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Expression of matrix metalloproteinases and their inhibitors during hepatic tissue repair in the rat

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Abstract Matrix metalloproteinases (MMPs) and their specific inhibitors (TIMPs) are thought to play an essential role in liver injury associated with tissue remodeling. However, their distinct expression profile in different liver repair models still remains to be established. Hepatic expression of collagenase (MMP-13), gelatinases A and B (MMP-2, -9), stromelysin-1 and -2 (MMP-3, -10), membrane-type MMP-1 (MMP-14), and TIMP-1 and -2 was studied following single and repeated CCl_4-mediated injury and after partial hepatectomy. Expression was analyzed by reverse transcription-PCR (RT-PCR), northern blot analysis, zymography, and immunohistochemistry. Following a single toxic liver injury, MMPs and TIMPs were induced in a distinct time frame in that expression of most MMPs was induced during the early phase of liver injury, was maximal during the inflammatory reaction, and was diminished in the recovery phase. In contrast, TIMP and MMP-2 steady state mRNA levels remained constant in the early phase, were strongly induced during tissue inflammation, and remained increased until the recovery phase. Interestingly, hepatic TNF-α expression paralleled the MMP induction profile, while the increase of TGF-β1 expression mapped to the increase of TIMPs. Chronic liver injury was accompanied by an increase in the steady state mRNA levels of MMP-2 and TIMPs, while other MMPs remained more or less unchanged or were diminished. Partial hepatectomy was followed by a dramatic increase of MMP-14 and to a lesser extent also of TIMP-1 expression; other MMPs and TIMPs were not significantly induced. Liver injury is accompanied by profound changes in hepatic MMP/TIMP expression, the latter being critically dependent on the type of injury. Single toxic injury resulting in complete restoration was characterized by a sequential induction of MMPs and TIMPs suggesting initial matrix breakdown and matrix restoration thereafter. Chronic liver injury leading to fibrosis displays overall diminished matrix degradation mainly through TIMP induction, while liver regeneration induced by partial hepatectomy caused an induction of MMP-14 and TIMP-1 only, which might be unrelated to matrix turnover but connected to pericellular fibrinolysis or fibrolysis required for hepatocellular replication.

Key words MMP · TIMP · Fibrosis · Inflammation · Regeneration · Liver

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Introduction

Matrix metalloproteinases (MMPs) comprise a family of zinc-dependent enzymes that specifically degrade extracellular matrix (ECM) components (Basbaum and Werb 1996; Birkedal-Hansen 1995; Cawston 1995; Ries and Petrides 1995). According to their substrate specificity, MMPs fall into three main groups; collagenases, gelatinases, and stromelysins. Furthermore, membrane-type MMPs (MT1-MMP – MT4-MMP) have been identified recently, which contain a transmembrane domain and localize proteolysis to the cell surface (Basbaum and Werb 1996). The activity of MMPs is regulated at several levels including gene transcription, proenzyme activation, and inhibition of activated enzymes by tissue inhibitor of MMP (TIMP) (Basbaum and Werb 1996; Birkedal-Hansen 1995; Cawston 1995; Ries and Petrides 1995).

Proteolytic degradation of ECM is an essential feature of tissue remodeling and has been implicated in a large range of processes. Current evidence indicates that matrix degradation is essential for liver repair reactions, such as hepatic fibrogenesis, which is characterized by an increase and altered deposition of newly formed ECM components (Arthur 1995a,b; Iredale 1996). Accumulation of ECM is caused by an increase of ECM synthesis but is basically the result of an imbalance between enhanced matrix synthesis and diminished or altered matrix

breakdown (Alcolado et al. 1997; Friedman 1993, 1997; Gressner and Bachem 1995; Knittel and Ramadori 1994). Indeed, recent studies indicated that in fibrotic liver collagenolytic activity was decreased accompanied by elevated TIMP-1 as well as TIMP-2 levels. This expression pattern is thought to result in a net inhibition of matrix breakdown and an accumulation of fibrillar collagens (Benyon et al. 1996; Herbst et al. 1997; Iredale et al. 1995, 1996; Maruyama et al. 1982; Milani et al. 1994; Perez-Tamayo et al. 1987; Roeb et al. 1997; Takahara et al. 1995). The basic importance of MMPs and TIMPs in the deposition of ECM components is further underlined by studies analyzing the expression patterns and the time kinetics during the recovery phase of chronic liver diseases (Emonard and Grimaud 1989; Iredale et al. 1998; Takahara et al. 1995). Following withdrawal of hepatotoxic compounds, expression levels of most MMPs were induced, again with the exception of that of elevated MMP-2. TIMP expression was reduced resulting in an increased fibrolysis characterized by removal of excessively deposited ECM.

Currently matrix degradation is usually investigated in view of liver fibrosis. However, matrix remodeling also takes place during liver regeneration. During liver regeneration in the rat induced by carbon tetrachloride (CCl_4) intoxication, hepatic stromelysin and TIMP expression were induced in a distinct time frame (Herbst et al. 1991, 1997; Iredale et al. 1996; Roeb et al. 1997). Although matrix degrading enzymes and their specific inhibitors play an important role during various physiological and pathological processes in the liver, only limited information is available with respect to their expression profile in different hepatic tissue repair models. To further investigate the latter issue, the present study was performed to analyze MMP and TIMP expression during liver injury induced by a single treatment with CCl_4, which is characterized by hepatocellular necrosis, inflammation, and complete restoration of liver tissue by regeneration. Furthermore, expression patterns were studied both after chronic liver injury induced by CCl_4, which results in replacement of damaged hepatocytes by connective tissue, and after partial hepatectomy, which is characterized by regeneration without concomitant hepatocellular necrosis and inflammation. To elucidate tissue breakdown after the differently induced injuries, expression of collagenase, gelatinases A and B, stromelysin-1 and -2, MT1-MMP (MMP-14), and TIMP-1 and -2 was studied simultaneously. TNF-α and TGF-β1 expression was analyzed in parallel to MMPs and TIMPs, because the inflammatory cytokine TNF-α and the growth factor TGF-β1 were identified as important regulators of MMP and TIMP expression in hepatic stellate cells (HSC) in vitro (Knittel et al. 1999b) and are known to be profoundly involved in hepatic tissue repair reactions (Fausto 1999; Friedman 1999; Thomson and Arthur 1999).

