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Comparison of matrix metalloproteinase expression in normal and cirrhotic human liver

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Abstract To study the extend of ongoing tissue remodelling in end-stage cirrhosis, the expression of different matrix metalloproteinases [interstitial collagenase (MMP-1), M_r 72000 gelatinase (MMP-2), stromelysin-1 (MMP-3) and stromelysin-3 (MMP-11)] and of TIMP-1 was studied in 13 cirrhotic livers explanted at transplantation. The results were compared with those obtained in normal liver. Western blot, northern blot, ELISA, RT-PCR and zymogram analysis were used. Proenzymes of stromelysin-1 and -3, interstitial collagenase and M_r 72000 gelatinase were positive in normal liver, while activated enzymes were not detectable in western blot analysis. In cirrhosis proenzyme levels of the studied MMPs were reduced to a mean of 60–70%, but mRNA expression and gelatin-degrading activity increased. TIMP-1 expression was detectable on mRNA level and by ELISA in normal liver and also increased in cirrhosis. Our results show that mRNA expression of certain matrix metalloproteinases is increased in end-stage liver cirrhosis, while the amount of proenzyme is decreased, indicating enhanced MMP proenzyme turnover. These data suggest that besides increased TIMP-1 activity, altered MMP expression may also play a part in fibroproliferation in liver disease.

Key words Liver cirrhosis · Matrix metalloproteinase · MMP · Collagenase · Stromelysin · TIMP

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Introduction

The development of hepatic cirrhosis is characterized by a substantial increase in the collagen content of the liver accompanied by major changes in liver architecture [22]. It can therefore be described as a process of tissue remodelling. The net accumulation of collagen in this process may reflect increased synthesis as well as decreased breakdown of collagenous proteins, so that the rate of extracellular deposition of collagen exceeds its rate of degradation [7, 11, 15].

Collagen turnover and extracellular matrix remodelling is largely the effect of matrix metalloproteinases, a family of neutral proteases requiring zinc ions for activity and digesting different collagens and gelatines (denatured collagen derivates) [2, 10]. Their activity is regulated both at the transcriptional level, by proenzyme activation, and finally by inhibitors, which are coexpressed and are present at the tissue sites of enzyme activity [20]. Earlier reports suggest that hepatic perisinusoidal cells (Ito cells), besides producing different collagens, are also capable of expressing matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) [4, 5, 19]. During the process of liver fibrosis and finally cirrhosis the Ito cells seem to dedifferentiate into myofibroblast-like cells. In this process the spectrum of proteins and enzymes secreted by these cells changes, to the effect that more collagens, but also more metalloproteinase inhibitors, are produced [11]. Little is known, however, about the changes that may occur in MMP production during the process of liver fibrosis and may also contribute to the tilting of the balance between matrix production and degradation.

We have recently raised antibodies against human MMPs and were able to show that in normal liver there is expression of quite a number of these enzymes, most notably stromelysin-1, stromelysin-3, interstitial collagenase and both gelatinases (M_r 72000 and M_r 92000) [17]. In the study reported here, we examined the expression of stromelysin-1, stromelysin-3, interstitial collagenase and M_r 72000 gelatinase in end-stage cirrhosis and compared it with the expression of TIMP-1.

Materials and methods

Polyclonal antibodies were raised against recombinant MMP fusion proteins (haemopexin-like domain) as described previously [17]. The fusion proteins consisted of the following segments of the native enzymes: stromelysin-1 (amino acids 263–327 [24]), stromelysin-3 (amino acids 227–347 [6]), interstitial collagenase (amino acids 257–340 [12]), and M_r 72000 gelatinase (amino acids 315–437 [9]).

Normal liver tissue was obtained from explanted livers that had been meant for liver transplantation but could not be utilized for reasons unrelated to this study. The cirrhosis samples were obtained from explanted livers from patients undergoing liver transplantation for end-stage liver disease. Liver slices were cut from the explanted liver immediately after excision and were kept sterile and placed on crushed ice. Samples were cut into aliquots and frozen to –70°C. Clinical data of the patients were taken from their clinical files; histological scores were done from routine slices of the explanted livers. Inflammation was graded from 0 to +++ as no, slight, moderate and severe lymphocytic infiltration. Fibrosis was assessed as 0 to +++ representing no, slight, moderate or severe fibrosis /cirrhosis, respectively. Histological slides were examined by two different investigators.

