Activation of hepatic stellate cells is associated with cytokine expression in thioacetamide-induced hepatic fibrosis in mice

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The pathophysiological mechanisms of thioacetamide (TAA)-induced hepatic fibrogenesis are not yet fully understood. In particular, the role of hepatic stellate cells (HSCs) remains unclear. We therefore examined proliferation and trans-differentiation of HSC as well as the underlying molecular mechanisms in TAA-induced fibrosis. Hepatic fibrogenesis was induced in mice by addition of TAA to drinking water. Liver damage was determined by assessment of alanine amino-transferase and aspartate aminotransferase levels, and measurement of collagen deposition. Additionally, expression patterns of α -smooth muscle actin, glial fibrillary acidic protein (GFAP, specific hepatic biomarker for HSC), cysteine- and glycine-rich protein 2 (CRP2, specific marker of HSC transdifferentiation), tissue inhibitor of metalloproteinases-1, matrix metalloproteinase-9 (MMP-9), interleukins (IL-1 β , IL-6), platelet-derived growth factors (PDGF-B, PDGF-D), tumor necrosis factor (TNF)- α , and (transforming growth factor (TGF)- β 1 were assessed by real-time PCR. Transcription of GFAP and CRP2 were transiently upregulated during TAA-induced fibrogenesis (punctum maxima (p.m.) week 10 for GFAP and week 14 for CRP2). Similar transient expression patterns were demonstrated for IL-1 β , IL-6, TGF- β 1, and PDGF-B (p.m. week 12) whereas TNF- α and PDGF-D continuously increased with ongoing liver injury. In particular, not only neutrophil granulocytes, but also macrophages and leukocytes served as a major source for MMP-9 expression. GFAP and CRP2 expression patterns demonstrated transiently increased HSC-activation during TAA-induced hepatic fibrogenesis. The rate of increase of transcription of GFAP correlated best with PDGF-B, whereas CRP2 levels correlated with PDGF-B, PDGF-D, and IL-1 β expression. This study demonstrates for the first time that transiently increased activation patterns of HSC are observed in toxically induced hepatic fibrosis. Thus, TAA in drinking water is an effective and elegant model to induce reproducible states of liver fibrosis without parenchymal damage in mice.

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The understanding of the cellular and molecular pathogenesis that eventually leads to liver fibrosis is of major importance. From a morphologic point of view, hepatic fibrosis is characterized by the accumulation of extracellular matrix (ECM), especially collagen, with hepatic stellate cells (HSCs) comprising the main collagen-producing cells during this process. Numerous cell culture experiments and rodent models of hepatic fibrosis have been performed to

elucidate the molecular mechanisms of HSC activation in fibrogenesis.¹

One of the most commonly used models to induce liver fibrosis chemically is the repetitive intraperitoneal (i.p.) injection of carbon tetrachloride (CCl₄), and the structural as well as molecular and biochemical changes in response to CCl₄ are well described in the literature.² A number of other approaches to induce liver fibrosis have been reported, eg the

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application of dimethylnitrosamine, thioacetamide (TAA), α -naphthyl-isothiocyanate, or 3,5-diethoxycarbonyl-1,4-dihydrocollidine.³

The hepatotoxin TAA has been applied in a variety of studies investigating the underlying mechanisms of liver fibrogenesis. TAA was administered orally,^{4,5} via i.p. TAA injections,⁶ or utilizing a combined approach, ie TAA injections together with alcohol feeding in drinking water.⁷ Compared to other experimental approaches, TAA-induced liver fibrosis most closely resembles alcoholic liver fibrogenesis as it shares a number of metabolic and histological alterations usually found in the livers of afflicted human beings.^{8–10}

In chronic liver injury, eg liver cirrhosis, hepatic wound healing is mediated by myofibroblasts that are potentially derived from a multiplicity of cells, with HSC being the most important cellular source.¹¹ In normal liver tissue, HSCs (desmin/glial fibrillary acid protein (GFAP)-positive cells-expressing synemin¹²) are perisinusoidal cells that usually reside in the space of Disse and contain numerous retinoid and lipid droplets. In contrast, liver myofibroblasts (desmin/ α -SMA-positive, GFAP-negative cells colocalized with fibulin-2) are located in the portal field, the walls of central veins, and only occasionally in the parenchyma.¹³ More recently, in rat liver, GFAP expression was detected in quiescent HSC *in vivo* during the acute phase response, whereas chronic liver injury led to a downregulation of the enzyme; and GFAP has therefore been suggested as an early marker of HSC activation.¹⁴ In the liver, cysteine- and glycine-rich protein 2 (CRP2) gene is exclusively expressed by HSC, with no transcripts detectable in hepatocytes, sinusoidal endothelial cells or Kupffer cells.¹⁵ Early activation of HSC induced by platelet-derived growth factor (PDGF) is accompanied by an enhanced expression of CRP2. During later stages of transdifferentiation, the expression of CRP2 in HSC is suppressed *in vitro*.¹⁵

Despite all efforts, the mechanisms underlying TAA-induced hepatic fibrogenesis still remain vague. Moreover, the potentially important role of HSC and their characterization during the development of TAA-induced liver fibrosis has not been determined. The aim of this study was to examine the cellular and molecular changes underlying TAA-induced fibrosis in mice, compared with the widely established and well-characterized CCl₄ model. Therefore, we focused on three aspects: first, fibrosis score in relation to liver tissue damage; second, expression of surrogate markers for HSC proliferation and transdifferentiation (especially GFAP, CRP2, and α -SMA); and third, cytokine regulation (especially IL-1 β , IL-6, PDGF-B, PDGF-D, tumor necrosis factor (TNF)- α , and TGF- β 1).

