 weeks of carbon tetrachloride, collagenase activity in the liver homogenates demonstrated an increase, peaking at 5 days of recovery (collagenase activities expressed as percentage of normal liver ± S.E. at each time point were as follows: day 0, 70 ± 1.9%; day 5, 147 ± 3.3%; day 15, 107 ± 1.6%).

Together these data demonstrate a strong correlation between persistence of activated HSC following fibrotic injury and TIMP-1 expression and a failure of matrix degradation with persistent inhibition of collagenase activity.

DISCUSSION

We have demonstrated that TIMP-1 promotes survival of activated hepatic stellate cells and have provided cogent evidence that this effect is specifically mediated via inhibition of MMP activity. Moreover, we have combined this functional data with evidence for a correlation of TIMP-1 expression and survival of activated HSC *in vivo* after withdrawal of a toxic injury.

During recovery from liver fibrosis in the rat carbon tetrachloride and bile duct ligation model of fibrosis, there is a diminution of HSC number mediated by apoptosis. At the same time, there is a reduced expression of TIMP-1. These studies and the data reported here have addressed a crucial question to our understanding of liver fibrosis: What determines whether a fibrotic liver injury recovers or fails to recover? The starting point for the studies reported in this paper is the observation that in recovery there is a net reduction in activated HSC and fibrotic matrix, whereas in progressive fibrosis, the activated HSC and neomatrix remain. Identification of factors promoting the survival of activated HSC is therefore essential to understanding the pathogenesis of fibrosis. TIMP-1 is an important potential candidate mediating HSC survival.

We have undertaken an exhaustive series of experiments using the established and robust model of activated stellate cells in tissue culture and analyzed the influence of TIMP-1 on HSC apoptosis induced by a variety of stimuli. Our results in tissue culture indicate that TIMP-1 has a direct, consistent, significant, and concentration-dependent antiapoptotic effect on both human and rat HSC. We have shown, using a series of complementary quantitative techniques, TIMP-1 reduces apoptosis induced by serum deprivation, cycloheximide exposure, and nerve growth factor stimulation and that this effect is shared by both rat and human HSC, suggesting that it is a biologically important phenomenon. Furthermore, despite the variety of means of induction of apoptosis, the antiapoptotic effect of TIMP-1 is remarkably consistent. TIMP-1 had no proproliferative effect on activated HSC. From a biological view, it would seem undesirable for a protein to both inhibit apoptosis and promote proliferation in the same cell type, since expression of such a protein would be potentially carcinogenic.

Because of the current evidence that TIMPs have divergent effects on proliferation and apoptosis in a number of different cell types (19–27), apparently independent of their matrix metalloproteinase activity, we went on to define the mechanism whereby TIMP-1 inhibits apoptosis. We approached this in two ways, by using the published K_i values of our reagents to use comparable inhibitory concentrations of synthetic inhibitor to recombinant TIMP-1 and by using the T2G mutant N-TIMP-1. Studies with the synthetic MMP inhibitor, MMPI-1, suggested that MMP inhibition was likely to be the mechanism mediating survival of HSC. Using the T2G mutant N-TIMP-1, we demonstrate directly that inhibition of apoptosis of HSC by TIMP-1 is in fact mediated via its effects on MMP activity. The T2G mutant N-TIMP-1 protein differs from the wild type protein by only a single amino acid substitution (threonine to glycine at amino acid position 2), which reduces the inhibition constant of TIMP-1 for MMP-1 and MMP-3 by a factor of over 1000. Moreover, the secondary structure of this mutant protein is not significantly different from the wild type. This makes it the best available reagent available to address the issue of MMP dependence in protection from apoptosis. At the dose of TIMP-1 used in these experiments (142.5 ng/ml), the mutant TIMP-1 would have effectively no MMP inhibitory activity, whereas the wild type TIMP-1 would be expected to significantly reduce MMP activity.

