Enhanced matrix degradation after withdrawal of TGF- β 1 triggers hepatocytes from apoptosis to proliferation and regeneration

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Abstract. TGF- β 1 is a profibrogenic cytokine participating in deposition of extracellular matrix in fibrotic disorders. In liver, its anti-proliferative/apoptotic effect on hepatocytes promotes fibrosis. The tetracycline-controlled double-transgenic TA^{LAP-2}/p_{tet}TGF- β 1 mouse provides a model for reversible liver fibrosis. In livers of TGF- β 1-expressing mice, hepatocytes showed synchronous apoptosis detected by DNA laddering and active caspase-3 staining that disappeared when expression of transgenic TGF- β 1 was switched off. In these ‘off’ mice, perisinusoidal liver fibrosis resolved within 21 days accompanied by elevated proliferation of hepatocytes. Here, we have specified the intermediary stages (2–3 days off and 6 days off) in terms of (i) proliferation (by immunohistochemical staining of proliferating cell nuclear antigen and expression of cyclin D1 mRNA) and (ii) extracellular matrix remodelling processes (by measuring mRNA expression of matrix metalloproteinases-2 and -13 (mmp-2 and mmp-13) and tissue inhibitor of matrix metalloproteinases 1 (timp-1) and quantitative morphometric analysis. In summary, we show a rapidly declining timp-1 mRNA level together with lastingly high mmp-2 and mmp-13 mRNA levels after 2–3 days, suggesting that high matrix-degrading potential represents a prerequisite for the markedly enhanced proliferation of hepatocytes in the early stages after switching off transgenic TGF- β 1.

INTRODUCTION

Fibrosis, as a conclusion to tissue repair following injury, resolves depending on the degree and structure of deposited fibrotic tissue (Hutterer *et al.* 1964; Abdel-Aziz *et al.* 1990; Iredale *et al.* 1998). The tetracycline-controlled double-transgenic TA^{LAP-2}/p_{tet}TGF- β 1 mouse represents a model for liver fibrosis (Ueberham *et al.* 2003). In these mice, fibrotic deposition of extracellular matrix in the liver, occurs in response to alternating liver-specific expression of transgenic TGF- β 1 as profibrogenic stimulus. An intermediary level of fibrosis is reached within 3–4 months.

Recently, we have been able to show reversibility of liver fibrosis in this model by depriving the mice of the fibrogenic factor TGF- β 1. An almost complete resolution of deposited extracellular

matrix occurs within 21 days. On day 6, an intermediate state can be characterized where matrix-degrading processes outbalance matrix-synthesizing ones (Ueberham *et al.* 2003). Another remarkable feature of this early regenerative phase is the level of hepatocyte proliferation (shown by PCNA staining). It is high and occurs between days 2 and 6, returning to the control level by day 21 (Ueberham *et al.* 2004). The rapid elevation of hepatocyte proliferative activity on the second day after switching off transgenic TGF- β 1 expression seems to compensate for apoptotic loss of many hepatocytes, demonstrated in mice in the TGF- β 1-on category. Although such compensation seems expedient, it does not provide immediately any molecular mechanisms leading to the sudden burst in proliferation. We deduce that an early time point (during the regenerative phase from fibrosis) might be particularly suitable for investigating any settings for control of proliferation in hepatocytes and their relation to matrix resolution.

An inverse relationship between hepatocyte proliferation and adhesion of cells to extracellular matrix was hypothesized many years ago (Tomomura *et al.* 1987), and the essential role of matrix metalloproteinases in liver growth and remodelling has been shown repeatedly (Haruyama *et al.* 2000; Knittel *et al.* 2000). Digestion of extracellular matrix has been identified as a possible means of priming hepatocytes to become competent, responsive to growth factors and subsequently to proliferate (Liu *et al.* 1994). Inversely, elevated expression of tissue inhibitor of matrix metalloproteinase 1 (timp-1) has been reported to impair hepatocyte proliferation (Martin *et al.* 1999). Direct influence of ectopic expression of human matrix metalloproteinase 1 on proliferation of rat hepatocytes has been demonstrated by Nishio *et al.* (2003) while our study was in progress.

Here, it has been the aim to characterize in detail the transition from massive apoptosis in the TGF- β 1 'expression-on' state to predominating proliferation of hepatocytes in the 'expression-off' state. Furthermore, oligonucleotide microarray analysis has been performed in order to identify molecular targets involved in this transition and specially at the onset of proliferation. Particular emphasis has been placed on the interrelationship between matrix-resolving activities and hepatocyte proliferation during the early regeneration phase after fibrotic injury.