MATERIALS AND METHODS

Animals

Liver fibrosis was induced by TAA or CCl₄ administration in 10-week-old wild-type C3H/He mice. The present study was performed with permission of the State of Hesse,

Regierungspräsidium Giessen, according to section 8 of the German Law for the Protection of Animals and conforms to the *Guide for the Care and Use of Laboratory Animals*.

Liver Damage

TAA (200 mg/l; Sigma-Aldrich, Munich, Germany) were orally administered in drinking water for 6, 10, 12, 14, or 16 weeks, respectively ($n = 7$ for control, $n = 4$ for each group of injured animals). CCl₄ (Merck, Darmstadt, Germany; 0.8 ml/kg as a 1:1 mixture with mineral oil) was injected i.p. twice a week for 4 weeks to constitute fully established liver fibrosis as a positive control. Animals were killed at least 2 days after the last CCl₄ injection.

Immunohistochemistry

Formaldehyde-fixed liver was paraffin-embedded, sliced in 6 μ m sections and stained with 0.1% Sirius red F3B in saturated picric acid (Chroma, Münster, Germany). Morphometric analysis of hepatic fibrosis was performed using semiquantitative fibrosis scores corresponding to the score system suggested by Batts and Ludwig.^{16,17} For α -SMA staining, 3 μ m sections were deparaffinized and hydrated through xylene and grade alcohol series and then incubated with 3% H₂O₂ to block peroxidase activity. Afterward, sections were digested with 0.1% trypsin for 10 min. Sections were then blocked in 5% bovine serum albumin (BSA; PAA, Pasching, Austria) and subjected to immunohistochemical staining with α -SMA monoclonal antibody (Progen, Heidelberg, Germany) and biotinylated secondary antibody (Vector Laboratories Inc., Burlingame, USA). Color reaction was performed with the VECTASTAIN[®] Elite ABC-kit (Vector Laboratories). Sections were co-stained with hematoxylin to visualize nucleus and finally Pertex mounted.

Immunofluorescence

Frozen sections (3 μ m) were blocked for 30 min with 5% BSA, 2% goat serum (Biomedica, Foster City, USA) and 0.1% cold fish skin gelatine (Sigma-Aldrich) in PBS with 0.1% Triton (Roth, Karlsruhe, Germany) and 0.05% Tween 20 (Serva, Heidelberg, Germany). Sections were then co-immunostained with rabbit anti-GFAP antibodies or rabbit anti-CRP2 antibodies, Alexa 488-conjugated goat anti-rabbit IgG as well as with mouse anti- α -SMA antibodies and Alexa 468-conjugated goat anti-rabbit IgG. Co-immunostainings were performed with goat anti-elasticin- and mouse anti-desmin antibodies (purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA). 4',6-diamidino-2-phenylindole dihydrochloride (Sigma-Aldrich) was used for nucleus staining.

Co-immunostainings were also performed with goat anti-MMP-9 and a set of IgGs for detection of cellular marker molecules: mouse anti- α -SMA, rat anti-GFAP, rat anti-CD45, rat anti-F4/80, mouse anti-myeloperoxidase.

Glial fibrillary acidic protein antibodies were from DAKO (Glostrup, Denmark) and α -SMA antibodies from Progen. CRP2 antibodies were raised against the epitope GIKPESAQPHRPTTN,

corresponding to amino acid 89–103 of murine CRP2, linked to KLH.¹⁸ MMP-9 IgGs were purchased from R&D Systems (Wiesbaden, Germany), CD45 IgGs from BD (Heidelberg, Germany), F4/80 IgGs from Dianova (Hamburg, Germany), and myeloperoxidase IgGs from Abcam (Cambridge, UK). Alexa 468- and Alexa 488-conjugated secondary antibodies were purchased from Molecular Probes (Eugene, OR, USA).