Pieces approximately 100 mg in weight were homogenized in 300 µl Tris buffered saline (50 mmol/l Tris/HCl, pH 7.5, 100 mmol/l NaCl) using an ultra-turrax (Kinematica). After centrifugation at 10,000 *g* the supernatant was stored at –70°C. The total protein concentration was measured using Biuret reagent (Boehringer Mannheim) [23] on a Hitachi 747 automatic analyser. Protein was diluted to 10 µg/µl.

The Amersham Biotrak TIMP-1, human ELISA system was used for determination of TIMP-1 concentrations in the tissue supernatants (20–100 µg total protein per assay) according to the manufacturer's instructions.

Aliquots (5 µl) of the tissue extracts (50 µg total protein/lane) were subjected to analytical SDS-PAGE (gel size: 6.0 × 8.5 cm) and then tranferred to nitrocellulose strips (BA85; Schleicher & Schüll) in a Sartoblot2 transfer apparatus (Sartorius) for Western blot analysis. A set of prestained protein standards was simultaneously run on SDS-PAGE and transferred to the excised strip. The blotted membrane was blocked overnight with 1% blocking reagent (Boehringer Mannheim) in Tris-buffered saline (50 mmol/l Tris/HCl, pH 7.5, 100 mmol/l NaCl). Subsequently, membrane strips were incubated for 2 h at room temperature with the primary antiserum diluted in 5 g/l blocking reagent in Tris-buffered saline (dilutions are indicated in legend to Fig. 1). Membrane strips were washed twice in Tris-buffered saline with 1 g/l Tween 20, twice in 5 g/l blocking reagent in Tris-buffered saline, and incubated for a further hour with peroxidase-conjugated sheep anti-rabbit IgG (Boehringer Mannheim; 1:1000 in 5 g/l blocking reagent in Tris-buffered saline). After four washing steps in Tris-buffered saline with 1 g/l Tween 20, immunoreactive bands were visualized using a luminol-based detection system (Boehringer Mannheim) and exposure to X-OMAT AR film (Kodak). Quantification on developed films was performed by scanning densitometry using a scientific digital documentation system (Kodak) and Sigmagel software (Jandel). Purified human MMP proenzymes were obtained from Biogenesis.

For northern blot analysis, frozen liver tissue was pulverized in liquid nitrogen using a mortar and pestle. The tissue powder was then dissolved in 4 mol/l guanidine isothiocyanate, 25 mmol/l sodium acetate, pH 6.0. Total RNA was isolated by pelletting through 5.76 mol/l cesium chloride, 25 mmol/l sodium acetate, pH 6.0. The concentration of RNA was determined by measuring the absorbance at 260 and 280 nm [18]. Then 10 µg RNA per lane was applied to formaldehyde, 1% agarose gels and electrophoresed before tranfer onto positively charged nylon membranes (Boehringer Mannheim). The RNA was UV cross-linked to the filters and hybridized using standard conditions [18]. Cloning of MMP-specific cDNAs was described previously [17]. The cDNA probes were labelled with a (^{32}P)dCTP using a random primer labelling kit (Boehringer Mannheim). Filters were exposed to Hyperfilm-MP (Amersham) at –70°C.