METHODS AND MATERIAL

Animals

Double-transgenic TA^{LAP-2}/p_{tet} TGF- β 1 mice were obtained by crossing individuals of the p_{tet} TGF- β 1 line (C57-BL/6-DBA background) with individuals of the transactivator line TA^{LAP-2} (NMRI background) (Ueberham *et al.* 2003). In accordance with the 'expression-on', 'expression-off' switching protocol, TA^{LAP-2}/p_{tet} TGF- β 1 mice expressing TGF- β 1 are designated TGF- β 1-on mice, and double-transgenic mice administered doxycycline (dox, a tetracycline-analogue) in drinking water preventing the expression of transgenic TGF- β 1 are designated TGF- β 1-off mice. In higher generations (F6, F7), an increasing instability of these transgenic mice was observed. Not all genotypic positively detected double-transgenic animals were inducible. In order to ensure proper development of fibrosis, only TGF- β 1-expressing animals detected by elevated TGF- β 1 serum levels in ELISA were included in the study presented here. TGF- β 1-on mice expressing TGF- β 1 in the first 'on' interval continue to express the transgene stably in all 12 cycles and were fully controllable by dox. To reach perisinusoidal fibrosis within their livers, animals were subjected to 12 repetitions of switch-on/-off cycles consisting of alternating periods of 3–5 days with access to plain water followed by 1–2 days of dox administration (10 μ g/ml). TGF- β 1-off mice (termed 2d-off, 3d-off, 6d-off and 21d-off) were induced 12-fold and finally dox (50 μ g/ml)

was continuously administered. Mice were killed after 2, 3, 6 or 21 days, respectively. No significant differences between 2d-off and 3d-off mice were found. Therefore, these mice were grouped in one group, 2–3d-off mice. TA^{LAP-2} mice housed in the same cage as TGF- β 1-on mice were used as controls. Animals were housed under a constant day–night cycle of 12 : 12 h and were fed a standard chow diet (Altromin 1324, Altromin Gesellschaft für Tierernährung, Lage, Germany), with access to water/doxycycline hydrochloride solution *ad libitum* under all conditions. Animal experiments were carried out in accordance with the European Council Directive of 24 November 1986 (86/609/EEC) and were approved by the appropriate local authorities.

DNA isolation

DNA from liver tissue was isolated using the Apoptotic DNA laddering kit (Roche Diagnostics, Mannheim, Germany) following the manufacturer's instructions. Briefly, 10 mg of liver tissue was homogenized in 200 μ l PBS (pH 7.4). Cells were lysed by adding 200 μ l binding buffer and incubating for 10 min at room temperature. Samples were then mixed with 100 μ l isopropanol and pipetted in a filter tube. Filters were centrifuged and washed twice in washing buffer. DNA was eluted in 200 μ l pre-warmed elution buffer. Equal amounts of DNA, estimated by measuring OD 260/280 nm, were electrophoretically separated on a 2% TAE agarose gel.

Histochemistry and immunohistochemistry

Immunohistochemical staining was performed on 4% paraformaldehyde-fixed, paraffin-embedded sections. Rabbit polyclonal anti-PCNA (proliferating cell nuclear antigen)-antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-active caspase-3 antibody and anti-mmp-2 antibody (Abcam, Cambridge, UK) were used as primary antibodies (1 : 50).

Immunoreactivity was visualized with 3,3'-diamino-benzidine (Sigma, Taufkirchen, Germany) after incubating with peroxidase-conjugated secondary antibody. For detection of anti-mmp-2 immunoreactivity the Vector M.O.M. Elite Immunodetection kit (Vector Laboratories, Burlingame, CA, USA) was used. Success of the mmp-2 staining procedure was verified by simultaneous staining of the kidney on the same slide as the positive control. In all cases, mouse renal tubules demonstrated a strong signal. Sections were counterstained with Mayer's haematoxylin. The number of PCNA positive cells was counted in five areas (310 $\times$ 310 μ m) selected randomly in sections of at least four mice per group (Ueberham *et al.* 2004). For Sirius red staining, the sections were incubated in 0.1% Sirius red F3B in picric acid (Sigma) and differentiated in 0.01 M HCl. After rinsing twice with distilled water, sections were dehydrated with ethanol and coverslipped.