Given that the antiapoptotic effect of TIMP-1 is mediated through MMP inhibition, it is beyond the scope of the current study to precisely characterize the relevant MMP and its substrates. Moreover, the potential mechanisms through which apoptosis may be regulated by TIMP-1 are legion and may involve more than one MMP. A major candidate mechanism through which TIMPs mediate survival is by preventing matrix degradation. HSC may gain direct signals from matrix. Moreover, matrix contains numerous matrix-bound cytokines that may have antiproliferative and/or proapoptotic effects on local cell populations (*e.g.* transforming growth factor β) that may be liberated by matrix degradation. In the context of the liver fibrosis recovery model (14), during the degradation of fibrotic tissue, release of matrix-bound cytokines may also be important in determining the pattern of recovery and apoptosis of activated HSC. If TIMP-1 reduces apoptosis via preventing matrix degradation, it may do this by preventing MMP degradation of some key targets. First, release of matrix-bound proapoptotic factors would be prevented. Second, intact matrix may provide direct cell survival signals and present matrix-bound survival signals in a spatially effective manner. TIMP would preserve such signals. In support of this hypothesis, we have recently demonstrated that a mutant collagen, resistant to collagenase digestion, will promote HSC survival in models of fibrosis (39). Moreover, the *in vivo* studies reported in this paper are compatible with TIMP-1 promoting HSC survival through MMP inhibition and protection of the fibrotic matrix.

Results from our previous 4-week model of rat liver fibrosis and the 6-week carbon tetrachloride model reported here indicate that spontaneous recovery is associated with a decrease in the number of α smooth muscle actin-positive cells. Furthermore, this is associated with a large decrease in TIMP-1 mRNA expression and an increase in collagenase activity that parallels the changes in α smooth muscle actin. In contrast, after 12 weeks of carbon tetrachloride cirrhosis results, and there is only minimal evidence of matrix remodeling, no increase in collagenase activity, and a persistence of activated HSC. TIMP-1 expression actually decreased modestly over 15 days of spontaneous recovery in the 12-week model. This result is to be expected, since TIMP-1 is expressed by inflammatory cells and in the acute response to injury (11). Nevertheless, after 15 days of recovery in the 12-week carbon tetrachloride model, significant expression of TIMP-1 remains. Taken together with our previous studies, this *in vivo* evidence strongly suggests that TIMP-1-mediated MMP inhibition is a unifying mechanism promoting survival of activated HSC and protecting the fibrotic matrix from degradation.

There are further mechanisms by which MMP inhibition may mediate survival *in vivo*. It is known that many cell surface proteins can be cleaved, provided their appropriate "sheddase" is present and active. In cases where MMPs mediate shedding of receptors (*e.g.* tumor necrosis factor receptor), TIMPs may indirectly regulate cell behavior. Recently, TIMP-3 has been demonstrated to induce apoptosis in human colonic

carcinoma cells by stabilizing tumor necrosis factor α receptors on the cell surface (40). Endothelial cells have been demonstrated to shed receptors for tumor necrosis factor following induction of apoptosis, which may be a mechanism to limit inflammation in response to apoptotic cell death (41). A further MMP-dependent cell surface protein system regulating apoptosis is the Fas/Fas ligand system. Hepatic stellate cells are known to express Fas and Fas ligand on their cell surface (16, 17). TIMP-1 did not have any effect on cellular Fas or Fas ligand protein levels in activated human hepatic stellate cells. It is also possible that TIMP-1 might inhibit apoptosis by preventing the shedding of a prosurvival receptor (*e.g.* insulin-like growth factor-1 receptor), which is known to prevent apoptosis in activated hepatic stellate cells and related cells (15, 42). A further MMP-cleaved cell surface receptor that regulates cell survival is cadherin. The cadherin and β-catenin pathway is known to impact on cellular Bcl-2 levels and thus the inherent tendency for a given cell to undergo apoptosis (38).