To establish whether there was any MMP mRNA in the examined tissues we performed reverse transcription-polymerase chain reaction (RT-PCR) analysis. To exclude possible amplification of DNA contamination, a prior DNase digestion was performed and the amplification products were characterized by their specific molecular sizes after polyacrylamide gel electrophoresis. Then 5 µg total RNA was incubated with 10 U RNase-free DNase (Boehringer Mannheim) for 1 h at 37°C. After phenol/chloroform extraction and ethanol precipitation reverse transcription was performed using First Strand Synthesis Kit (Pharmacia) with random priming. PCR was performed under standard conditions [21]. The oligonucleotide primer sequences were described previously [17]. Nested oligonucleotide primers were used for interstitial collagenase (5'-CAA AAT CCT GTC CAG CCC ATC G-3', nucleotides 856–877; 5'-CGG CAA CTT CGT AAG CAG CTT C-3', nucleotides 1051–1072), for M_r 72000 gelatinase (5'-CTC TCC TGA CAT TGA CCT TGG CAC-3', nucleotides 1294–1317; 5'-CAA GGT GCT GGC TGA GTA GAT CAC-3', nucleotides 1574–1597) for stromelysin-1 [5'-GTA CCC ACG GAA CCT GTC CCT C-3', nucleotides 867–887; 5'-AGC TTC CTG AGG GAT TTG CGC C-3', nucleotides 988–1008) and for stromelysin-3 (5'-CAA CAC CTA TAT GGC CAG CCC TGG-3', nucleotides 769–792; 5'-AGG GCT GGG CAG TCC CTG CCA GTG-3', nucleotides 1012–1035). PCR products were run on polyacrylamide gel and were silver stained [16].

Gelatin-degrading activity of the liver samples (50 µg total protein/assay) was assessed by electrophoresis on nonreducing SDS-10% polyacrylamide gels incorporating 1.5 mg of gelatin/ml [14]. MMP activity was detected by an overnight incubation step in 5 mmol/l $CaCl_2$, 100 mmol/l Tris/HCl, pH 8.0 at 25°C before Coomassie Brilliant Blue staining of the gels.

Results

Polyclonal antibodies were raised against the haemopexin like domain of different MMPs. The amino acid sequences in these domains of the enzymes show a lesser extent of sequence homology than other domains, especially in the case of the stromelysins. Thereby cross-reactivity of the antibodies with other members of the MMP family could be avoided. Antibody specificity was assessed by ELISA and western blot as previously described [17].

Clinical data of the cirrhotic patients whose livers were analysed and the histological gradings of the explanted livers are given in Table 1. Three patients (5, 7, and 11) had histologically incomplete cirrhosis. They had been transplanted for small HCCs (5 and 7) and and for pronounced peripheral wasting (11).

Figure 1 shows the results of the western blot assays. In the cirrhotic livers studied (lanes 1–13), the amount of detectable matrix metalloproteinases stromelysin-1, stromelysin-3, interstitial collagenase and M_r 72000 gelatinase was quite variable. In general there was a tendency towards a reduction in the detectable amount of all of these MMPs compared with normal liver. Figure 2 summarizes the intensities of the detected bands in all western blots. For each enzyme the percentage deviation between the mean peak area of four normal livers and the individual area of all normal and diseased livers was calculated. For all MMPs we found a mean intensity reduction of about 60–70% in end-stage cirrhosis.

To assess whether the molecular masses of the detected bands correspond to the inactive proenzyme or the active forms of the MMPs we compared immunoreactive bands

Table 1 Clinical data of patients and histological scores of the explanted livers studied (*AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *AP* alkaline phosphatase, *γ-GT* γ-glutamyl transferase, *Alc* alcoholic liver cirrhosis, *HBV* hepatitis B-induced liver cirrhosis, *HCV* hepatitis C-induced liver cirrhosis, *PBC* primary biliary cirrhosis, *PSC* primary sclerosing cholangitis)

No.[a]	Age (years)	AST	ALT	Bilirubin	AP	γGT	Prothrombin time[b]	Fibrosis[c]	Inflammation[d]	Diagnosis
1	27	70	35	17	458	53	48	III	I	Alc
2	42	112	55	89	267	75	35	III	II	Alc
3	43	75	43	594	147	29	18	III	II	HBV
4	34	39	37	21	151	58	60	III	I	HBV
5	50	24	10	20	194	17	85	II	I	HBV
6	51	68	77	50	288	27	49	III	II	HCV
7	39	31	43	107	162	41	55	II	I	HCV
8	61	25	16	127	296	28	41	III	0	PBC
9	52	303	70	129	151	32	22	III	III	PBC
10	54	43	39	55	492	79	82	III	II	PBC
11	50	17	22	7	318	94	94	II	0	PBC
12	60	263	80	36	690	63	61	III	III	PBC
13	37	82	48	44	1044	168	56	III	II	PSC

