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Antifibrotic effect of silymarin in rat secondary biliary fibrosis is mediated by downregulation of procollagen α1(I) and TIMP-1

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Background/Aims: **Silymarin reduces hepatic collagen accumulation by 35% in rats with secondary biliary cirrhosis. The aim of the present study was to explore its antifibrotic mechanism.**

Methods: **Thirty female adult Wistar rats were allocated to (1) bile duct occlusion, (2) bile duct occlusion and oral silymarin at 50 mg/kg per day, and (3) sham operation and oral silymarin at 50 mg/kg per day. Steady-state mRNA levels for procollagen α1(I), tissue inhibitor of metalloproteinases-1 (TIMP-1), and transforming growth factor (TGF) β1 were determined by multi-probe ribonuclease protection assay.**

Results: **After 6 weeks of bile duct occlusion, liver collagen content was increased 12-fold, when compared with the sham-operated controls. These animals displayed 17-, 6.5- and 16-fold higher transcript levels for procollagen α1(I), TIMP-1 and TGFβ1 ($P < 0.01$). Silymarin downregulated elevated procollagen α1(I), TIMP-1 and TGFβ1 mRNA levels by 40-60% ($P < 0.01$). These lowered hepatic profibrogenic transcript levels correlated with decreased serum levels of the aminoterminal propeptide of procollagen type III.**

Conclusions: **Silymarin suppresses expression of profibrogenic procollagen α1(I) and TIMP-1 most likely via down-regulation of TGFβ1 mRNA in rats with biliary fibrosis. The serum procollagen type III propeptide level mirrors profibrogenic mRNA expression in the liver.**

Keywords: **Antifibrotic therapy; Cirrhosis; Collagen; Fibrosis; Fibrogenesis; Liver, Matrix metalloproteinase-3; Matrix metalloproteinase-13; Aminoterminal propeptide of procollagen type III; Procollagen; Silymarin; Tissue inhibitor of metalloproteinases-1; Transforming growth factor β1**

1. Introduction

Silymarin (SIL) is a standardized extract of the milk thistle *(Silybum marianum)* that has a long tradition as a herbal remedy [1]. The main active compounds of SIL are the flavonoids silibinin, silychristine and silydianin, with silibinin amounting to approx. 60% of all ingredients. SIL as well as silibinin activates hepatocyte RNAse polymerase II, restores their ATPase activity and glutathione content, and prevents (oxidative) membrane damage [2,3]. SIL is an attractive drag to treat liver disease, since it virtually lacks toxic side effects [4,5]. It prevents or attenuates acute Liver injury caused by carbon tetrachloride [6,7], paracetamol [8,9], D-galactosamine [10], ischemia/reperfusion [11] or radiation [12,13]. Two reports showed that SIL prevents fibrosis induced by carbon tetrachloride [14,15]. However, in this and related animal models, the antifibrotic effect of SIL may rather results from its antioxidant and radical scavenging properties.

Using the model of secondary biliary fibrosis after complete bile duct occlusion (BDO), which results in progressive fibrosis in the virtual absence of inflammation and necrosis [16], we previously showed that SIL prevented

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both total and relative hepatic collagen accumulation by 30-35% [17].

Fibrosis results from increased synthesis and deposition, i.e. fibrogenesis, and/or decreased degradation and removal, i.e. fibrolysis, of extracellular matrix components [18]. Transforming growth factor β (TGFβl) plays an important role as profibrogenic factor in chronic liver disease, triggering the expression of procollagen I and tissue inhibitor of metalloproteinases-1 (TIMP-l), key effectors of fibrogenesis [19,20]. In biliary fibrosis hepatic expression of TGFβ1, procollagen α 1(I) and TIMP-l is highly upregulated [21-24].

Therefore, we further explored the hitherto unknown antifibrogenic mechanism of SIL by using multi-probe ribonuclease protection assays (RPA) for procollagen α1(I), TIMP-l and TGFβ1 mRNA. Their hepatic mRNA levels were correlated to serum levels of the aminoterminal propeptide of procollagen type III (PIIINP), a surrogate marker of hepatic fibrogenesis.