Morphometric analysis

The level of Sirius red-stained collagen was determined on randomized sections containing at least one blood vessel within the visual field. Readings were digitized by use of an Axiophot microscope (Zeiss, Oberkochen, Germany) equipped with an AxioCam HRC camera (Zeiss). Ten fields each from four different mice were analysed and quantified with ImageTool software version 3 (UTHSCSA; The University of Texas Health Science Center in San Antonio, TX, USA).

Isolation of RNA

Total RNA was isolated using the PeqGOLD RNA Pure isolation system (Peqlab, Erlangen, Germany). A clean-up of total RNA was performed using the Qiagen RNeasy Total RNA isolation kit (Qiagen, Hilden, Germany). Quality of RNA was assessed by electrophoresis in denaturing formaldehyde agarose gels and purity was estimated by ratio A260/280 nm spectrophotometrically. Concentration was adjusted to 1 mg/ml and RNA was stored at -80°C until use.

Microarray

Murine Genome Arrays U74Av2 (Affymetrix®) were used. Following Affymetrix recommendation, first-strand synthesis was performed using Superscript II RT (Gibco, Rockville, MD, USA) and second-strand synthesis using T4 polymerase. Phenol/chloroform-extracted cDNA was used as template for synthesis of biotinylated antisense cRNA with the Enzo Bioarray High Yield RNA Transcript Labelling kit. Hybridization and washing were performed according to Affymetrix recommendation with fluidics station (Affymetrix®) and scanned with a laser scanner (Agilent Tech®) at 570 nm.

Real time RT-PCR

RT-PCR for real time quantification was performed as previously described (Ueberham *et al.* 2003, 2004). Cyclin D1 mRNA levels were quantified using mouse-specific cyclin D1 forward primer (GTGCCCTCTCCCCCAACAAC) and reverse primer (GACATCCCCATCCATTCCATT) (GeneBank accession number BC044841).

Statistical analysis

All data are expressed as mean $\pm$ SEM. Statistical analysis was performed by Student's *t*-test using Sigma plot 8 (SSP Science, Chicago, IL, USA). The accepted level of significance was set at $P < 0.05$.

Correlation analysis was performed using FigureP software (Biosoft, Cambridge, UK) and statistical significance was determined with the associated Student's *t*-test.

RESULTS

Induced expression of TGF- β 1 (on-state) in tetracycline-controlled TGF- β 1 mice was shown to result in enhanced apoptosis and impaired proliferation of hepatocytes (Ueberham *et al.* 2003). After turning off TGF- β 1 expression (off-state) unexpectedly high proliferation of hepatocytes was detected after 2–3 days by staining for PCNA (Ueberham *et al.* 2004), while it remained undetermined whether apoptosis decreased or continued in this period. Thus, we have investigated the occurrence of apoptosis in the on-state and several off-states by means of DNA laddering. To this end, DNA was extracted from liver tissue and was separated on agarose gels. As shown in Fig. 1, the on-state was characterized by clearly synchronous DNA-fragmentation, corroborating our previous observation of frequent apoptotic hepatocytes (labelled by the TUNEL reaction) during induced expression of TGF- β 1 (Ueberham *et al.* 2003). In DNA extracts of 2–3d-off mice, only slightly persisting DNA fragmentation was found, while DNA from 6d-off and 21d-off mice showed the normal pattern, as in control mice (Fig. 1). A similar temporal pattern was found for caspase-3 activation which, in contrast to DNA laddering, is an early but likewise irreversible apoptotic event (Huppertz *et al.* 1999; Schuchmann & Galle 2001). Hepatocyte staining was detectable only in on-state but not in any of the off-states (Fig. 2), indicating that apoptosis in hepatocytes is not newly induced after switching off TGF- β 1 expression. As previously noted for a further cohort of TGF- β 1-on mice (Ueberham *et al.* 2004), caspase-3-positive hepatocytes were located preferentially around large blood vessels in zones 1 and 3 of liver lobules (Fig. 2). These results demonstrate that apoptosis of hepatocytes is strictly dependent on the presence of TGF- β 1 and is not seen during regeneration from induced fibrosis. However, a slight staining of smaller non-hepatocyte cells with anti-active caspase-3 antibody was still detectable throughout the parenchyma in 2–3d-off mice (Fig. 2) which might contribute to the persisting minor DNA fragmentation shown in Fig. 1.