[a] Same numbers as in Fig. 1
[b] Percentages of normal values
[c] *0* no fibrosis to *III* fibrosis with destruction of normal organ architecture (i.e. cirrhosis)
[d] *0* no relevant lymphocytic infiltration and no cell necrosis to *III* severe lymphocytic infiltration of portal and lobular areas accompanied by multiple cell necrosis

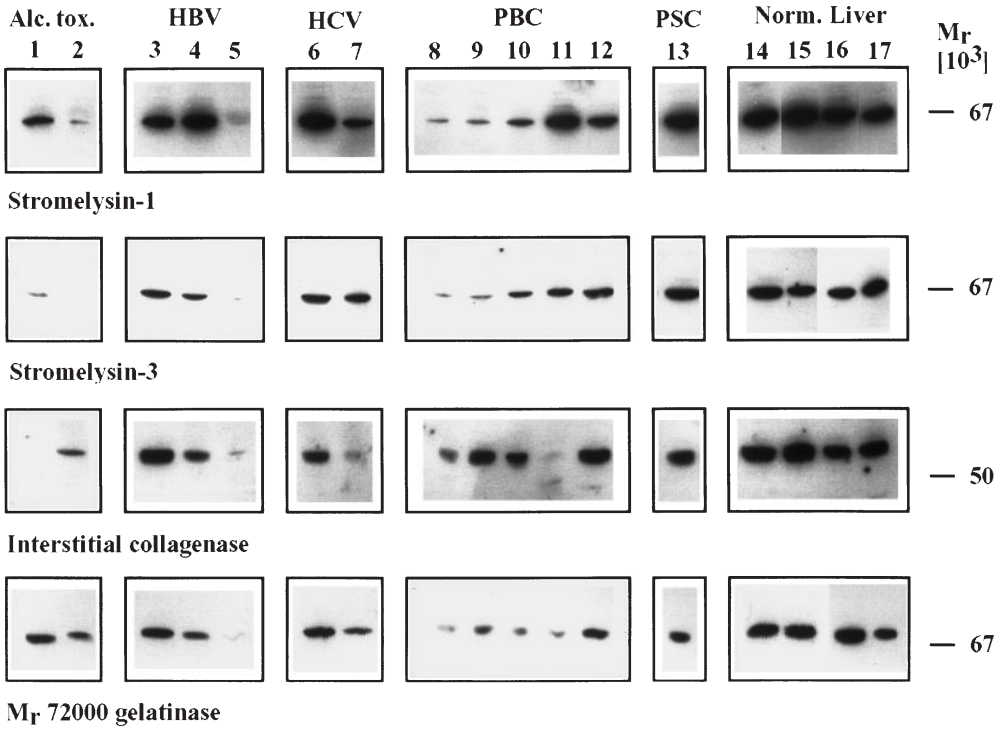

Fig. 1 Western blot assay of different liver samples for matrix metalloproteinases (MMPs). Crude tissue preparations (see "Materials and methods" for details) were separated by 9% SDS polyacrylamide gel electrophoresis and immunoblotted using polyclonal antibodies against stromelysin-1 (dilution 1: 2000), stromelysin-3 (1: 1000), interstitial collagenase (1: 500) and M_r 72000 gelatinase (1: 2000). Tissue preparations were from: alcoholic liver cirrhosis (*1*, *2*), hepatitis B-induced cirrhosis (*3–5*), hepatitis C-induced cirrhosis (*6*, *7*), primary biliary cirrhosis stage IV (*8–12*), primary sclerosing cholangitis (*13*) and normal liver (*14–17*). Immunoreactive bands were visualized using a luminol-based detection system. No bands of any significance were observed in other regions of the gels. Molecular mass markers were a set of prestained proteins [prestained SDS-PAGE standard solution (Sigma)] with molecular masses of native proteins (M_r, 10^3) of 45 and 58 with apparent molecular masses of 50 and 67