Together with our previous reports, the data described in this study provide strong evidence that TIMP-1 is mechanistically important in promoting fibrosis: first by directly inhibiting MMPs, thus promoting matrix accumulation, and second, by inhibiting the apoptosis of activated hepatic stellate cells by a process that we have shown is also MMP-dependent. This observation highlights TIMP-1 as an important potential therapeutic target in the treatment of liver cirrhosis in the future.

REFERENCES

1. Alcolado, R., Arthur, M. J. P., and Iredale, J. P. (1997) *Clin. Sci.* **92,** 103–112
2. Friedman, S. L. (1993) *N. Engl. J. Med.* **328,** 1828–1835
3. Friedman, S. L., Roll, F. J., Boyles, J., and Bissell, D. M. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 8681–8685
4. Bachem, M. G., Meyer, D., Melchior, R., Sell, K.-M., and Gressner, A. M. (1992) *J. Clin. Invest.* **89,** 19–27
5. Maher, J. J., and McGuire, R. F. (1990) *J. Clin. Invest.* **86,** 1641–1648
6. Milani, S., Pinzani, M., Casini, A., Schuppan, D., Herbst, H., Grappone, C., Pellegrini, G., Gentilini, A., and Surrenti, C. (1992) *Hepatology* **16,** 186 (abstr.)
7. Herbst, H., Heinrichs, O., Schuppan, D., Milani, S., and Stein, H. (1991) *Virchows Archiv. B Cell Pathol.* **60,** 295–300
8. Vyas, S. K., Leyland, H., Gentry, J., and Arthur, M. J. P. (1995) *Cells of the Hepatic Sinusoid*, Vol. 5, p. 399, The Kupffer Cell Foundation, Leiden
9. Winwood, P. J., Schuppan, D., Iredale, J. P., Kawser, C. A., Docherty, A. J. P., and Arthur, M. J. P. (1995) *Hepatology* **22,** 304–315
10. Arthur, M. J. P., Friedman, S. L., Roll, F. J., and Bissell, D. M. (1989) *J. Clin. Invest.* **84,** 1076–1085
11. Iredale, J. P., Benyon, R. C., Arthur, M. J. P., Ferris, W. F., Alcolado, R., Winwood, P. J., Clark, N., and Murphy, G. (1996) *Hepatology* **24,** 176–184
12. Benyon, R. C., Iredale, J. P., Goddard, S., Winwood, P. J., and Arthur, M. J. P. (1996) *Gastroenterology* **110,** 821–831
13. Yoshiji, H., Kuriyama, S., Miyamoto, Y., Thorgeirsson, U. P., Gomez, D. E., Kawata, M., Yoshii, J., Ikenaka, Y., Noguchi, R., Tsujinoue, H., Nakatani, T., Thorgeirsson, S. S., and Fukui, H. (2000) *Hepatology* **32,** 1248–1254
14. Iredale, J. P., Benyon, R. C., Pickering, J., McCullen, M., Northrop, M., Pawley, S., Hovell, C., and Arthur, M. J. P. (1998) *J. Clin. Invest.* **102,** 538–549
15. Issa, R., Williams, E., Trim, N., Kenall, T., Arthur, M. J. P., Reichen, J., Benyon, R. C., and Iredale, J. P. (2001) *Gut* **48,** S48–S57
16. Saile, B., Knittel, T., Matthes, N., Schott, P., and Ramadori, G. (1997) *Am. J. Pathol.* **151,** 1265–1272
17. Gong, W., Pecci, A., Roth, S., Lahme, B., Beato, M., and Gressner, A. M. (1998) *Hepatology* **28,** 492–502
18. Wright, M. C., Issa, R., Smart, D. E., Trim, N., Murray, G. I., Primrose, J. N., Arthur, M. J. P., Iredale, J. P., and Mann, D. A. (2001) *Gastroenterology* **121,** 685–698