2. Material and methods

2.1. Rat model of secondary biliary cirrhosis

Thirty adult female Wistar rats (Schoenwalde, Germany) weighing approx. 250 g were maintained 12:12-h light/dark cycles and at 23 ± 2°C and a humidity of 60 ± 10%. Bile duct occlusion (BDO) was performed as reported previously [16,17]. In brief, after midline abdominal incision and proper isolation, the common bile duct was injected with sodium amidotriazoate (Ethibloc 0.2 ml/kg body weight, Ethicon, Germany) in retrograde direction using a Teflon catheter (Abbocath-T 26 G, Abbott, Chicago, IL, USA), followed by double ligation and scission in-between. Sham operation was a midiine abdominal incision, isolation of the common bile duct and wound closure, SIL (50 mg/kg per day; Madaus, Cologne, Germany) was given by oral route For 6 weeks after BDO [17]. Groups of ten rats received: (1) sham operation with SIL, (2) BDO without SIL, and (3) BDO with SIL. After 6 weeks, animals were killed and aliquots of right and left liver lobes snap-frozen in liquid nitrogen.

All animal experimentation was carried out in accordance with our institutional and governmental regulations on the use of experimental animals.

2.2. *Determination of hepatic hydroxyproline content and serum aminoterminal procollagen 111 peptide levels*

Hepatic hydroxyproline (HYP) content was determined in duplicates from 0.2 g of formalin-fixed rat liver tissue using the method of Jamall et al. [25] with minor modifications [16,17]. The serum aminoterminal procollagen III peptide (PIIINP) was measured by a radioimmunoassay based on rat PIIINP, a monospecitic rabbit antiserum to rat PIIINP and a goat antiserum to rabbit IgG as detailed before [16,17].

2.3. RNA isolation

Total RNA was extracted from liver by the single-step method of Chomezynski and Sacchi [26] with minor modifications. In brief, approx. 300 mg of snap-frozen rat liver was powdered in liquid nitrogen and immediately homogenized in 4 ml guanidine-thiocyanate with an Ultra-Turrax T25 Basic Dispersion Unit (IKA, Staufen, Germany) at 20 000 rpm for 1 min. To remove insolubles, the homogenate was centrifuged at 9000 x *g* at 4°C for 20 min, followed by acid-phenol-chloroform extraction and isopro panol precipitation. Concentration and purity of RNA were determined by spectrophotometry at 260/280 nm and integrity of RNA was verified by visualization of the 18S and 28S rRNA bands after agarose electrophoresis and ethidium bromide staining.

2.4..Sources of plasmids

To create the rat procollagen α1(I) probe a 1.3 kb *PstI/Hind* III-fragment of plasmid α1R1 (a kind gift of Dr. C.G. Rowe) [27] was subcloned into pGEMI (Promega, Madison, WI, USA). Rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [28] and TGFβ1 [29] plasmids were gifts from Dr. X.L. Tian (Max Delbrueck Center for Molecular Medicine, Berlin-Buch, Germany). The rat TIMP-I containing plasmid was generated by reverse transcription or" rat liver RNA using an oligo-dT primer and PCR amplification with primers according to positions 109-133 and 415-439 of a published partial cDNA (GenBank accession no. L29512) [30]. The blunt-ended amplicon was cloned into the *Eco*RV site of pZErO-1 (Invitrogen, Leek, Netherlands) and orientation and identity confirmed by restriction map analysis. Rat MMP-3 and MMP-13 plasmids were obtained from Dr. L.M. Matrisian (Molecular Genetics, University of Strasbourg, France) [31] and Dr. CO. Quinn (Cardinal Glennon Children's Hospital, St. Louis, MO, USA) [32], respectively.