Material and methods

Animals

Two-month-old Wistar rats were provided by Charles River (Sulzfeld, Germany). For animal experiments, the *Principles of Laboratory Animal Care* (NIH publication number 85–23, revised 1985) and the current version of the German law on the protection of animals were followed.

Antibodies

The monoclonal anti-MMP-9 antibody (Ab-1, clone 6–6B; Calbiochem, Bad Soden, Germany) was used for immunohistochemistry of liver tissue samples and displayed univocally a specific staining reaction. The latter antibody was generated by immunizing mice with MMP-9 protein partially purified from the conditioned media of PMA-stimulated HT-1080 human fibrosarcoma cells. The antiserum directed against mouse or rabbit IgGs and the APAAP complex were from Dako (Copenhagen, Denmark).

Primers and cDNA probes

Primers and cDNAs used to detect rat MMP- and rat TIMP-specific transcripts are described in detail elsewhere (Knittel et al. 1999b). Furthermore, PCR generated cDNAs directed against rat TNF-α and rat TGF-β were employed (Knittel et al. 1999a). In addition, a cDNA probe specific for human albumin (Kurnit et al. 1982), a clone carrying the rat GAPDH cDNA (Fort et al. 1985), and, to validate northern blot results, an oligonucleotide (5′ AAC GAT CAG AGT AGT GGT ATT TCA CC 3′) complementary to 28S RNA were used (Barbu and Dautry 1989).

CCl_4-induced liver injury and partial hepatectomy

Rats were given CCl_4/maize oil solution (50%, v/v) orally as described earlier (Knittel et al. 1995, 1996, 1999a; Neubauer et al. 1995, 1996, 1998). Acute liver damage was induced by administration of a single dose of 150 µl CCl_4/100 g body weight and control animals were given olive oil only. For induction of liver fibrosis, animals were treated between 12 and 16 weeks with CCl_4 at weekly intervals according to Proctor and Chatamra (1982), as described elsewhere (Knittel et al. 1996; Neubauer et al. 1996). To induce chronic liver injury, animals were pretreated with drinking water containing phenobarbital (0.03%) for 2 weeks and were then exposed to CCl_4 in a dose escalation regimen. The CCl_4 regimen started with a dose of 0.04 ml in the 1st week and an additional 0.04 ml CCl_4 were added weekly. Control animals were given olive oil only. As assessed by histological analysis of liver tissue, rats developed hepatic fibrosis displaying septa connecting portal tracts and central veins.

Animals were killed and the liver was perfused with saline solution (0.9%), removed, and snap frozen in liquid nitrogen for immunohistochemistry. In the case of fibrotic livers, organs were removed 7 days after CCl_4 administration. Partial hepatectomy (70%) was performed according to Higgins and Anderson (1931). In the sham operation, the abdomen was opened and the liver mobilized. Livers were removed following 0.5–72 h and processed as described above.

Immunohistochemistry

Cryostat sections (5 µm) of normal or diseased livers were fixed in methanol/acetone (5 min/10 s at –20°C) and were examined by the APAAP staining procedure as described earlier (Knittel et al. 1996, 1999a; Neubauer et al. 1996, 1998). Tissue sections were incubated with specific primary antibodies for 1 h at 37°C and were then covered for 1 h at 37°C with an antiserum directed against

mouse IgG followed by incubation with the APAAP complex. Antibodies directed against MMP-9 were used at dilutions of 1/200. The primary antibody was linked to the APAAP molecule (Dako; 1:25) by a rabbit anti-mouse antibody (Dako; 1:25) according to the manufacturer's recommendations (Dako). Non-specific staining was controlled for by incubation with mouse Ig instead of the specific primary antibody. Concerning the liver sections, MMP-9-specific immunoreactivity was quantitated in that the number of MMP-9-positive cells per field of vision at 250× magnification was counted. The number of MMP-9-positive cells within two different liver samples was determined within 12 different fields of vision by two independent investigators. Statistical analysis was performed using Mann-Whitney *U*- and ANOVA tests.

RNA extraction, northern blot analysis, and RT-PCR

Liver tissue samples were lysed with guanidinium isothiocyanate and total RNA was extracted as reported (Knittel et al. 1997a,b, 1999a,b). In some experiments poly-A^+ mRNA was prepared from total RNA using the Oligotex mRNA minikit (Qiagen, Hilden, Germany).

For northern blot analysis, RNA was resolved by agarose gel electrophoresis, transferred to Hybond membranes (Amersham Buchler, Braunschweig, Germany) and hybridized with specific 32P-labeled cDNA probes. Hybridization was performed for 2 h at 68°C using the QuickHyb kit (Stratagene, La Jolla, Calif., USA). Posthybridization washes were performed twice for 15 min at room temperature and once for 5–15 min at 60°C in 2×SSC containing 0.1% SDS. Nylon filters were washed, dried, and exposed to X-ray films at –80°C. Northern data and results of the substrate gel analysis presented in the figures of this report are representative for at least two independent experiments. In each experiment two different animals were studied independently for each time point following a single CCl_4 application. In the case of controls and fibrotic livers more than three different animals were studied by the latter methods.

For RT-PCR, 1 µg total RNA was reverse transcribed using 200 U MMLV (Gibco BRL, Karlsruhe, Germany) at 37°C. Out of 20 µl total RT reaction, 1 µl was used as template DNA for PCR. PCR was performed with specific primers mostly for 35 cycles with cycle times of 1 min at 95°C, 1.30 min at 50–55°C, and 2 min at 72°C, as described previously (Knittel et al. 1999b). The final elongation time was 10 min at 72°C. In parallel, PCR reactions were performed with primers coding for G3PDH. Out of 50 µl total PCR reaction, 10 µl were analyzed in a 2% agarose gel. Specificity of PCR products was confirmed by digestion using appropriate restriction enzymes and by sequencing of cloned PCR products, as described previously (Knittel et al. 1999b).