19. Guedez, L., Stetler-Stevenson, W. G., Wolff, L., Wang, J., Fukushima, P., Mansoor, A., and Stetler-Stevenson, M. (1998) *J. Clin. Invest.* **102,** 2002–2010
20. Li, G. Y., Fridman, R., and Kim, H. R. C. (1999) *Cancer Res.* **59,** 6267–6275
21. Ree, A. H., Florenes, V. A., Berg, J. P., Maelandsmo, G. M., Nesland, J. M., and Fodstad, O. (1997) *Clin. Cancer Res.* **3,** 1623–1628
22. Docherty, A. J. P., Lyons, A., Smith, B. J., Wright, E. M., Stephens, P. E., Harris, T. J. R., Murphy, G., and Reynolds, J. J. (1985) *Nature* **318,** 66–69
23. Kikuchi, K., Kadono, T., Furue, M., and Tamaki, K. (1997) *J. Invest. Dermatol.* **108,** 281–284
24. Barasch, J., Yang, J., Qiao, J., Tempst, P., Erdjument-Bromage, H., Leung, W., and Oliver, J. A. (1999) *J. Clin. Invest.* **103,** 1299–1307
25. Lim, M. S., Guedez, L., Stetler-Stevenson, W. G., and Stetler-Stevenson, M. (1999) *Ann. N. Y. Acad. Sci.* **878,** 522–523
26. Baker, A. H., Zaltsman, A. B., George, S. J., and Newby, A. C. (1998) *J. Clin. Invest.* **101,** 1478–1487
27. Valente, P., Fassina, G., Melchiori, A., Masiello, L., Cilli, M., Vacca, A., Onisto, M., Santi, L., Stetler-Stevenson, W. G., and Albini, A. (1998) *Int. J. Cancer* **75,** 246–253
28. Meng, Q., Malinovskii, V., Huang, W., Hu, Y., Chung, L., Nagase, H., Bode, W., Maskos, K., and Brew, K. (1999) *J. Biol. Chem.* **274,** 10184–10189
29. Iredale, J. P., Goddard, S., Murphy, G., Benyon, R. C., and Arthur, M. J. P. (1995) *Clin. Sci.* **89,** 75–81
30. Boulton, R. A., and Hodgson, H. J. F. (1995) *Clin. Sci.* **88,** 119–130
31. Trim, N., Morgan, S., Evans, M., Issa, R., Fine, D., Afford, S., Wilkins, B., and Iredale, J. (2000) *Am. J. Pathol.* **156,** 1235–1243
32. Stahelin, B. J., Marti, U., Solioz, M., Zimmermann, H., and Reichen, J. (1998) *Mol. Pathol.* **51,** 204–208
33. Hengartner, M. (2000) *Nature* **407,** 770–776
34. Evan, G. I., Wyllie, A. H., Gilbert, C. S., Littlewood, T. D., Land, H., Brooks, M., Waters, C. M., Penn, L. Z., and Hancock, D. C. (1992) *Cell* **69,** 119–128
35. Krammer, P. (2000) *Nature* **407,** 789–795
36. Tanaka, M., Suda, T., Haze, K., Nakamura, N., Sato, K., Kimura, F., Motoyoshi, K., Mizuki, M., Tagawa, S., Ohga, S., Hatake, K., Drummond, A., and Nagata, S. (1996) *Nat. Med.* **2,** 317–322
37. Saile, B., Matthes, N., Knittel, T., and Ramadori, G. (1999) *Hepatology* **30,** 196–202
38. Herren, B., Levkau, B., Raines, E. W., and Ross, R. (1998) *Mol. Biol. Cell* **9,** 1589–1601
39. Trim, N., Issa, R., Krane, S., Benyon, R. C., and Iredale, J. P. (2000) *Hepatology* **32,** 183 (abstr.)
40. Smith, M. R., Kung, H., Durum, S. K., Colburn, N. H., and Sun, Y. (1997) *Cytokine* **9,** 770–780
41. Madge, L. A., Sierra-Honigmann, M. R., and Pober, J. S. (1999) *J. Biol. Chem.* **274,** 13643–13649
42. Baker, A. J., Mooney, A., Hughes, J., Lombardi, D., Johnson, R. J., and Savill, J. (1994) *J. Clin. Invest.* **94,** 2105–2116