Hydroxyproline Content

Total collagen was determined by hydroxyproline quantification as described before¹⁹ with only minor modifications.²⁰ Briefly, mouse liver tissue was hydrolyzed with 6 N HCl at 110°C for 14 h. Hydrolysates were filtered through 45- μ m pore filters (Sartorius, Göttingen, Germany), dried and the sediment was redissolved in 50% isopropanol. Samples and hydroxyproline standard were incubated with chloramine-T buffer (Merck) for 10 min at room temperature. Ehrlich's reagent was added and the samples were again incubated for 45 min at 65°C. Absorbance of each sample was measured at 450 nm using a microplate reader (Packard BioScience, Meriden, CT, USA). Hydroxyproline levels were calculated against standard curves of 4-hydroxy-L-proline (Sigma) and expressed as μ g hydroxyproline per gram liver tissue.

RNA Extraction, cDNA Synthesis, and Quantitative Real-Time PCR

Total liver RNA was isolated using RNeasy Kit (QIAGEN, Hilden, Germany) from liver tissue ground to a powder in liquid nitrogen. RNA (1 μ g) was utilized for the reverse transcriptase reaction (Omniscript, QIAGEN) according to the manufacturer's instructions. Aliquots of 40 ng cDNA were used as template for quantitative real-time PCR using the Platinum SYBR Green qPCR Kit from Invitrogen (Karlsruhe, Germany) according to the manufacturer's protocol. Optimization was performed for each gene-specific primer prior to the experiment to confirm the absence of any nonspecific amplification product. Primers were purchased from MWG Biotech (Ebersberg, Germany) and primer sequences are presented in Table 1.

qRT-PCR was performed on the Mx3000P (Stratagene, La Jolla, CA, USA) by using 3-stage program parameters as follows: (1) 10 min at 96°C, (2) 40 cycles of 10 s at 95°C, 30 s at 57°C, and 30 s at 73°C, (3) 10 min at 73°C. The specificity of the PCR was confirmed by examination of the dissociation reaction plot subsequent to qRT-PCR. PCR products were separated on a 1.5% TAE agarose gel and visualized by staining with ethidium bromide to confirm the appearance of a single band of the correct molecular size. qRT-PCR-data were analyzed using the $\Delta\Delta C_t$ model.²¹

Immunoblotting

Liver tissue was ground to powder in liquid nitrogen and proteins were isolated by lysing cells with buffer (20 mmol/l Tris (pH 7.4), 150 mmol/l NaCl, 2 mmol/l EDTA, 1% Triton,

Table 1 SYBR Green real-time qPCR primer sequences

Target gene	Primer	Sequence
r18S	Forward	5'-gaataatggaataggaccgcgg-3'
	Reverse	5'-ggaactacgacggatctctgac-3'
α 1-(I)-collagen	Forward	5'-caaggctcttctggatcaagt-3'
	Reverse	5'-cctttatgcctctgtcaccttg-3'
GFAP	Forward	5'-cgtgtggatttgagagagaag-3'
	Reverse	5'-gtgaggctgcaacttagacc-3'
CRP2	Forward	5'-gctacggaagaagttaggacc-3'
	Reverse	5'-ctcagtcagagttgtagatcc-3'
IL-1 β	Forward	5'-catcagcttcaaatctcgcag-3'
	Reverse	5'-cacacaccagcaggttatcatc-3'
IL-6	Forward	5'-catgttctctggaaatcgtgg-3'
	Reverse	5'-gtactccaggtagctatggtac-3'
PDGF-B	Forward	5'-gtcaggttggaagctcatctc-3'
	Reverse	5'-gagatgagctttccaactcgac-3'
PDGF-D	Forward	5'-caattcggactagaggaagcag-3'
	Reverse	5'-cttcgggttggaatcttccac-3'
TGF- β 1	Forward	5'-gcggactactactgtaagagg-3'
	Reverse	5'-gtagagtccacatgtgtctcc-3'
TNF- α	Forward	5'-ggcaggtactcaagacaacc-3'
	Reverse	5'-caggtatatgggctcataccag-3'

Abbreviations: CRP2, cysteine- and glycine-rich protein 2; GFAP, glial fibrillary acidic protein; IL, interleukin; PDGF, platelet-derived growth factor; TGF, transforming growth factor; TNF, tumor necrosis factor.

and protease inhibitors (Complete mini, Roche, Mannheim, Germany) for 15 min on ice. Debris was removed by centrifugation for 30 min at 20 000 g (4°C) and protein content was determined according to the Bradford method. Homogenates (50 μ g per lane) were separated on 10–12% SDS-polyacrylamide gels and proteins were blotted to PVDF membranes. After blocking with BSA, membranes were incubated with antibodies to matrix metalloproteinase-9 (MMP-9, R&D Biosystems), tissue inhibitor of metalloproteinase-1 (TIMP-1; Biodesign International, Maine, MA, USA), α -SMA (Progen), and tubulin (NeoMarkers, Thermo Fisher, Fremont, USA).

Liver Function Tests

Blood of each mouse was collected and serum separated for analyzing the activity of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the Institute of Clinical Chemistry, RWTH-University Hospital Aachen, utilizing a Roche P module analyzer.