in human liver with purified proenzymes of stromelysin-1, interstitial collagenase and M_r 72000 gelatinase. The control enzymes were commercial preparations purified from cultured fibroblasts, which secrete these proteins in two individual proenzyme forms of different sizes [2]. Figure 3 shows the results of this comparison. The immunoreactive bands of stromelysin-1, interstitial collagenase and M_r 72000 gelatinase in human liver comigrated with the largest corresponding secreted fibroblast proenzyme.

The gelatin-degrading activity of the different liver samples was assessed by zymogram analysis as shown in Fig. 4. In normal liver only bands with molecular masses of approximately [M_r 10^3] 95 were detectable. The inten-

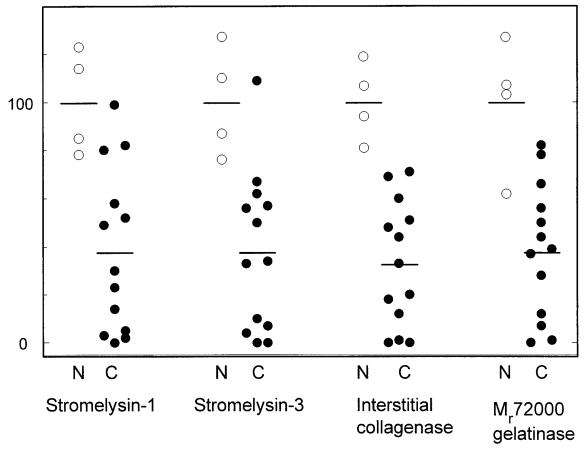

Fig. 2 Semiquantitative analysis of MMP expression in different samples from cirrhotic and normal livers. The scanned areas of immunoreactive bands (see "Materials and methods") were expressed as percentage deviations of the mean values of the normal liver samples. [*N* normal liver samples (m), *C* cirrhotic liver samples (l), *bars* mean values of each group]

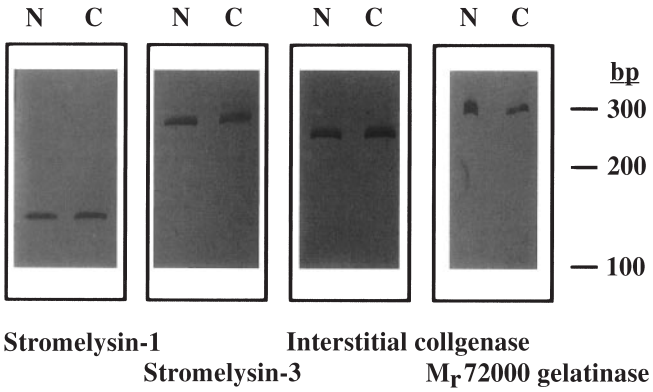

Fig. 5 Qualitative identification of MMP transcripts in total RNA from normal and cirrhotic human liver using RT-PCR technique. Fragments, which are size specific only for mRNA of stromelysin-1 (141 bp), stromelysin-3 (266 bp), interstitial collagenase (216 bp) and M_r 72000 gelatinase (303 bp) were amplified with specific oligonucleotide primers using cDNA templates copied from DNase-digested total RNA after reverse transcription and random priming. Total RNA samples were from normal liver (lane *N*) and primary biliary cirrhosis (lane *C*). Molecular mass marker was a 100-bp ladder (Pharmacia)