Substrate gel analysis (zymography)

One hundred milligrams liver tissue was solubilized in 2 ml extraction buffer containing 10 mM cacodylic acid, 0.5 M NaCl, 20 mM $CaCl_2$, 1.5 mM NaN_3, and 0.01% Triton X-100, and incubated in the latter buffer for 48 h at 4°C under agitation. Samples were centrifuged and the protein content of the supernatants was measured using the Coomassie protein assay (Pierce, Rockford, Ill., USA). Furthermore, conditioned media of HSC were used, which were obtained after a 24-h incubation of 2-days cultured HSC in DME supplemented with 0.3% FCS as described (Knittel et al. 1999b). Eighty micrograms liver tissue extracts or 30 µl media were analyzed by 9% SDS-PAGE according to Laemmli (1970) containing 1 mg/ml gelatin. Gels were rinsed twice for 30 min in 2.5% Triton X-100, incubated for 24 h at 37°C in 2.5% Triton X-100, 5 mM $CaCl_2$, and 50 mM TRIS (pH 8.0), stained overnight with 0.25% Coomassie blue in 50% methanol and 10% acetic acid, and destained. Gelatinase A and B standards were purchased from Novus Molecular (San Diego, Calif., USA).

Results

Expression of MMP- and TIMP-specific transcripts during acute liver injury

Using the CCl_4 model of acute liver damage, hepatic expression of MMPs/TIMPs, in detail collagenase (MMP-13), gelatinases A and B (MMP-2, -9), stromelysin-1 and -2 (MMP-3, -10), membrane-type MMP-1 (MMP-14), and TIMP-1 and -2 (Table 1), was studied by RT-PCR (data not shown), by northern blot analysis (Fig. 1), by zymography (Fig. 2), and by immunohistochemistry (Figs. 3, 4).

In normal rat liver, basal expression of MMPs and TIMPs was low, however, as assessed by RT-PCR (data not shown) and northern blot analysis (Fig. 1), transcripts specific for the MMPs and TIMPs analyzed in this study were present. Following a single toxic liver injury, MMPs and TIMPs were induced in a distinct, time-dependent pattern (Fig. 1). Expression of MMPs, such as collagenase (MMP-13) and stromelysin-1 and -2 (MMP-3, -10), was induced during the early phase of liver injury (3–12 h following intoxication), was maximal during the inflammatory reaction (12–24 h), and was diminished in the recovery phase (72–96 h; Fig. 1). A different pattern was noted in the case of MMP-14 which was already induced at 3 h and was elevated until 96 h. In contrast to most MMPs, MMP-2 displayed a different pattern in that MMP-2-specific transcripts were elevated at later stages of tissue injury starting at 24 h and being maximal at 72 h. Gelatinase B (MMP-9)-specific messengers were undetectable by northern blot analysis of diseased livers, even when poly-A^+ RNA was tested, however, as evidenced by RT-PCR MMP-9 coding mRNAs relative to G3PDH specific messengers were induced during liver injury and displayed an expression profile similar to MMP-13 and stromelysins. TIMP steady state mRNA levels remained constant in the early phase, became increased at later time points, namely at 9 h (TIMP-1) or 24 h (TIMP-2), and remained increased until 72 h.

Interestingly, hepatic TNF-α expression paralleled the MMP induction, while TGF-β1 expression mapped to the increase of TIMPs and MMP-2. As shown previously (Knittel et al. 1999a) TNF-α-specific messengers of 1.6 kb in size became induced at 3 h post CCl_4 application, showed highest levels at 9–12 h, and were down-

Table 1 Nomenclature of the matrix metalloproteinases (MMPs) analyzed in this study

MMP number	Synonym
MMP-13	Collagenase
MMP-2	Gelatinase A
MMP-9	Gelatinase B
MMP-3	Stromelysin-1
MMP-10	Stromelysin-2
MMP-14	Membrane-type MMP-1

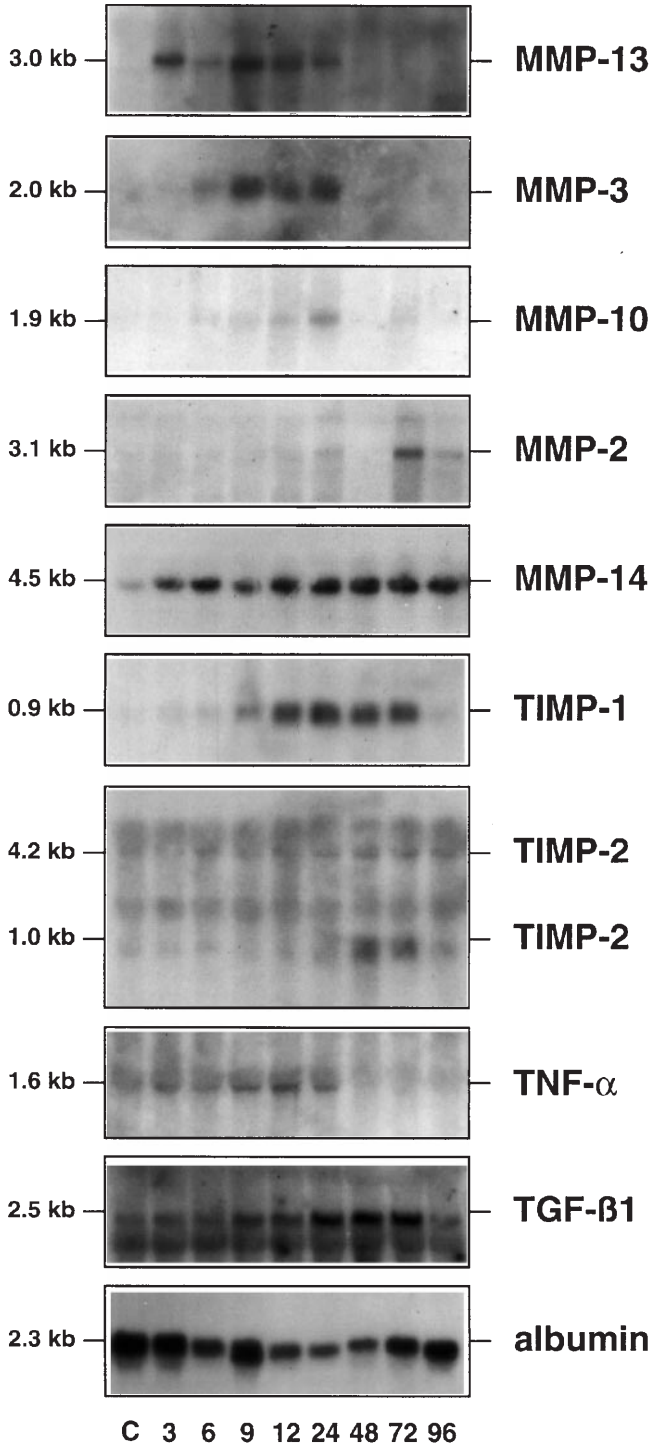


Fig. 1 Induction of matrix metalloproteinases (MMPs) and their specific inhibitors (TIMPs) during single, CCl_4-induced liver injury. Northern blot analysis of 10 µg, pooled total RNA purified from two control livers (*C*) and from two acutely damaged livers analyzed 3 (*3*), 6 (*6*), 9 (*9*), 12 (*12*), 24 (*24*), 48 (*48*), 72 (*72*), and 96 h (*96*) after a single carbon tetrachloride (CCl_4) administration. RNA was size selected by 1% agarose gel electrophoresis and filters were hybridized using specific cDNA probes, subsequently. In the case of MMP-10 and MMP-2 poly-A^+ RNA was used, which was obtained from 100 µg total RNA