Statistical Analysis

Statistical analysis was performed with SPSS 12.0 software applying Mann-Whitney *U*-test and correlations were

Figure 1 Increased matrix deposition after liver damage with CCl₄ or TAA. **(a)** For the detection of collagen, paraffin liver sections of control mice as well as of CCl₄ (4 weeks, w) and TAA (10, 16 w)-treated mice were stained in 0.1% Sirius red F3B, hematoxylin and eosin (H&E) and α -SMA. (original magnifications: SR, $\times 50$; H&E, $\times 400$, α -SMA, $\times 100$). **(b)** Fibrosis grade was assessed by a semiquantitative fibrosis score.^{16,17} Two trained technicians (RS and AT) blinded for the group of animals determined the fibrosis scores. $*P < 0.05$. **(c)** Entire collagen was assessed by measurement of hydroxyproline in mice liver. $*P < 0.05$. **(d)** The mRNA expression of COL1A1 was determined by qRT-PCR. The histogram shows relative gene expression of COL1A1 mRNA vs untreated mice. All results were normalized to r18S content. (mean $\pm$ s.e.m., $n = 4$, $*P < 0.05$). **(e)** ALT and AST increased following CCl₄ treatment whereas no elevation was observed after TAA treatment. $*P < 0.05$.

analyzed with Spearman's rank correlation. Results are presented as mean $\pm$ s.e.m. A two-sided $P < 0.05$ was considered significant. The agreement between the two scorers with regard to the fibrosis score was assessed using κ and weighted κ for ordinal scales. The calculation of weighted κ assumes the categories are ordered and accounts for how far apart the two scorers are. Given a scale consisting of four subsequent levels, eg A, B, C, and D, homogeneity between two assessors is larger if one classifies a subject into group B and the other into group C, in contrast to an allocation into A and D.

RESULTS

Characterization of Liver Fibrosis

In untreated control mice, staining of collagen with Sirius Red revealed collagen deposition surrounding the central veins and enclosing the portal triads, thus representing a typical morphologic pattern of collagen distribution in healthy liver tissue. In contrast, mice treated with CCl_4 over 4 weeks and with TAA over 16 weeks displayed a periportal fibrosis characterized by portal-portal septa surrounding the hepatic lobules (Figure 1a). We observed only minimal fibrosis during the first 6 weeks of TAA treatment with a considerable progression of collagen deposition thereafter (10, 12, and 14 weeks, respectively). To assess fibroblastic liver cells, paraffin sections were subjected to immunohistochemical α -SMA staining. TAA-treated mice depicted a strongly time-dependent development of α -SMA expression. The amount and expression pattern of α -SMA in mice treated with TAA for 16 weeks were similar to that observed in CCl_4 -induced fibrosis (Figure 1a).

A semi-quantitative grading method was applied to assess the amount of fibrosis with 0 representing no fibrosis and 3 characterizing completely fibrotic tissue.²² After four weeks of CCl_4 treatment the fibrosis score yielded 2.2 ($P = 0.011$). The grade of fibrosis in TAA-treated mice increased gradually over time with only minimal to moderate fibrosis after six and ten weeks of TAA treatment (0.2, $P > 0.05$ and 1.0, $P = 0.047$), intermediate fibrosis after twelve weeks (1.4, $P = 0.014$), and significant fibrosis after 14 and 16 weeks with a fibrosis score of 2.1 ($P = 0.011$ and $P = 0.013$ respectively) (Figure 1b). The concordance in the grading of fibrosis between the two assessors was moderate with a κ of 0.45 (95% confidence interval 0.2–0.7). However, calculating a weighted κ of 0.66 the strength of agreement is considered to be good.

In addition, the collagen content of the liver was determined quantitatively via hydroxyproline measurements. Hydroxyproline levels increased by twofold ($252.8 \pm 10.5 \mu\text{g/g}$ liver, $P = 0.021$) in CCl_4 -treated mice with a considerable increase starting upon the 10th week of TAA treatment ($216.6 \pm 35.2 \mu\text{g/g}$, $P = 0.043$). After 16 weeks hydroxyproline content was augmented more than threefold ($403.8 \pm 96.3 \mu\text{g/g}$, $P = 0.021$, control $136.1 \pm 14 \mu\text{g/g}$) (Figure 1c).

Assessment of collagen $\alpha 1$ (COL1A1) expression via RT-PCR mRNA levels revealed a fourfold ($P = 0.011$)

upregulation following six weeks TAA intoxication. After 12 weeks of TAA treatment, COL1A1 mRNA increased by 6.3-fold ($P = 0.010$) and remained nearly constant up to the 16th week (5.7-fold, $P = 0.019$), thus confirming the progression of liver fibrosis in this model over time (Figure 1d). CCl_4 injections caused a 26-fold increase in COL1A1 expression ($P = 0.019$).

After four weeks of CCl_4 -treatment clinically relevant surrogate parameters for liver function, ie ALT and AST rapidly increased (21.5- and 14.7-fold increase, respectively, both $P = 0.020$), whereas in TAA-treated mice nonsignificant (2.2- and 1.8-fold) increase in transaminases levels peaked at week 12 (Figure 1e).