2.5. *Preparation of riboprobes*

The cDNA templates for procollagen α1(I), TIMP-l, TGFβ1, MMP-3, MMP-13 and GAPDH were linearized with *RsaI, Ncol, Accl, Hsp92I, Kpn1* and Msel, respectively. In vitro transcription was carried out with 5 units of bacteriophage T7 RNA polymerase (Promega) in 10 µl of a mixture containing 0.5 µg of cDNA template, 50 µM each of ATP, CTP and GTP, 5 µM of UTP (50 µM UTP for GAPDH), 50 µCi of [α-^{32}PJUTP (800 Ci/mmol, 10 mCi/ml; NEN Life Science Products, Boston, MA, USA), 1 mM of dithiothreitol, 20 U of RNAsin (Promega, Madison, Wl, USA), 1 X transcription buffer. After incubation at 37°C for 60 min, the transcription mixture was digested with I unit of RNase-free DNase (Promega) at 37°C for 30 min. The riboprobes were purified by electrophoresis through a denaturing polyucrylamide gel (5%, 8 M urea) and the radioactivity of eluted probes was measured by liquid scintillation.

Table 1
Parameters of fibrogenesis in biliary fibrotic rats and controls[a]

	HYP (mg/liver)	HYP (µg/g liver)	PIIINP (ng/ml)	TGFβ1 mRNA[b]	Procoll, αl(I) mRNA[b]	TIMP-1 mRNA[b]
Sham/SIL	1.74 ±0.26**	234.21 ± 13.54**	3.77 ± 0.87**	1.11 ± 1.01**	0.147 ±0.052**	0.111 ±0.076**
BDO/SIL	14.50 ± 5.65**	574.57 ± 120.04*	10.73 ± 3.66*	6.32 ±4.07**	0.942 ± 0.446**	0.394 ±0.121**
BDO	22.44 ± 7.54	758.71 ± 171.64	13.90 ±4.82	17.45 ± 7.23	2.407 ±1.283	0.675 ±0.189

[a] Sham/SIL, sham operation and daily oral administration of silymarin (50 mg/kg per day) for 6 weeks; BDO, bile duct occlusion; BDO/SIL, rats with BDO treated with silymarin (50 mg/kg per day) from weeks i to 6; PIIINP, aminoterminal propeptide of procollagen type III. Values are mean ± SD. Significance: *P < 0.05 and **P < 0.01 vs. BDO alone.

[b] Normalized to GAPDH mRNA-levels; ten rat livers were analyzed per group.

2.6. *Multi-probe ribonuclease protection assay*

Ribonuclease protection assay was carried out with RPA II kit (Ambion. Austin, TX, USA) according to the manufacturer's instructions. Briefly, 20 μg of total RNA. 30 000 cpm of each probe (20 000 cpm for GAPDH) were hybridized in 20 μl of hybridization buffer containing 80% formamide at 45°C for 16 h. As negative control, 20 μg of yeast tRNA was used instead of rat liver RNA. Then 200 μl of digestion buffer containing 20 units of RNase Tl was added to the hybridization mixture and incubated at 37°C for 1 h. The ethanol-precipitated pellets were resuspended in 6 μl of loading buffer, denatured at 90°C for 5 min and run on a 5% polyacrylamide/8 M urea gel at 8 W for 60 min. The gel was exposed to X-ray film (Kodak, Rochester, NY, USA) at −70°C for 16 h. Expected sizes of protected sequences for procollagen α1(I), TIMP-1, TGFβ1, MMP-3, MMP-13 and GAPDH were 206, 264, 250, 400, 450 and 102 nucleotides, respectively.

2.7. *Signal quantification and statistical analysis*

The autoradiographs were analyzed with a public domain NIH Image program (developed at the US National Institutes of Health) on a Power Macintosh 7500/100 computer. The target mRNA signals were normalized to the signal of GAPDH mRNA and expressed as relative abundance (arbitrary units). The differences in hepatic hydroxyproline content, serum PIIINP and relative abundance of mRNA among groups were analyzed by one-way ANOVA and confirmed by Student's-Newman-Keuls test. $P < 0.05$ was regarded as statistically significant. Pearson correlation of serum PIIINP with hepatic hydroxyproline content and mRNA levels of procollagen α1(I), TIMP-1 and TGFβ1 was also calculated after double-logarithmic transformation.