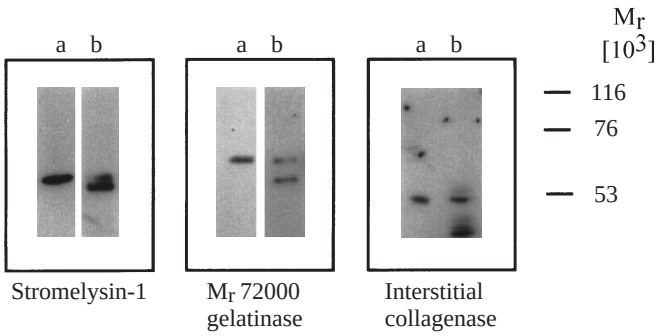

Fig. 3 Comparison of MMPs from human liver and the corresponding proenzymes purified from cultured fibroblasts. Crude tissue preparations from normal human liver (*a*) and purified proenzymes (stromelysin-1, M_r 72000 gelatinase, interstitial collagenase) (*b*) from cultured fibroblasts were separated by 9% SDS polyacrylamide gel electrophoresis and immunoblotted using polyclonal antibodies against haemopexin-like domain of the corresponding MMPs. For antibody dilutions see legend to Fig. 1. For molecular mass markers see legend to Fig. 4

sities of these bands were increased in cirrhosis. Moreover main bands of approximately [M_r 10^3] 61 and 67 were only detectable in cirrhotic liver samples.

RT-PCR clearly demonstrated the presence of mRNA for all MMPs studied. Figure 5 shows that both in normal and in cirrhotic livers PCR yielded amplification products of the appropriate mRNA sizes for MMP-1, MMP-2, MMP-3 and MMP-11. In contrast, classic northern analysis only detected signals for M_r 72000 gelatinase and stromelysin-3, while mRNA levels for interstitial collagenase and stromelysin-1 were apparently too low to be detected without prior amplification. In Fig. 6 the results of Northern analyses with stromelysin-3 and M_r 72000 gelatinase-specific probes are shown. Stromelysin-3 was not detectable in normal liver samples, whereas it was strongly positive in the placenta control. A slight increase in some of the cirrhosis samples was notable. M_r 72000 gelatinase mRNA was weakly detectable in normal liver and stronger positive in total RNA from cirrhotic liver samples. Stromelysin-1 and interstiti-

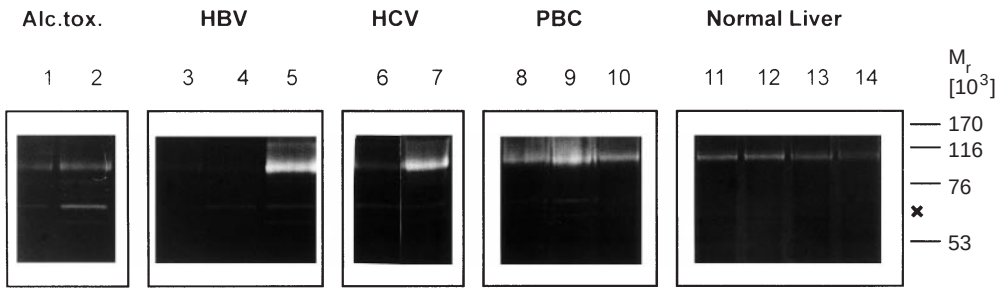

Fig. 4 Zymogram analysis of samples from normal and cirrhotic human livers. Aliquots of the prepared liver samples were subjected to SDS-PAGE in 10% acrylamide gels containing 0.15% gelatin (see "Materials and methods"). Gelatin-degrading activity in four normal liver samples (*11–14*) was compared with different cirrhotic samples like alcoholic liver cirrhosis (*Alc.tox.; 1, 2*), hepatitis B-induced cirrhosis (*HBV; 3–5*), hepatitis C-induced cirrhosis (*HCV; 6, 7*), and primary biliary cirrhosis stage IV (*PBC; 8–10*). Molecular mass markers were a set of proteins with molecular sizes [M_r, 10^3] of 53, 76 and 116 and 170 (Sigma)