Expression of MMP-9 and TIMP-1

MMP-9 protein expression increased significantly in CCl_4 -injured liver (2.2-fold, $P = 0.014$). By contrast, in the initial six weeks of TAA-administration no differences in MMP-9 expression were observed. Continuation of TAA treatment led to a comparable MMP-9 protein expression after twelve and 16 weeks (1.9-fold, $P = 0.014$) (Figure 2a).

Western blot analysis of liver homogenates revealed a time-dependent TIMP-1 expression pattern similar to that observed for MMP-9 with a 1.9-fold increase after CCl_4 -

Figure 2 Protein expression of MMP-9 and TIMP-1. (a) MMP-9 was increased in CCl_4 - and TAA-injured mouse liver. (b) Progressive increase of TIMP-1 after liver injury. Representative western blots are presented. The bar chart depicts the densitometric quantification of all western blots. All data were normalized to tubulin content. Fold increase is shown (mean $\pm$ s.e.m., $n = 4$, * $P < 0.05$).

treatment and a 2.5-fold increase ($P=0.014$) after 16 weeks of TAA intoxication (Figure 2b).

Activation of Hepatic Stellate Cells

To assess the role of fibroblastic liver cells, protein expression analysis of α -SMA was carried out. Western blot analysis showed a time-dependent upregulation of α -SMA beginning with the 10th week of TAA treatment (1.4-fold increase, $P=0.014$) with the peak at week 14 (2.1-fold increase, $P>0.05$) that was comparable to that in CCl₄-induced fibrosis (2.3-fold, $P=0.014$; Figure 3A).

Aimed toward the characterization of the development of fibrosis, the transcription level and immunofluorescence

staining of GFAP and CRP2 in HSCs were assessed. During TAA-intoxication mRNA expression of GFAP increased continuously up to week 10 (GFAP: 20-fold increase, $P=0.020$) whereas CRP2 peaked at the 14th week (CRP2: 6.1-fold increase, $P=0.011$). Thereafter, expression declined, although CRP2 transcription levels at week 16 were still elevated (4.7-fold for CRP2) compared with control mice. Chronic CCl₄ treatment led to an 8.8-fold increase of GFAP ($P=0.039$) but did not change CRP2 expression (Figure 3B).

Both HSC-activation markers, GFAP, and CRP2 were positively correlated with COL1A1 transcription in TAA-treated animals (GFAP: $r=0.72$, $P<0.001$, and CRP2: $r=0.70$, $P<0.001$; Figure 3C and D). In contrast to these HSC-

Figure 3 Expression of HSC markers. (A) Representative Western blot detecting α -SMA after 4 weeks of CCl₄ and after 6–16 weeks of TAA treatment. $*P<0.05$. (B) The transiently upregulation of GFAP (dark gray shaded columns) and CRP2 (light gray shaded columns) gene expression indicates HSC proliferation and transdifferentiation, respectively. $*P<0.05$. (C) Correlation between COL1A1 and GFAP (ΔCt depicts the crossing point of fluorescent threshold with amplification curve in quantitative PCR. ΔCt indicates a measurement of gene transcription.). (D) Correlation between COL1A1 and CRP2 (mean $\pm$ s.e.m., $n=4-7$, $P<0.05$). (E, F) Distribution of HSC in portal tracts expressing GFAP, α -SMA, and CRP2 in CCl₄- and TAA-injured mouse liver. (a) Triple staining for α -SMA (green), GFAP (red), and nuclei (blue) of liver sections from male mice treated with CCl₄ for 4 weeks (b) or with TAA for 10 (c) and 16 weeks (d), respectively. Arrows indicate co-staining. (F) Triple staining for α -SMA (green), CRP2 (red), and nuclei (blue) of liver sections from male mice treated with CCl₄ for 4 weeks (b) or with TAA for 10 (c) and 16 weeks (d), respectively. Arrowheads indicate co-staining. The decrease of GFAP and CRP2 together with higher numbers of single α -SMA-positive cells demonstrates almost complete transdifferentiation of HSCs following 16 weeks of TAA treatment (bar, 50 μ m, original magnification $\times 400$, PV = portal vein). Representative single stains and overlays are shown. (G) Triple staining for elastin (green), desmin (red), and nuclei (blue) around portal tracts. (a) Normal liver, (b) 4 weeks CCl₄ treated, and (c) 16 weeks TAA treated. Arrows indicate co-staining (bar, 50 μ m, original magnification $\times 400$, PV = portal vein).

Figure 3 Continued.

specific markers, α -SMA was not correlated with the expression of COL1A1 ($r = -0.45$, $P = 0.029$, data not shown).

Co-immunostaining for α -SMA/GFAP and α -SMA/CRP2 on liver-sections in control mice depicted a weak expression of α -SMA around blood vessels and typical dendritic-GFAP staining for quiescent HSCs in intact parenchyma, but almost no expression of CRP2 (Figure 3E and F, panels a).