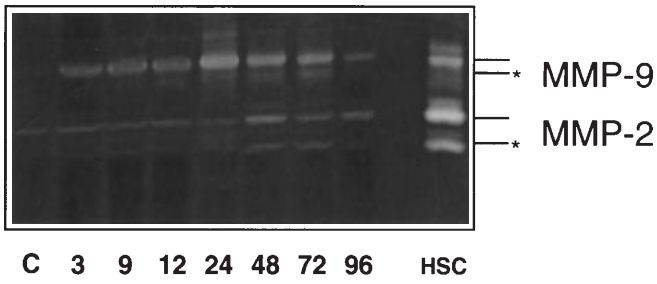


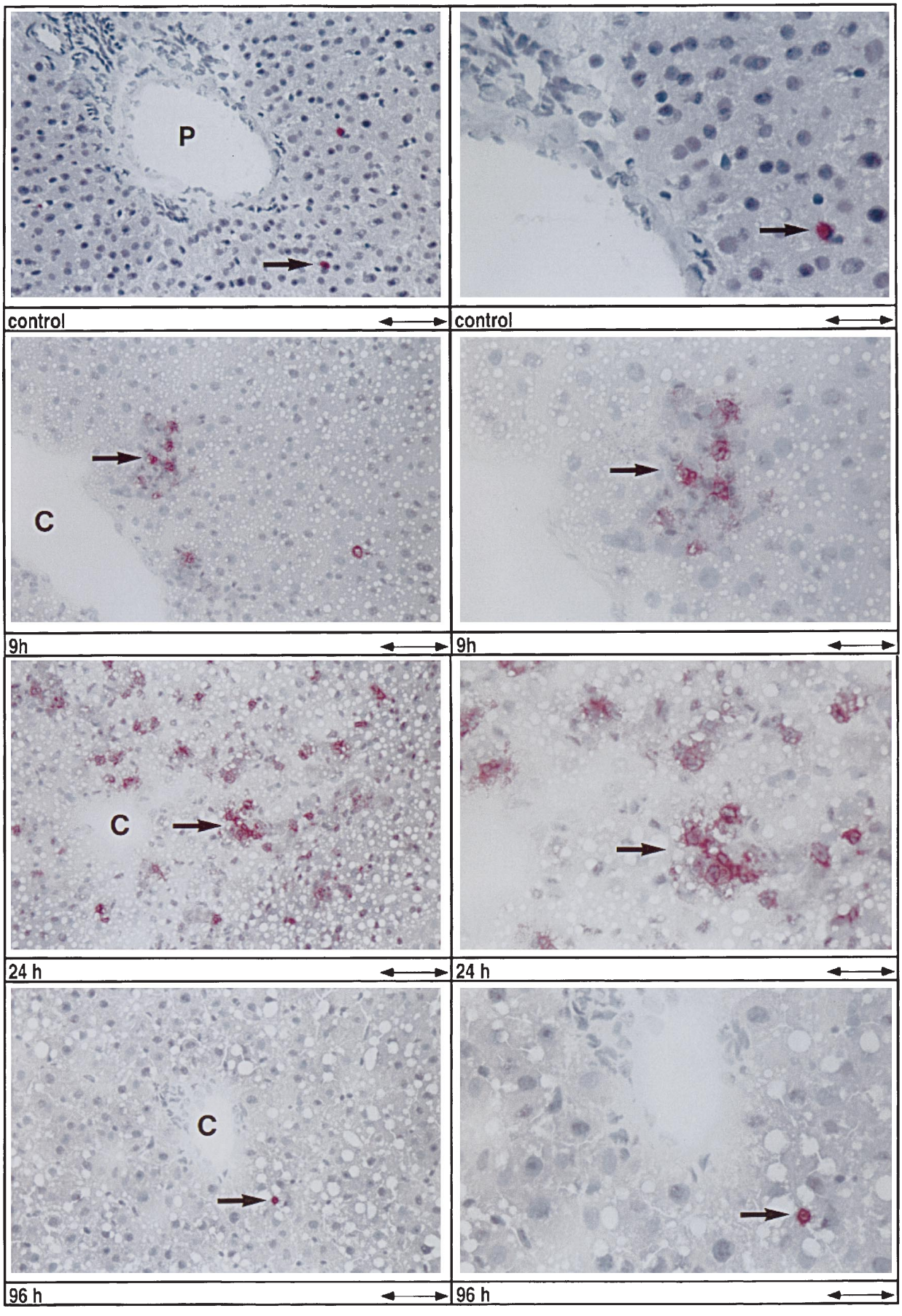
Fig. 2 Gelatinase activity present in liver tissue following single, CCl_4-induced injury. Tissue extracts were obtained from two control livers (*C*) and from acutely damaged livers analyzed 3 (*3*), 9 (*9*), 12 (*12*), 24 (*24*), 48 (*48*), 72 (*72*), and 96 h (*96*) after a single CCl_4 administration. Furthermore, conditioned media were obtained from hepatic stellate cells (*HSC*) displaying prominent MMP-2- and MMP-9-specific protease activity. Eighty micrograms liver tissue extracts or 30 µl cell supernatant were analyzed by substrate gel analysis. *Asterisks* indicate active forms of MMP-9 and MMP-2

regulated later on thereby matching the MMP induction kinetics. In contrast, as reported Knittel et al. (1999a), hepatic expression of TGF-β1-specific transcripts of 2.5 kb in size became elevated at 9 h, was increased to highest quantities at 24–72 h, and matched the TIMP/MMP-2 induction profile. In control hybridizations, albumin-specific mRNAs decreased in a time-dependent manner to lowest levels at 24–48 h after CCl_4 administration following the course of necrosis. Due to hepatic regeneration, albumin expression increased with the onset of the recovery phase at 72 h and reached baseline levels of normal livers at 96 h (Fig. 1).