3. Results

3.1. *Silymarin decreases hepatic collagen content and serum PIIINP levels*

Rats with BDO developed histological cirrhosis within 6 weeks (not shown). Total (per liver) and relative (per g of liver) collagen content, as quantitated by hydroxyproline-determinations, increased 12.5- and 3.5-fold, respectively. In accord with a previous study [17], SIL significantly decreased total and relative hepatic collagen by 35% and 25%, respectively (Table 1). Serum PIIINP levels, which were elevated 3.5-fold in untreated BDO-animais, were lowered by 25% under SIL treatment (Table 1)

3.2, *Silymarin downregulates profibrogenic mRNAs*

Hepatic procollagen α1(I), TIMP-1 and TGF-β1 steady state mRNA levels were determined by multi-probe ribonuclease protection assays. The protected bands showed the expected sizes and there were no equivalent bands in the negative controls. After BDO the relative abundances of hepatic procollagen α1(I) and TIMP-1 and TGFβ1 transcripts were highly increased relative to the sham-operated controls ($P < 0,01$, Fig. 1 and Table 1). SIL significantly lowered procollagen α1(I), TIMP-1 and TGFβ1 mRNA levels by 40-60% ($P < 0.05$, Fig. 1 and Table 1). Transcripts encoding MMP-3 and MMP-13 were not detectable in any group of rats (data not shown).

3.3. *Correlation of serum PIIINP levels with hepatic parameters of fibrogenesis*

After 6 weeks serum PIIINP showed a good correlation with hepatic total and relative collagen content, and with