Until the 10th week of TAA-treatment GFAP and CRP2 were increasingly found in portal and periportal areas (Figure 3E and F, panels c, arrows indicate co-staining). From the 10th to the 16th week GFAP expression declined whereas simultaneously α -SMA and CRP2 expression continuously increased. (Figure 3E and F, panels d, arrows indicate co-staining).

In CCl_4 -injured livers, enhanced expression of α -SMA was observed. The number of α -SMA/GFAP-positive cells was similar to that in TAA mice. In CCl_4 -treated mice coexpression of α -SMA together with CRP2, however, was only occasionally detected (Figure 3E and F, panels b, arrows indicate co-staining).

Co-immunostainings of elastin and desmin were undertaken to define the role of portal fibroblasts in TAA- and CCl_4 -induced fibrosis (Figure 3G).²³ Both, desmin- and elastin-positive cells were induced by chronic liver injury. The majority of fibrotic lesions around portal tracts and septae were positive for both markers.

Cytokine Regulation

To analyze the transient course of HSC activation considering the expression patterns of fibrogenic and inflammatory cytokines, transcription levels of IL-1 β , IL-6, TGF- β 1, PDGF-B, PDGF-D, and TNF- α were quantified by real-time PCR (Figure 4a and b). In TAA-treated mice transcription levels of IL-1 β , TGF- β 1, and PDGF-D significantly increased beginning with week 10, and declined after week 12. IL-6 was found increased in week 12 only. The amount of mRNA observed for the individual cytokine differed considerably from 3.4- to 8.6-fold. In contrast, in livers of CCl_4 -treated animals only IL-1 β expression was significantly increased (3.7-fold, $P = 0.021$).

Figure 3 Continued.

Compared with control, higher PDGF-B and TNF- α expression were found from week 6 to 16. After 16 weeks of TAA treatment, PDGF-B was upregulated 2.8-fold ($P=0.011$) and TNF- α 6.8-fold ($P=0.0034$). Again, differences in the magnitude of upregulation between the cytokines were pronounced.

In TAA-treated animals, significant correlations were found for the gene expression of GFAP and PDGF-B (Figure 5a), CRP2 and PDGF-B, PDGF-D, and IL-1 β , respectively (Figure 5b–d). Transcription of α -SMA was not correlated to any of the cytokines analyzed within this study.

Cellular Sources of MMP-9 in TAA-Treated Mice

As MMP-9 has been described as one of the most important proteases-mediating ECM turnover during fibrogenesis that is also capable of controlling HSC activation, the cellular sources of enhanced hepatic MMP-9 in TAA-injured liver were detected.^{24,25} Immunostaining with different marker

proteins was performed to disclose the cellular sources of MMP-9. MMP-9 was detected in neutrophil granulocytes, leukocytes, and Kupffer cells (Figure 6a–c). Following TAA-treatment liver tissue was interspersed with neutrophil granulocytes, most likely representing the major source of MMP-9 in liver fibrosis (Figure 6a). Interestingly, most of the neutrophil granulocytes were surrounded by ceroid-laden macrophages (arrowheads: yellow autofluorescent cells in Figure 6a, middle panel). In unstained liver slices, autofluorescent ceroid macrophages became visible in fibrotic areas (pictures not shown). MMP-9 expressing neutrophil granulocytes were accumulated in direct neighborhood with ceroid macrophages (Figure 6a). To distinguish between co-immunostaining and autofluorescence, Figure 6 provides the original micrographs of red and green channels (Figure 6, middle and right panels). By contrast, only a small number of MMP-9-expressing leukocytes (Figure 6b) and Kupffer cells (Figure 6c, arrowhead depicts yellow autofluorescent Kupffer cell) was detectable.

Figure 3 Continued.

DISCUSSION

In this study, we examined the role of HSC in hepatic fibrogenesis induced by oral TAA treatment in mice. In addition to the negative control mice, CCl₄-treated mice—representing a well-established and thoroughly described animal model for liver fibrosis—served as positive control.

The main findings of this study were threefold: First, collagen deposition in liver tissue of CCl₄- and TAA-treated mice confirmed fibrosis in both models with significant expression of α -SMA, MMP-9, and TIMP-1, respectively. Second, fibrosis-dependent upregulation of GFAP and CRP2 on mRNA and protein level demonstrated a transiently increased activation of HSCs during hepatic fibrogenesis induced by TAA treatment. Third, in TAA-treated mice proliferation of HSCs is correlated with PDGF-B whereas transdifferentiation is associated with PDGF-B, PDGF-D, and IL-1 β , respectively.