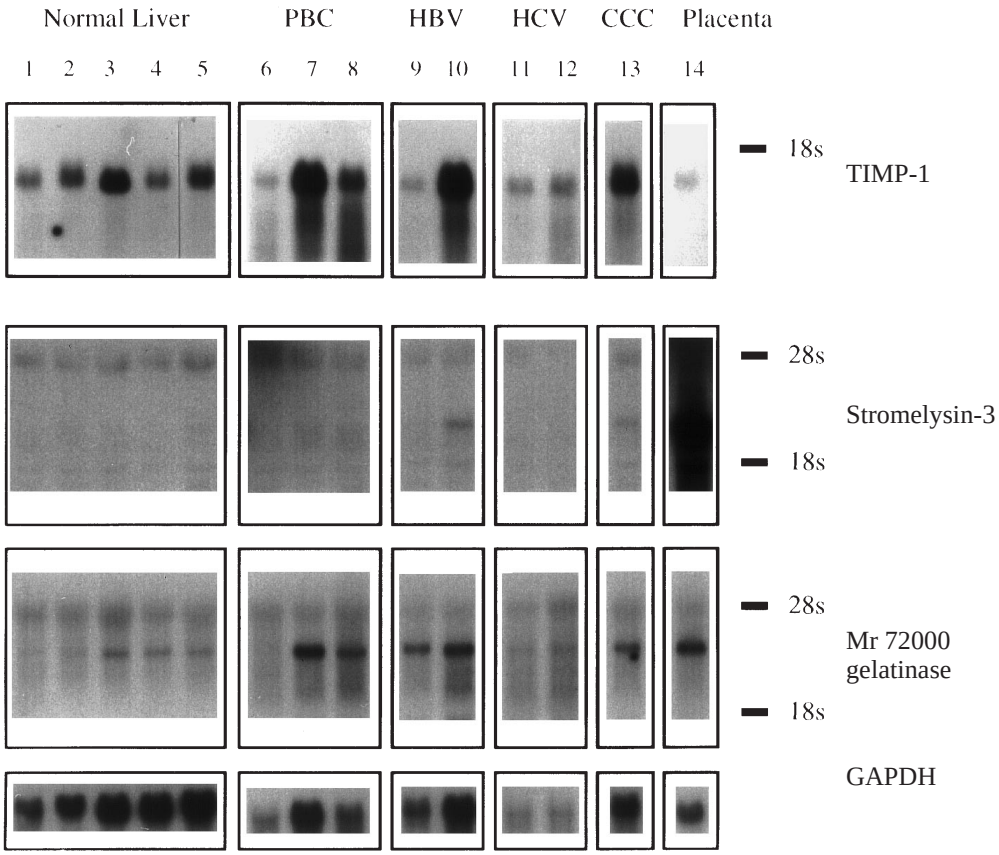

Fig. 6 Northern blot analysis of mRNA encoding for stromelysin-3 and M_r 72000 gelatinase in total RNA prepared from normal and cirrhotic human liver. Results with total RNA from normal liver (*lanes 1–5*) and cirrhotic liver samples (*lanes 6–12*) are shown. Cirrhotic liver samples are divided into primary biliary cirrhosis (*PBC*), hepatitis B-induced cirrhosis (*HBV*) and hepatitis C-induced cirrhosis (*HCV*). Preparations from cholangiocellular carcinoma (*CCC, lane 13*) and from placenta (*lane 14*) are shown as positive controls. Hybridization with a GAPDH-specific probe was performed as an external standard. Relative positions of the 28S and 18S ribosomal RNA bands are indicated as molecular weight standards. For a comparison of the cirrhotic samples with Western analysis in Fig. 1 the corresponding numbers are put in parentheses: 6(12), 7(11), 8(8), 9(3), 10(5), 11(6) and 12(7)