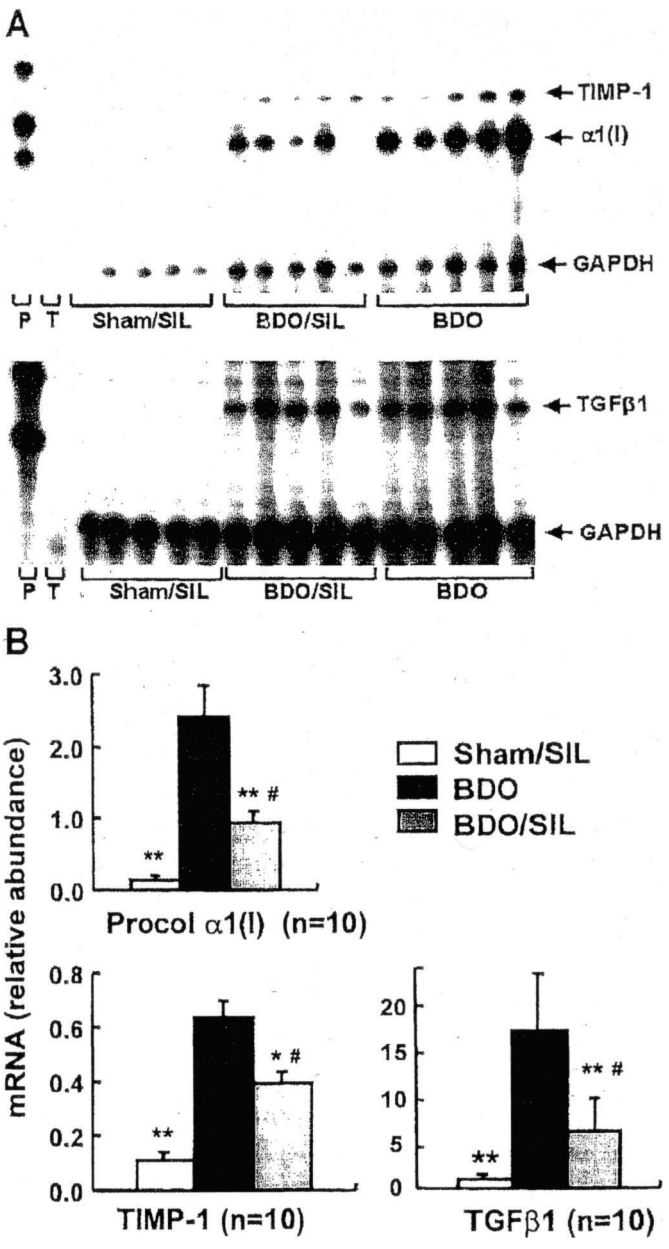

Fig. 1. (A) Representative examples of the multiprobe ribonuclease protection assays for TIMP-1, procollagen α1(I) and GAPDH (upper panel), and for TGF β1 and GAPDH (lower panel). Twenty micrograms of total RNA from rat livers were hybridized with various anti-sense RNA probes at 45°C overnight. After digestion with RNase Tl the protected probes were separated by electrophoresis through a denaturing polyacrylamide gel and subjected to autoradiography overnight at −70°C for 16 h. P, full-length probes, from top to bottom, TIMP-1, procollagen α1(I) and GAPDH (upper panel), and TGFβ1 and GAPDH (lower panel); T, complete probe degradation in the specificity control with yeast tRNA. (B) Downregulation of steady-state procollagen α1(I), TIMP-1 and TGF β1 mRNA-levels in rat biliary fibrosis by silymarin. Signals were normalized to GAPDH and expressed in arbitrary units. The error bars represent standard deviations. *$P < 0.05$, **$P < 0.01$ vs. BDO alone; #$P < 0.05$ vs. sham/SIL.

transcript levels of procollagen α1(I), TIMP-1 and TGFβ1. Among profibrogenic transcripts the best correlation were found between TGFβ1 and procollagen α1(I) mRNA and between TIMP-1 and procollagen α1(I) mRNA (Fig. 2 and Table 2).

4. Discussion

Biliary fibrosis in rats after complete bile duct occlusion (BDO) is progressive and leads to an 8-12-fold increased total hepatic collagen content after 6 weeks [16,17]. We used a multi-probe RNAse protection assay (RPA) to show that, after 6 weeks of BDO, hepatic mRNA levels of procollagen type α1(I), TIMP-1 and TGFβ1 were upregulated 16-, 6- and 16-fold, respectively. RPA is a sensitive method for detection and quantification of mRNAs [33], and the used multi-probe design [34] eliminates errors that would be caused by running separate parallel reactions for each target transcript. Our results expand and confirm previous semi-quantitative data for the model of BDO obtained by in situ hybridization or Northern blot analysis [19,21,22,35].

The reproducible 25-35% reduction of both total and relative liver collagen by silymarin (SIL) is paralleled by a significant suppression of procollagen α1(I) mRNA, which encodes the major collagen type in fibrosis, and of TIMP-1 mRNA, which encodes the most important physio-