Utilizing well-established surrogate markers, ie histological grading, hydroxyproline content, and expression of COL1A1, liver fibrosis in orally treated TAA-treated mice was confirmed. The obvious variation between histological score (Figure 1b), hydroxyproline levels (Figure 1c), and COL1A1 transcription (Figure 1d) may well reflect the limitations of each assay to capture all aspects for the development and progression of liver fibrogenesis. Although the histological score (Figure 1b) of collagen-stained liver-slices primarily depicts distribution pattern of fibrillar collagen, the hydroxyproline (Figure 1c) more closely reflects the amount

of overall-collagen accumulation in fibrotic liver tissue. COL1A1 transcription analysis (Figure 1d), mainly contributes to the characterization of the grade of fibrosis primarily depicting the transcriptional level that is not necessarily consistent with protein content. Although inconsistency or varying output of the different methods may at first glance seem confusing, we consider the presentation of these slight variations important to emphasize the complexity of liver fibrogenesis that cannot be fully resembled by a single analytic approach alone.

Our study specifically addressed the time-dependent *in vivo* behavior of HSCs in TAA mice. Therefore, we monitored cell-specific markers for HSC proliferation and transdifferentiation, ie GFAP, CRP2, and α -SMA, respectively.

In liver tissue, GFAP is expressed in quiescent or partially activated HSCs but not in perivascular myofibroblasts.^{26–28} Hence, GFAP expression levels reveal the overall number of HSC and can therefore be utilized as a specific HSC proliferation marker. *In vitro*, CRP2 expression increases during activation of HSC and is suppressed in later stages of transdifferentiation, thereby displaying a specific marker for HSC activation/transdifferentiation.^{15,29} α -SMA is not only a marker for activated HSCs, but also for portal myofibroblasts.³⁰ Thus, despite very popular, α -SMA represents a rather unspecific marker with respect to HSC activation.

In TAA-treated mice, transcription levels of GFAP steadily increased until week 10 and declined thereafter, suggesting

Figure 4 Expression pattern of fibrogenic cytokines over time. (a) Transiently upregulated cytokines IL-6 (open columns), IL-1 β (light gray columns), TGF- β 1 (dark gray columns), PDGF-D (solid columns) in the course of TAA-induced hepatic fibrosis. Expression of IL-1 β (4.7-fold, $P = 0.021$), IL-6 (3.4-fold, $P = 0.021$), TGF- β 1 (7.0-fold, $P = 0.021$), and PDGF-D (8.6-fold, $P = 0.021$) peaked after 12 weeks of TAA treatment. IL-1 β and PDGF-D expression increased after 10 weeks (IL-1 β , 4.0-fold, $P = 0.021$; PDGF-D, 6.0-fold, $P = 0.021$) and declined after 14 weeks (IL-1 β , 3.9-fold, $P = 0.021$; PDGF-D, 5.9-fold, $P = 0.021$). * $P < 0.05$. (b) Transcription levels of PDGF-B (light gray columns) and TNF- α (dark gray columns) were increased with fibrogenesis. All data were normalized to r18S content. (mean $\pm$ s.e.m., $n = 4$, $P < 0.05$). Expression of PDGF-B (1.9-fold, $P = 0.042$) and TNF- α (3.6-fold, $P = 0.021$) after 6 weeks, and after 16 weeks of TAA treatment (PDGF-B 2.8-fold, $P = 0.011$, and TNF- α 6.8-fold, $P = 0.0034$). * $P < 0.05$.

HSC proliferation. We also observed an elevated expression of CRP2, which peaked after 14 weeks of TAA treatment (Figure 3B). Given the simultaneous increase of α -SMA expression (Figure 3A), the peak of HSC activation most likely occurred after 10 to 14 weeks of TAA treatment. Until now, transiently increased CRP2 expression during HSC activation was observed *in vitro* and *in vivo* in bile duct-ligated mice only.^{15,29} This is the first time that transiently increased CRP2 expression, and thus transiently increased transdifferentiation of HSC, has been described *in vivo* during toxically induced fibrogenesis. As both GFAP and CRP2, correlated well with COL1A1 expression, HSCs may be responsible for increased matrix production in TAA-induced liver fibrosis (Figure 3C and D). Between weeks 10 and 14 of TAA treatment, a large amount of cells exhibited coexpression of GFAP and α -SMA, a phenomenon recently referred to as 'semi-activation' of HSCs²⁷ (Figure 3E).

Figure 5 Correlation between HSC markers and expression of fibrogenic cytokines (ΔC_t depicts the crossing point of fluorescent threshold with amplification curve in quantitative PCR. ΔC_t indicates a measurement of gene transcription). (a, b) PDGF-B gene expression is significantly correlated with both, GFAP and CRP2 transcription levels. PDGF-D (c) and IL-1 β (d) levels are correlated to CRP2 expression. (mean $\pm$ s.e.m., $n = 4$, $P < 0.05$).

Immunostaining of elastin and desmin may differentiate between portal fibroblasts and HSC, at least *in vitro* and in healthy liver *in vivo*.²³ To elucidate the contribution of portal tract myofibroblasts to TAA or CCl₄-induced hepatic fibrosis, appropriate co-staining was performed. Both desmin- and elastin-positive cells were induced by chronic CCl₄ and TAA treatment. Fibrotic lesions around portal tracts and fibrotic septae were almost always co-stained with both markers. Elastin is a part of the ECM that accumulates in fibrotic scars. In contrast, desmin constitutes an intermediate filament. The staining shown in Figure 3G put the role of portal fibroblasts compared with HSC into perspective. The accumulation of extracellular elastin within the fibrotic scars rather depreciates this assay with respect to the quantification of different amounts of portal fibroblasts and HSC in fibrotic lesions.