Figure 1. TGF- β 1 treatment results in DNA laddering in whole liver extracts. Double-transgenic TA^{LAP-2}/P_{tet} TGF- β 1 mice were induced by 12 successive TGF- β 1 expression cycles and livers were prepared 4–6 days after the last withdrawal of dox ('on'). Other members of the same animal cohort were induced 12 times and then switched-off finally by dox (50 μ g/ml) over a period of 2 days (2d-off and 3d-off, respectively), 6 days (6d-off) and 21 days (21d-off). TA^{LAP-2} mice housed in the same cage were used as controls. DNA from liver tissue was isolated and equal amounts of DNA were electrophoresed on 2% TAE agarose gels. Lanes 1, 2 and 11 represent DNA molecular weight markers (lanes 2 and 11, 100 bp DNA ladder and lane 1, 1 kb DNA ladder; Peqlab). Lanes 3 and 10 show a positive control (DNA of apoptotic U937 cells; Roche Diagnostics). The white arrow in the lower panel points to reduced higher chromosomal DNA in 2–3d-off mice compared with controls.

Switching from TGF- β 1 expression to the non-expressing state (that is from deposition of matrix components to their resolution during regeneration) is accompanied by the onset of massive proliferation of hepatocytes which compensate for the apoptotic loss during the on-state. As depicted in Fig. 2, PCNA staining was already found in 2–3d-off mice, but was most pronounced in the 6d-off mice. In order to learn more about the molecular changes in livers with enhanced hepatocyte proliferation rates, we performed oligonucleotide microarrays (Affymetrix) comparing expression patterns of TGF- β 1-on mice with 6d-off mice. These data were further compared with previously collected microarray data showing the expression profile of TGF- β 1-on mice versus control mice (to be reported elsewhere). Analysis of these data resulted in elevated levels of cyclins D1, B1, and E2. Cyclins A2 and B2 were only slightly elevated in 6d-off mice, and expression of cyclins A1, D2, C and G seemed not to be changed (data not shown).

Cyclin D1 expression has been shown to be sufficient to promote replication of adult hepatocytes *in vivo* (Nelsen *et al.* 2001). Furthermore, cyclin D1 activity is known to be an inaugural participant for the G₁ phase of cell cycle re-assigning quiescent hepatocytes (Coqueret 2002),

Figure 2. Proliferation and apoptosis in TGF- β 1-on mice and -off mice. Paraffin embedded sections of livers of TGF- β 1-on mice (a, e), 2-3d-off mice (b, f), 6d-off mice (c, g) and 21d-off mice (d, h) were incubated with either anti-PCNA antibody (a, b, c, d) or an anti-active caspase-3 antibody (e, f, g, h) and a peroxidase-conjugated secondary anti-rabbit antibody. Immunoreactivity was visualized with 3,3' diaminobenzidine (brown) and slides were counterstained with haematoxylin (blue). Caspase-3-positive staining was only detected in hepatocytes surrounding the major blood vessels of on-mice (e) and faintly in smaller non-hepatocyte cells in 2-3d-off mice (f; arrowhead in the inserted rectangle showing stained cells in detail). The magnified area is indicated by the black rectangle in the original photomicrograph). Images of control mice were identically to 21d-off (not shown). Bar in (h) represents 50 μ m.

Figure 3. Quantitative analysis of proliferation by determination of cyclin D1 mRNA. RNA was extracted from liver tissue of TGF- β 1-on mice (on) and off-mice (2-3d-off, 6d-off and 21d-off). Cyclin D1 mRNA levels were determined by real time RT-PCR experiments (Rotorgene 2000, Corbett Research, Mortlake, Vic., Australia) using cyclin D1-specific primers. Each group was composed of at least six animals. Data were displayed as percentages of the level in control mice. Differences between on-status and off-status groups were statistically significant with $P < 0.02$ (Student's *t*-test). Dashed line represents the reference 100%.

and cyclin D1 acts as growth factor sensor (Sherr 1996). Therefore, we have quantified cyclin D1 mRNA level by RT-PCR at three different stages of reversion of liver fibrosis (Fig. 3). Elevated levels of cyclin D1 mRNA were detected in two stages, amounting to 1.9-fold and 2.8-fold of controls for 2-3d-off and 6d-off mice, respectively. The considerably reduced values for