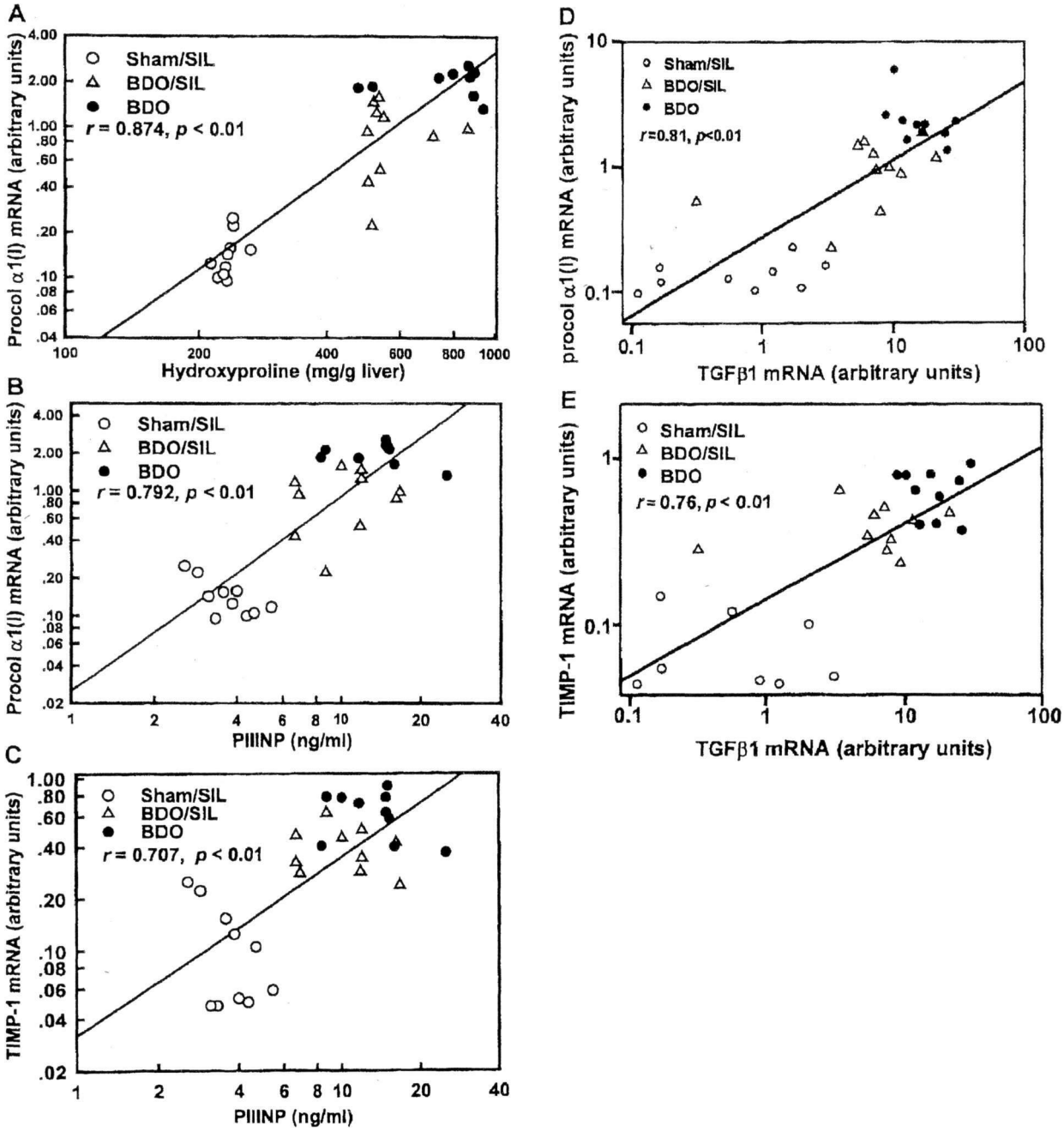

Fig. 2. Correlations between profibrogenic transcripts and hepatic hydroxyproline content or serum PIIINP levels.

Table 2
Correlation between parameters of hepatic fiorogenesis[a]

	Procoll. α1(I) mRNA	TIMP-1 mRNA	TGFβ1 mRNA	Total HYP	Relative HYP
PIIINP	0.792	0.707	0.699	0.939	0.929
Procoll. α1(I) mRNA		0.869	0.810	0.894	0.874
TIMP-1 mRNA			0.760	0.853	0.818
TGFβ mRNA				0.782	0.772
Total HYP					0.989

[a] Values of all treatment groups were correlated ($n = 30$). Pearson correlation was carried out after logarithmic transformation. All correlations are significant at the level of $P < 0.01$.

logical inhibitor of various MMPs. This allows to draw two conclusions: (1) SIL exerts its antifibrogenic effect either on a transcriptional level or by decreasing profibrogenic mRNA stability, and (2) SIL has a dual beneficial action by inhibiting fibrogenesis (procollagen α1(I) expression) and by relieving the suppression of ECM degradation (blocking TIMP-I mRNA expression). In this regard SIL resembles on oral endothelin A receptor antagonist that also decreases rat biliary fibrosis by downregulation of both procollagen α1(I) and TIMP-1 mRNA [36]. However, concomitant downregulation of these profibrogenic mRNAs by potential antifibrotic agents is not the rule, as exemplified by pentoxifylline which potently decreased procollagen α1(I) mRNA (8-fold), but upregulated TIMP-I mRNA 2-fold, resulting in an only moderate net inhibition of collagen accumulation in rat biliary fibrosis [37].