In vivo activation of HSC is regulated by a complex network of interactions between growth factors, cytokines, chemokines, products of oxidative stress, and changes in composition and organization of the ECM. To analyze major fibrogenic cytokines, we quantified hepatic transcription of IL-1 β , IL-6, TGF- β 1, PDGF-B, PDGF-D, and TNF- α during TAA-induced fibrogenesis. The cytokines were grouped upon their transient and constantly rising character during the course of fibrogenesis (Figure 4a and b). Interestingly, the maximal expression of transiently elevated cytokines IL-1 β , IL-6, TGF- β 1, and PDGF-D was observed in between the maximum of HSC proliferation (GFAP, 10th week) and the

Figure 6 Cellular sources of MMP-9 in TAA-treated mice exposed via co-immunostaining of MMP-9 with cellular marker proteins. **(a)** Triple staining for myeloperoxidase (neutrophil granulocytes, green), MMP-9 (red), and nuclei (blue). Neutrophil granulocytes appear to be the major source of MMP-9 in mouse liver chronically exposed to TAA. MMP-9 expressing neutrophil granulocytes (arrows) accompanying ceroid-laden macrophages (arrowheads) appears in fibrotic portal and septal areas of fibrotic liver. The granular ceroid-pigmented Kupffer cells are characterized by intensive autofluorescence and thus appear yellow in the green channel (arrowheads, middle panel). **(b)** Triple staining for CD45 (leukocytes, green), MMP-9 (red), and nuclei (blue). Leukocytes represent a minor group of MMP-9 expressing cells in TAA-induced liver fibrosis. **(c)** Triple staining for F4/80 (macrophages, green), MMP-9 (red), and nuclei (blue). Like leukocytes, also Kupffer cells are rarely MMP-9 positive in chronically TAA-injured liver (bar, 10 μ m, original magnification $\times$ 400, PV = portal vein). Representative single-stains and overlays are shown.

peak of HSC transdifferentiation (CRP2, 14th week). Nevertheless, PDGF-B transcription fits best with HSC markers GFAP and CRP2 in TAA-induced liver fibrosis in mice. This is in accordance with previous results from HSC cell culture experiments.³¹

IL-1 β is one of the most significant inflammatory mediators in liver injury.³² The positive correlation between IL-1 β and CRP2 may reflect the inflammation-driven transdifferentiation of HSC during TAA-induced liver injury. However, a direct effect of IL-1 β on HSC transdifferentiation has not been demonstrated.

Especially neutrophils, but also Kupffer cells and leukocytes contribute to MMP-9 expression in a chronic model of TAA-injured mouse liver (Figure 6a–c). These observations differ from reports ascribing Kupffer cells the major source in acute and chronic CCl₄-induced liver injury.^{33,34} Furthermore, we demonstrated colocalization of MMP-9 producing neutrophil granulocytes together with ceroid-laden macro-

phages. This aspect was not observed in the liver of CCl₄-treated animals.³³

Following CCl₄ injection, morphologic changes were accompanied by a notable increase of ALT and AST, most likely representing toxic hepatocyte injury. Interestingly, nonsignificantly raised ALT and AST were observed after TAA, irrespective of the duration of treatment and the grade of liver fibrosis. However, liver fibrosis may develop with little or no elevation of transaminases.³⁵ Fibrosis models with low ALT/AST levels may reproduce the clinical state of chronic hepatitis, with only mild elevation of AST/ALT still carrying the risk of developing fibrosis. Thus, the modus of intoxication or tissue damage—here eg the route of administration—might be of importance. Intraperitoneal injections of TAA, ie repetitive high dose treatment with TAA, regularly exhibit an increase of ALT (fourfold) and AST (2.5-fold), possibly evoked by the induction of acute phase proteins.³⁶ Combined TAA injections and ethanol feeding again

provoked 25-fold increased ALT levels after 10 weeks followed by almost normal values at week 15 despite ongoing treatment.⁷ Obviously tissue damage is the result of both, the hepatotoxin and the application method. Thus both, toxin and application need to be considered for the determination of an appropriate animal model of liver fibrosis.

The transiently increased expression of GFAP and CRP2, their positive correlation to collagen expression, and the fibrosis-dependent enhanced expression of α -SMA emphasize an important role of HSCs in TAA-induced liver fibrosis. PDGF-B seems to be the most important cytokine for activation of HSC in TAA-induced liver fibrosis.

Notably, the induction of liver fibrosis with TAA in drinking water serves as an effective and elegant model mimicking human liver fibrosis while preventing hepatocyte damage.

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