Gelatinolytic activity during acute liver injury

To study whether the induction of mRNA steady state levels is reflected by changes of corresponding proteins, gelatinolytic activity present in normal and diseased livers was studied by zymography (Fig. 2). Gelatinolytic activity specific for MMP-9 was near the detection limit in normal livers and was already severalfold induced 3 h after CCl_4 administration. Thereafter MMP-9 activity increased to maximal levels at 24 h and declined subsequently reaching significantly lower levels at 96 h, which were slightly above the baseline level present in normal livers. In the case of MMP-9, mostly the latent form of MMP-9 (Mr 95 kDa) was found, while only mi-

Fig. 3 Distribution of MMP-9-positive cells in normal and acutely damaged rat livers. Sections of normal rat livers and livers 9, 24, and 96 h following CCl_4 administration were incubated with antibodies directed against MMP-9 or mouse immunoglobulins used as negative controls (data not shown). Immunoreactive material was detected using the APAAP staining method. Note the portal vein (*P*) and central vein (*C*). *Right panels* show higher magnifications of *left panels*. *Arrows* indicate MMP-9-positive cells present in each panel. *Bar* represents 166 (*left panel*) and 83 µm (*right panel*) ▶


P
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9h
9h
C
24 h
24 h
C
96 h
96 h

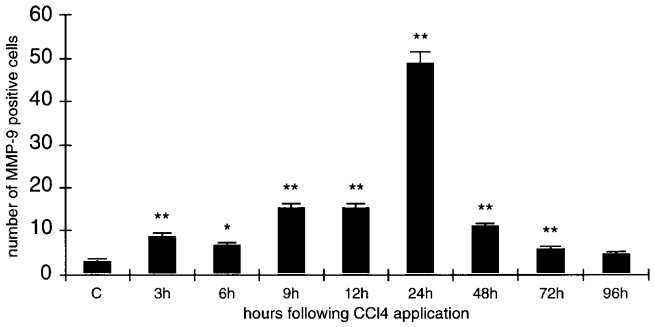


Fig. 4 Kinetics of MMP-9-positive cells during the course of acute, CCl_4-induced liver injury. Rats were given a single dose of CCL_4 and livers were analyzed at 3 (*3h*), 6 (*6h*), 9 (*9h*), 12 (*12h*), 24 (*24h*), 48 (*48h*), 72 (*72h*), and 96 h (*96h*) after a single CCL_4 administration. The average total number of MMP-9-positive cells present in a field of vision at 250× magnification including the SEM is provided. Compared to controls, the increase in the number of MMP-9-positive cells was statistically significant at $P<0.01$ (**) and $P<0.05$ (*) of diseased versus control livers

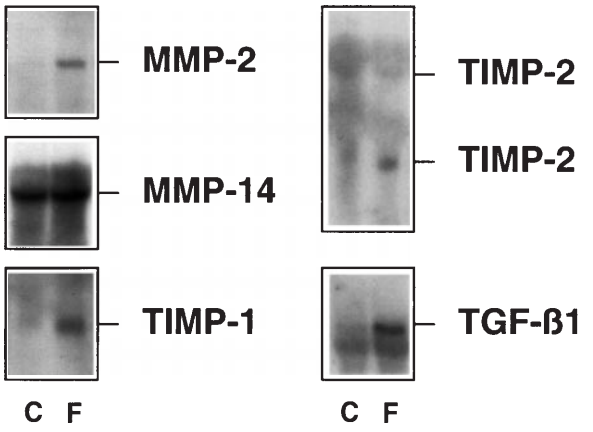


Fig. 5 Expression of MMPs and TIMPs following chronic liver injury resulting in liver fibrosis. Northern blot analysis of 10 µg total RNA purified from control liver (*C*) and chronically damaged, fibrotic liver (*F*) 7 days after termination of long-term CCl_4 intoxication. Total RNA was size selected by 1% agarose gel electrophoresis and filters were hybridized using specific cDNA probes, subsequently

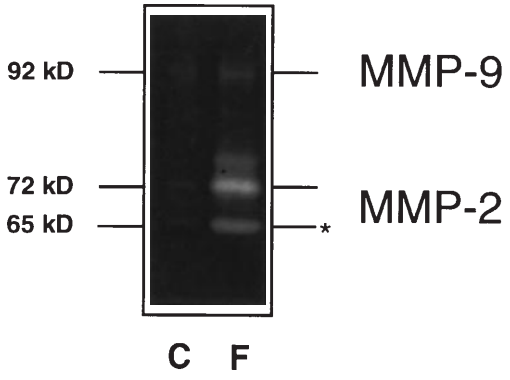


Fig. 6 Gelatinase activity present in liver tissue following chronic liver injury. Tissue extracts were obtained from control liver (*C*) and chronically damaged, fibrotic liver (*F*) 7 days after termination of long-term CCl_4 intoxication. Eighty micrograms liver tissue extracts were analyzed by substrate gel analysis. *Asterisk* indicates active forms of MMP-2

nor amounts of active MMP-9 migrating at 82 kDa were detectable (Fig. 2, 12–72 h, asterisk). In contrast to MMP-9, MMP-2-specific activity was unchanged until 24 h following CCl_4 treatment but was increased from 48 h onwards. As in the case of MMP-9, liver extracts contained mainly the latent form of MMP-2 at about 72 kDa, however at 48 h and 72 h considerable amounts of active MMP-2 were detectable (Fig. 2, asterisk).