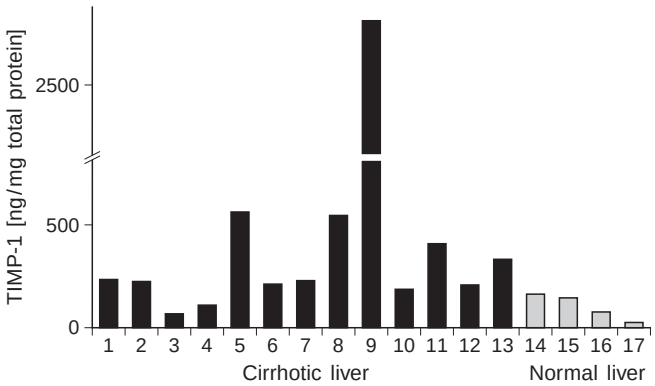

Fig. 7 ELISA quantification of TIMP-1 in samples from normal and cirrhotic human livers. TIMP-1 concentrations in tissue preparations from 13 cirrhotic human livers and 4 normal livers (see legend to Fig. 1) was performed according to the manufacturer's instructions (Amersham). Measured mean values were in normal livers 100 ng/mg total protein with a standard deviation of 65. In cirrhotic livers we found 455 (270) ng/mg total protein with a standard deviation of 690 (156). The values in parentheses are those obtained when sample 9 is excluded

al collagenase were negative using Northern technique (data not shown).

In summary, we found a reduced amount of MMP proenzymes in cirrhotic human liver, while the gelatin-degrading activity in cirrhotic livers was increased.

TIMP-1 mRNA was clearly detectable in all normal livers, and increased amounts were seen in cirrhosis (see Fig. 5). Correspondingly increased amounts of TIMP-1 protein were detected in the cirrhotic sampled by ELISA (see Fig. 7).

Discussion

The emerging hypothesis concerning matrix metabolism in liver cirrhosis suggests that changes in collagen production and a reduction in collagen breakdown contribute to the net accumulation of extracellular matrix [3, 8, 11]. Recent results suggest that this is mainly due to increased TIMP expression in chronic liver disease [7, 15].

Our results confirm that TIMP-1 mRNA and protein are increased in cirrhotic human liver. Furthermore, we find increased mRNA levels of MMP-2 and -11 and increased gelatin-degrading activity in cirrhosis. At the same time, proenzyme levels for the MMPs studied were unchanged or even lower in cirrhotic than in normal livers. These findings suggest increased activation and turnover of MMPs in cirrhosis. In cultured rat kidney mesangial cells, Ailenberg et al. [1] described enhanced steady state mRNA levels for gelatinase A (MMP-2) following increasing activation of MMP-2 by cytochalasin D treatment. We propose that in fibroproliferative liver diseases a similar mechanism might be operative (increased activation of MMPs accompanied by elevated mRNA levels).

MMPs are secreted as inactive proenzymes, and after activation they are rapidly degraded. It is thus not surprising that only the proenzyme can be assessed at protein level. The proenzyme pool functions as a reservoir from which active enzyme can be recruited rapidly. We found reduced amounts of MMP proenzymes in some of our cirrhotic samples, suggesting that the proposed increase in transcription does not compensate the increased degradation of MMPs in all cases.

While all four metalloproteinases analysed in our study were clearly detectable on protein level and using RT-PCR also on mRNA level, the high variability of the results obtained in the samples analysed shows that there are substantial interindividual differences in the extent to which tissue remodelling persists in the late stages of liver diseases. The clinical term "end-stage cirrhosis" does not reflect a precise and well-defined stage in the fibrotic process.

Histological and biochemical analysis revealed no correlation between inflammatory activity and the gelatinolytic activity demonstrated by zymography. Also there was no correlation between the amount of fibrosis and the proenzymes detected in western blot analysis. We are therefore unable to speculate about mechanisms inducing MMP-regulatory processes, which have been viewed by others as a counterregulatory mechanism against increasing collagen synthesis [11, 13, 22].

Earlier we demonstrated that in normal human liver, apart from matrilysin (MMP-7) virtually the whole family of MMPs can be detected [17]. Our present findings suggest that matrix degradation and tissue remodelling continue even in very late stages of organ fibrosis. The level of MMP expression and activity is not significantly different from that found in hepatic tumour tissue, but it is rather low compared with the levels in highly dynamic tissues, such as placenta.

Further studies must establish whether measurement of MMPs in liver tissue leads to a better understanding of the pathophysiology of fibroproliferation and allow an assessment of the prognosis in individual cases.

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