Figure 4. Correlation analysis between cyclin D1 mRNA levels and PCNA staining. Data for cyclin D1 mRNA levels from Figure 3 (displayed as ag mRNA per μ g total RNA) and quantitative data on the percentage of PCNA-positive hepatocytes published recently (Ueberham *et al.* 2004) were analysed for possible correlation. The correlation coefficient was determined to $r = 0.9566$ ($P < 0.01$). Correlation analysis was performed using Figure-P software (Biosoft) and statistical significance was determined with the associated Student's *t*-test. The five points represent the five different investigated states: control, on, 2–3d-off, 6d-off and 21d-off.

on-mice (0.075-fold of controls) matched clearly with the blockage of proliferation observed in this state (Ueberham *et al.* 2003). Cyclin D1 mRNA data of all states also corresponded to the PCNA staining of hepatocyte proliferation. Despite the fact that PCNA shows an overlapping expression in several phases of the cell cycle, rising in G_1 , culminating in S, and decreasing in the G_2 phase (Morris & Mathews 1989), quantitative estimation of PCNA staining correlated well with cyclin D1 mRNA expression ($r = 0.9566$, $P < 0.01$; Fig. 4).

In order to characterize the 2–3d-off state in terms of matrix remodelling, procollagen I mRNA was quantified along with mmp-2, mmp-13, and timp-1 mRNA levels (Fig. 5). The TGF- β 1 on-state as well as the 6d-off and the 21d-off states were also included in this analysis to ensure an identical time course for fibrosis and its reversal in the new animal cohort subjected to the on-off induction protocol for TGF- β 1 expression. Unexpectedly, expression of mmeps in 2–3d-off mice persisted (mmp-2) or was only slightly reduced (mmp-13), whereas that of timp-1 was considerably reduced (Fig. 5). Thus, a short period of 2–3 days of doxycycline re-feeding was sufficient to switch off an almost 12-fold induction of timp-1 mRNA and to reduce it to less than 2-fold compared with controls. These data indicate a high matrix degrading potential even at early time points after switching-off TGF- β 1 expression, which agrees well with the rapid resolution of fibrosis noted previously (Ueberham *et al.* 2003). Mmp-2 was detected in small cells around blood vessels in liver sections of on-mice, 2–3d-off mice (Fig. 6) and to a limited extent in 6d-off mice (not shown). Weak staining of hepatocytes was only found in 2–3d-off mice. No mmp-2 immunoreactivity was detected in controls (Fig. 6) or 21d-off mice.

Alterations in expression of extracellular matrix components, matrix proteins, mmeps and timp in off-states lead to degradation of deposited matrix within the liver as shown by Sirius red-stained sections (Fig. 7). In TGF- β 1-expressing on-mice, an 8-fold increase of collagen deposition was quantified morphometrically. In 2–3d-off mice, only a 3.4-fold and in 6d-off and 21d-off mice a 1.7-fold elevated deposited collagen content remained in test liver compared with controls. Elevated levels in 6d- and 21d-off mice resulted from thickened vessel walls and was not as a result of perisinusoidal fibrosis prominent in on-mice.

Figure 5. Changes in expression of matrix metalloproteinases 2 and 13, their inhibitor and procollagen I $\alpha 1$ in relation to induction and suppression of TGF- $\beta 1$ expression. Steady-state mRNA levels of matrix metalloproteinases 2 (mmp-2), 13 (mmp-13), tissue inhibitor of matrix metalloproteinase 1 (timp-1) and procollagen I $\alpha 1$ (col I) were measured by real time RT-PCR (Rotorgene 2000, Corbett Research). TGF- $\beta 1$ -on mice (on) were compared with the three different off-mice groups (2-3d-off, 6d-off, and 21d-off). Six animals were grouped for every time point. Data were expressed as percentage of the level in control mice and are represented by means $\pm$ SEM. *Significantly different from the on-state ($P < 0.05$, Student's t -test). The dashed line represents the reference 100%.

DISCUSSION

Our results indicate that the early phase, around days 2 and 3 after switching off transgenic expression of TGF- $\beta 1$, marks a very important breakpoint for regeneration from induced liver fibrosis. Both active caspase-3 staining and DNA fragmentation, demonstrated in agarose gels, point to completed hepatocyte apoptosis by this time and, thus, no simultaneous occurrence of apoptosis and proliferation of hepatocytes. The switch from an apoptotic, non-proliferating condition, characterizing the TGF- $\beta 1$ on-state (Ueberham *et al.* 2003), to the non-apoptotic, highly proliferating condition, dominating already in the 2-3d-off state, is reflected also by cyclin D1 mRNA levels. Although our microarray studies, likewise, suggested elevated cyclin B1 and E2 expression in livers of 6d-off mice, we focused on cyclin D1 mRNA levels for the following reasons. Cyclin D1 has been proposed to be a key regulator of hepatocyte proliferation and its induction corresponds to the G₁ restriction point (Albrecht & Hansen 1999). Hepatic cyclin D1 is induced during the G₁ phase of the cell cycle and is able to sequester cell cycle inhibitors, preventing the inhibition of cyclin E/Cdk complexes (Albrecht *et al.* 1999). Besides functional activation of cyclin E/Cdk, the transcription of cyclin E itself is regulated by cyclin D1 and is