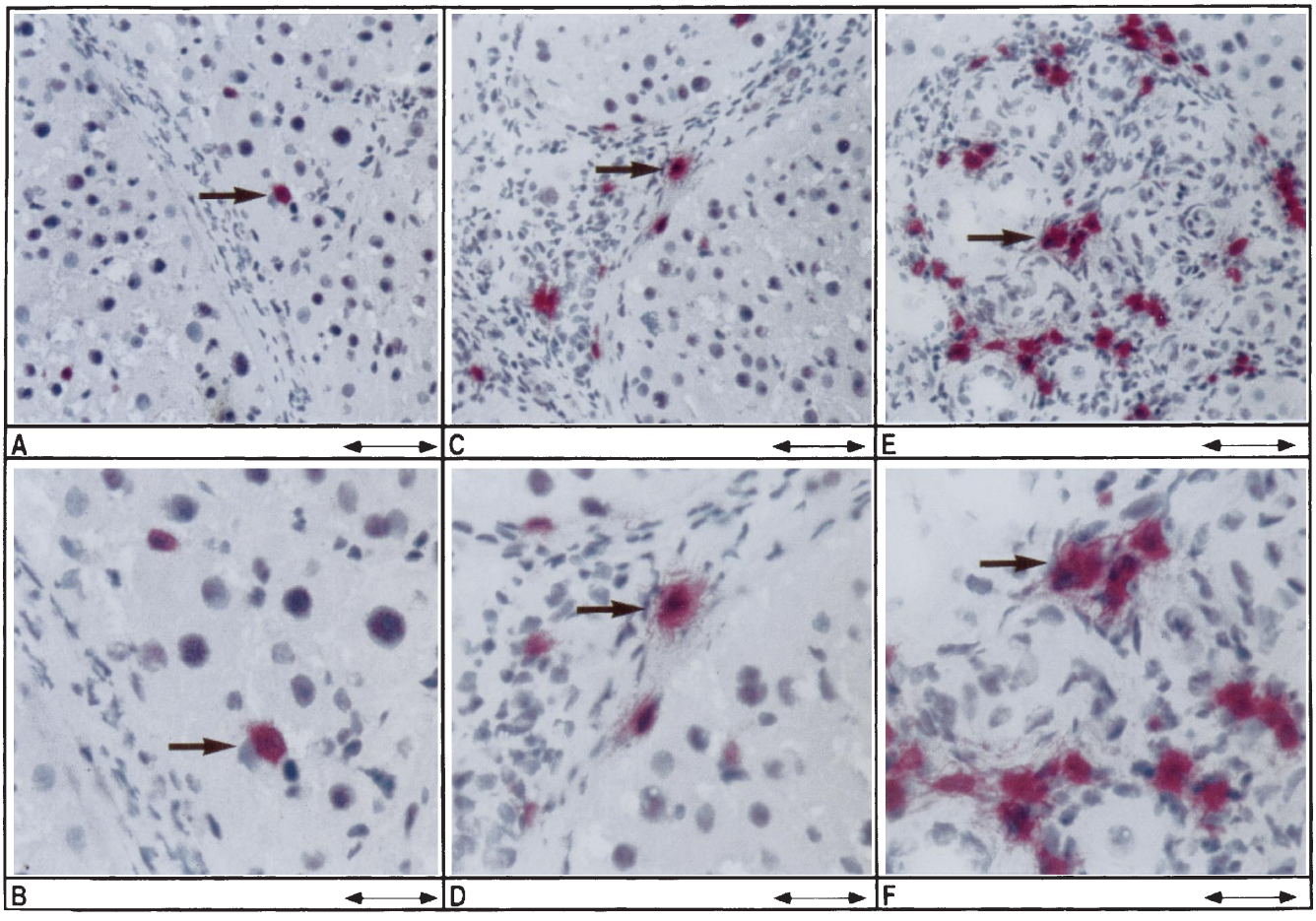
Immunolocalization of gelatinase B during acute liver injury

In accordance with the previous data demonstrating low MMP-9 activity in normal livers, only a few MMP-9-positive cells were found in control livers in the hepatic parenchyma (Fig. 3). Based on the size, shape, and distribution of the cells, the MMP-9-positive cells do not correspond to hepatocytes but to non-parenchymal cells. During acute liver injury, MMP-9-positive cells increased in number, which started 3 h following CCl_4 application, reached maximal levels at 24 h, being about 20-fold higher compared to control livers, and returned to baseline levels at 96 h (Figs. 3, 4). While at 3 h and 6 h MMP-9-positive cells were located uniformly in the hepatic lobule, MMP-9-positive cells were present at 9 h often in clusters located near vascular structures. At 24 h and 48 h, representing the stages of prominent hepatocellular necrosis, MMP-9-positive cells were predominantly located within the necrotic area with a ratio of five times more cells in the necrotic area compared to the surrounding tissue.

In an attempt to clarify what cell population expressed MMP-9, the MMP-9 staining pattern was compared with immunostainings of adjacent sections against desmin/GFAP reacting with HSC (Neubauer et al. 1996; Niki et al. 1996) and immunostainings against ED1/ED2 identifying resident macrophages, the Kupffer cells (ED2), and other monocyte/macrophage/dendritic cells (ED1) (data not shown) (Dijkstra et al. 1985). Only in the case of ED1 some overlap with the MMP-9 immunoreactivity was noted in normal and in acutely as well as chronically injured livers. However, as constantly more ED1-positive cells than MMP-9-positive cells were present, the data suggest that only a subpopulation of the ED1-positive cells could be MMP-9 positive.

Expression of MMPs and TIMPs in liver fibrosis

Chronic liver injury resulting in hepatic fibrosis was accompanied by a severalfold increase in the steady state mRNA levels of MMP-2, TIMP-1, and TIMP-2, while MMP-14-specific messengers were only slightly increased (Fig. 5). Expression of other MMPs in fibrotic



livers was not detectable by northern blot analysis. However, as assessed by RT-PCR technology, MMP-3- and MMP-10-specific transcripts relative to G3PDH-specific messengers were diminished in fibrotic livers compared to controls (data not shown).

In accordance with the mRNA data, MMP-2 activity was increased severalfold in fibrotic livers compared to controls using gelatin zymography analysis (Fig. 6), while gelatinase B activity was elevated only in minor quantities. In the case of MMP-2, both the latent form and the active form of the enzyme were present (Fig. 6, asterisk). Applying immunohistochemistry, MMP-9-positive cells were detectable in fibrotic scars (Fig. 7C–F) and the hepatic parenchyma (Fig. 7A,B). In the fibrous septa, MMP-9-positive cells were located at the scar–parenchyma interface (Fig. 7C,D) and within the scar (Fig. 7C,D) especially in fibrotic nodules exhibiting proliferating vascular structures such as bile ductules (Fig. 7E,F).