Since SIL did not influence matrix turnover in quiescent stellate cells [381 or in normal rat livers [39], the decrease of profibrogenic mRNA expression under treatment with SIL seems to be closely linked to a suppression of hepatic stellate cell activation, as previously shown by a reduced number of desmin positive cells both in vitro [38] and in the BDO model in vivo [39]. Thus in the present study SIL induced a remarkable reduction of the highly elevated hepatic TGFβl transcript levels in rats with BDO, As a key cytokine in fibrogenesis, the overall effect of TGFβ1 is promotion of matrix deposition. However, the role of TGFβ1 as a pure profibrogenic cytokine is not undisputed [20,21], since it can suppress immune responses and thereby inhibit profibrogenic effects of inflammation. Furthermore, high concentrations of TGFβl can block mesenchymal migration and proliferation. Thus elevated bioactive TGFβl in arteries can prevent intimal fibrosis in a rat model of atherosclerosis [40]. Nonetheless, since TGFβl upregulates the expression of both procollagen α1(I) [41] and TIMP-1 [42], the downregulation of procollagen α1(I) and TIMP-1 by SIL is most likely mediated via suppression of TGFβ1. It remains to be shown on the protein level if the amount of bioactive TGFβl is also reduced by SIL.

In order to assess the modulation of matrix proteolysis by SIL, we additionally focused on two key proteases, namely MMP-3 and MMP-13. MMP-3 degrades several noncollagenous matrix proteins and serves as a proactivator of other MMPs, and MMP-13 serves as the major interstitial collagenase in rat [43]. We could neither detect MMP-3 nor MMP-13 expression in normal rats or rats with BDO, whereas clearly positive signals were found in the stomach of rats with experimental gastric ulcers which served as positive controls (data not shown). These findings are in agreement with previous studies using in situ hybridization that showed a lack of MMP-3 mRNA from livers of normal rats or rats with BDO, and only a brief upregulation, mainly in hepatocytes, after a single dose of CCl_4 [44]. Similarly, MMP-13 was undetectable in livers of normal rats and in rats after partial hepatectomy [45]. Since RPAs are more sensitive than in situ hybridization or Northern blotting, MMP-3 and -13 appear to play no or only a minor role in hepatic fibrolysis. Our findings are in conflict with a study that showed a weak MMP-13 mRNA signal in normal and cirrhotic rat liver which remained unchanged during development of liver fibrosis caused by CCl_4 or BDO [30]. Though technical differences in the applied RPAs may account for this difference, we rather suggest that MMP-3 and MMP-13 do not play a prominent role in hepatic fibrolysis of the rat, and that the main ECM-degrading enzymes remain to be identified.

Furthermore, we found that hepatic TGFβl mRNA correlated well with procollagen α1(I) and TIMP-1 mRNA. More importantly, serum PIIINP, a surrogate marker of fibrogenesis, reflected hepatic mRNA levels for TGFβ1, procollagen α1(I) and TIMP-1. These results not only suggest that serum PIIINP can mirror the extent of ongoing hepatic fibrogenesis and thus matrix deposition in rat biliary fibrosis, but also lends further support for its clinical use as a noninvasive marker for the monitoring of hepatic fibrogenesis in man.

We conclude that SIL downregulates the expression of both procollagen α1(I) and TIMP-1 mRNA in livers of rats with secondary biliary cirrhosis. These beneficial effects appear to be mediated via inhibition of TGF-βl expression. PIIINP predicts the antifibrotic effect of SIL.

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