Fig. 7A–F Distribution of MMP-9-positive cells in fibrotic rat livers. Sections of fibrotic rat liver were incubated with antibodies directed against MMP-9 or mouse immunoglobulins used as negative controls (data not shown). Immunoreactive material was detected using the APAAP staining method. The *lower panel* displays higher magnifications of the corresponding sections presented in the *upper panel*. *Arrows* indicate corresponding MMP-9-positive cells. *Bar* represents 166 (**A,C,E**) and 83 µm (**B,D,F**)

Expression of MMPs and TIMPs in liver regeneration following partial hepatectomy

Partial hepatectomy was followed by a dramatic increase in MMP-14 coding mRNAs and to a lesser extent also of TIMP-1-specific transcripts (Fig. 8). In contrast, no major induction of other MMPs such as MMP-13, -3, -10, -2, and -9 as well as TIMP-2 was detectable as assessed

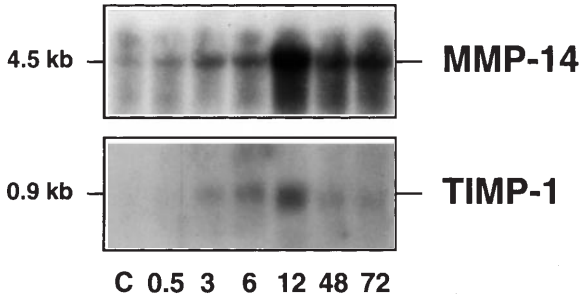


Fig. 8 Expression of MMPs and TIMPs following partial hepatectomy. Northern blot analysis of 10 µg total RNA purified from livers of sham-operated animals (*C*) and livers prepared 0.5 (*0.5*), 3 (*3*), 6 (*6*), 12 (*12*), 48 (*48*), and 72 h (*72*) following partial hepatectomy. Total RNA was size selected by 1% agarose gel electrophoresis and filters were hybridized using specific cDNA probes, subsequently

by northern blot analysis (data not shown). In the case of MMP-14 and TIMP-1, induction started 3 h following partial hepatectomy, was maximal at 12 h, and decreased thereafter to levels still above the baseline defined by control livers. Compared to normal animals, no induction of MMP-14 or TIMP-1 expression was observed in livers of sham-operated animals covering the complete time course presented in Fig. 8.

Discussion

The data presented in this paper indicate that liver injury is accompanied by profound changes in hepatic MMP and TIMP expression, the latter being critically dependent on the type of injury. Single toxic injury resulting in complete restoration was characterized by a sequential induction of MMPs and TIMPs, which was related to the hepatic expression of TNF-α and TGF-β1. Presently only limited information is available on the expression of MMPs and TIMPs during the course of a single toxic injury (Herbst et al. 1991; Roeb et al. 1997), although this model appears quite attractive since it is characterized by necrosis, inflammation, and complete restitution. Herbst and co-workers (1991) examined the expression of stromelysin (MMP-3) following CCl_4-mediated, single injury by in situ hybridization and showed that stromelysin displayed cell type-specific spatial and temporal RNA expression patterns with high transcript levels in small proportions of hepatocytes and non-parenchymal cells. The data presented in this paper are in agreement with the previous study illustrating an induction of MMP-3 expression with two peaks, which might correspond to the expression of hepatocytes and non-parenchymal cells detected by in situ hybridization in the former report.

In another study, Roeb and co-workers (1997) demonstrated that TIMP-1 coding mRNAs were increased during toxic liver injury. TIMP-1-specific messengers peaked at 24 h following CCl_4 administration and occurred predominantly in areas of inflammation, in hepatocytes, and in mesenchymal and endothelial cells by in situ hybridization. However, only the time points at 1, 24, and 72 h after CCl_4 application were analyzed and therefore the precise TIMP-1 expression profile, namely its induction at 9 h, its peak from 12 until 72 h, and the downregulation at 96 h illustrated in this paper, was not evident in the former report (Roeb et al. 1997). Furthermore, the increase of TIMP-2 expression at 48 and 72 h presented in this study was not mentioned in the former publication. In another report, TIMP-1 and -2 expression was studied by in situ hybridization and illustrated that TIMP-1 and -2 transcripts appeared in rat livers within 1–3 h after intoxication, increased at 12–24 h, and peaked at 72 h in a few hepatocytes and mesenchymal cells located in the perisinusoidal, perivenular, and portal tract region (Herbst et al. 1997).

Apart from MMP-3 and TIMP, no experimental data are published on the expression of MMPs in the CCl_4 model of single liver injury. As shown in this report, all members of the MMP family, in detail collagenase (MMP-13), gelatinases A and B (MMP-2, -9), stromelysin-1 and -2 (MMP-3, -10), and membrane-type MMP-1 (MMP-14), were induced following single CCl_4 administration thereby matching the data obtained in other injury models, for example in the brain, peripheral nerves, skin, or lung (Lafleur et al. 1996; Madlener et al. 1998; Pagenstecher et al. 1998; Swiderski et al. 1998). Furthermore, MMP expression was induced in a distinct time frame characterized by an early increase of MMP-3, MMP-14, and MMP-9 expression and an induction of stromelysin coding messengers at somewhat later stages.

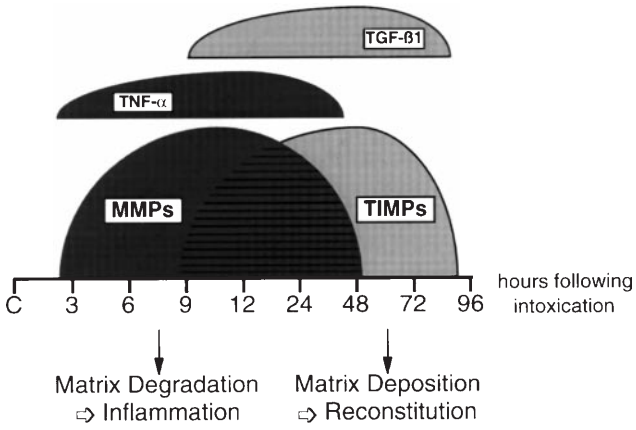


Fig. 9 Proposed model for the role of MMPs and TIMPs during hepatic tissue injury

The authors are aware that MMP and TIMP expression was studied mainly at the level of specific transcripts in this report, which might not necessarily reflect MMP or TIMP protein or even matrix degrading activity. However, as shown in the case of gelatinases, the induction/deinduction of MMP-2 and MMP-9 coding messengers were truly accompanied by corresponding changes on the protein level as evidenced by zymography or immunohistochemistry in the acute and chronic CCL_4 liver injury model. Furthermore, the presence of active MMP-2 at 48 and 72 h following injury (Fig. 2, asterisk) provides indirect evidence that this applies also for MMP-14 and TIMP-2. The latter proteins are crucial for MMP-2 activation (Yu et al. 1996), which coincided with elevated levels of MMP-14- and TIMP-2-specific messengers at 48 and 72 h.

The MMP induction might be driven by TNF-α and should result in enhanced matrix degradation as evidenced, for example, by increased gelatinolytic activity specific for MMP-9 in liver extracts from 3 h postintoxication onward. At this stage of tissue injury, proteolytic degradation of ECM might be of essential importance for a number of events including the invasion of the liver with mononuclear cells or migration of HSC (Fig. 9). In contrast, TIMP and MMP-2 steady state mRNA levels remained constant in the early phase, were induced during tissue inflammation, and remained increased until the recovery phase. The expression profile of the latter pro-

tease inhibitors in combination with the downregulation of the major MMP family members should diminish matrix degradation thereby promoting matrix deposition to achieve reconstitution of the tissue repair reaction. Interestingly enhanced TGF-β1 expression levels mapped to this phase of tissue injury, which is in agreement with the general concept elaborated in other types of tissue repair in that TGF-β is the principal cytokine in the resolution of tissue repair reactions (Mutsaers et al. 1997).

Currently matrix degradation is mostly investigated in view of liver fibrosis indicating that in fibrotic liver collagenolytic activity was decreased and that fibrogenesis is accompanied by elevated gelatinase A (MMP-2) and TIMP-1 as well as TIMP-2 levels in the fibrotic tissue and in the serum (Benyon et al. 1996; Greenwel and Rojkind 1997; Herbst et al. 1997; Iredale et al. 1995, 1996; Kasahara et al. 1997; Lichtinghagen et al. 1998; Maruyama et al. 1982; Milani et al. 1994; Murawaki et al. 1997; Perez-Tamayo et al. 1987; Roeb et al. 1997; Takahara et al. 1995, 1997). The data illustrated in this paper are in perfect agreement with the former result, however, in the case of MMP-14 the results contrast with others (Takahara et al. 1997). As assessed by northern hybridization of human fibrotic liver, Takahara and coworkers (1997) recently showed that MMP-14 expression increased simultaneously to MMP-2 steady state mRNA levels, which was not evident in this report and might be explained by the different source of tissue studied. Additionally the present study demonstrated that the expression of other MMPs, such as stromelysins, were downregulated in fibrotic livers compared to controls in agreement with others (Lichtinghagen et al. 1998).

Overall it appears noteworthy that the MMP/TIMP expression patterns present in fibrotic livers strongly resembled those of the recovery phase following a single liver injury mediated by CCl_4. Since chronic liver injury is caused by repeated single tissue injury events, it is tempting to suggest that the TGF-β-driven induction of MMP-2 and TIMPs dominates the tissue repair reaction, which lacks an appropriate TNF-α-mediated induction of MMPs, as observed in the early phase of the single CCl_4 injury model. A possible explanation for this hypothesis might be provided by the fact that in HSC, an important cellular source of MMPs, MMP and TIMP expression as well as its regulation by TNF-α and TGF-β1 are highly dependent on the differentiation status of these cells (Knittel et al. 1999b). In vitro, resting HSC and HSC at an early stage of activation, which are believed to correspond to HSC present in normal liver or at the onset of tissue injury, are characterized by high MMP (exception MMP-2) and low TIMP expression levels and respond to TNF-α with a strong induction of MMP expression collectively mapping to the early phase of the single injury model. In contrast, activated HSC, which are believed to correspond to HSC present in fibrotic liver, display low MMP (exception MMP-2) and high TIMP expression and respond to TGF-β1 with further upregulation of TIMP mRNA steady state levels thereby reflecting the MMP/TIMP expression pattern detected in fibrotic livers. However, in vitro the TNF-α-mediated induction of MMPs present in resting HSC is completely missing in activated HSC suggesting that during fibrogenesis diminished matrix degradation is caused by inappropriate MMP expression due to general lower basal MMP expression of activated HSC and lack of MMP inducibility by TNF-α in activated HSC.

The present study localized MMP-9-positive cells in normal and injured livers illustrating that in normal liver only a few MMP-9-positive cells were present, which increased in number in acutely as well as chronically injured livers. Although our previous studies indicate that Kupffer cells in primary culture appear to represent an important source of MMP-9 (Knittel et al. 1999b), at least in normal livers Kupffer cells do not display MMP-9 positivity. Based on the absolute number of MMP-9-positive cells and their tissue localization in diseased livers as well as the lack of colocalization with Kupffer cells, the MMP-9-positive cells might correspond primarily to inflammatory, mononuclear cells including lymphocytes or neutrophils, as shown in other disease models (Kherif et al. 1999; Trocme et al. 1998; Zaoui et al. 1996).

Finally the present report analyzed MMP/TIMP expression following partial hepatectomy to study the role of matrix degradation in a liver regeneration model lacking necrosis and inflammation. In contrast to the acute CCl_4 intoxication model, MMPs and TIMPs were generally not induced in this model, however a strong induction of MMP-14 expression and a minor stimulation of TIMP-1-specific transcripts were noted. As known so far, major matrix turnover does not take place in the early stages of hepatic regeneration, while the deposition of the ECM protein laminin within hepatocyte clusters at later stages is known to play an important role in the formation and reorganization of the regenerating liver (Martinez-Hernandez and Amenta 1995).

However, it has been reported recently that laminin, entactin, and fibronectin decreased shortly after partial hepatectomy and were restored later suggesting that a rapid reorganization of selected ECM components is important for hepatocyte proliferation at the early stages (0.25–24 h) of liver regeneration (Kim et al. 1997). Since no major MMP induction was present in this report analyzing the latter time interval, blood-derived protease activity might account for the ECM degradation. Alternatively MMP-14 induction might be involved in this process, since recent reports indicate that MMP-14 (MT1-MMP), apart from its role in MMP-2 activation, on its own can directly degrade a number of matrix macromolecules at high efficiency. MMP-14 (MT1-MMP) present on the cell surface caused focal degradation of gelatin films (Dortho et al. 1998) and regulated neovascularization by acting as pericellular fibrinolysins (Hiraoka et al. 1998). Therefore the induction of MMP-14 expression following partial hepatectomy could be connected to fibrinolysis or matrix degradation in the pericellular compartment of hepatocytes and might be required for hepatocellular replication, in particular as

the first round of DNA synthesis occurring 12–16 h following partial hepatectomy is concomitant with maximal MMP-14 induction (Fausto and Webber 1993; Fausto et al. 1995).

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