Figure 6. Mmp-2 localization in livers of TGF- β 1 expressing mice and after withdrawal of TGF- β 1. Mmp-2 was detected using anti-mmp-2 antibody (clone ab3158; Abcam) and the Vector Elite M.O.M. kit (Vector Laboratories). Clear staining of small cells around vessels (arrow) was observed in on-mice (d) and 2–3d off mice (b, c, e and f) and in 6d-off mice (not shown). Additionally, slight staining of hepatocytes was detected only in 2–3d-off mice (arrowhead). No mmp-2 immunoreactivity was found in control mice (a) or 21d-off mice (not shown). Bar represents 50 μ m.

linked to Cdk activity (Harbour *et al.* 1999). Therefore, cyclin D1 represents an early key regulator of cyclin E. The expression of cyclin B1, in turn, starts at late S phase (Clute & Pines 1999) and its up-regulation in livers of 6d-off mice shows that in the 6d-off state most hepatocytes have entered the cell cycle. Nevertheless, cyclin D1 expression seems to reflect best the proliferative situation at the early time point, 2–3 days after TGF- β 1 down-regulation. Several pathways may be relevant for signal transduction leading to elevated cyclin D1 expression, including activation of the cyclin D1 promoter, by transcription factors such as the STAT proteins, Egr-1, CREB or TCF/LEF (Coqueret 2002). TCF/LEF activation of the cyclin D1 promoter is because of nuclear accumulation of β -catenin. β -Catenin accumulation has also been shown to be involved in proliferation of hepatocytes after ectopic mmp-1 expression in mouse liver (Nishio *et al.* 2003). However, although in our off-mice cyclin D1 was up-regulated, no accumulation of β -catenin was observed within the parenchyma. Only in 6d-off mice, induced for one time only, could we show an enrichment of cytoplasmic β -catenin in cells around the pericentral vessels (Ueberham *et al.* 2004). We assume that an overall induction of β -catenin takes place only under abnormal conditions such as ectopic expression of collagenase or carcinogenic events (Gotoh *et al.* 2003) and not in normal, even injured, liver parenchyma.

The early phase of switching off was also characterized by unexpected maintenance or down-regulation of factors involved in matrix degradation. The still high level of collagenase (mmp-13) in 2–3d-off mice could be expected from previously published results showing a continuously increased level in the 6d-off state (Ueberham *et al.* 2003). However, the unchanged level of gelatinase A (mmp-2) in 2–3d-off mice was unexpected, because of its strong decrease in 6d-off mice. This finding suggests that mmp-2 might play a role in signals leading to proliferation of hepatocytes which already starts at this early time point. Mmp-2 elevation has also been detected in recovery of fibrosis on day 3 in the CCl₄ model, where it was suggested that it destroyed even

Figure 7. Remodelling of collagen depositions. Morphometric quantification of fibrosis (a) and representative micrographes of liver sections stained with Sirius red F3B (b) as described in MATERIALS and METHODS. The Sirius red-stained collagen quantified via image analysis (ImageTool software, version 3; for details see MATERIALS and METHODS) was set 100% for control values. Bars in (a) represent percentage of stained collagen compared with control mice. The dashed line represents the reference 100%. *Statistically significantly different from controls; †statistically significantly different from on-mice; ‡statistically significantly different from 2-3d-off mice (Student's *t*-test; $P < 0.001$). Bar represents 50 µm.

pericellular fibrosis (Takahara *et al.* 1995). Furthermore, elevated expression and activation of mmp-2 in early stages of regeneration after partial hepatectomy (Kim *et al.* 2000) supports the hypothesis that it may stimulate hepatocyte proliferation by hepatic matrix remodelling.

At first glance, our findings are in agreement with the results of Nishio *et al.* (2003) showing elevated levels of hepatocyte proliferation in phases with high ectopic collagenase expression. However, in our model, endogenous collagenases, mouse mmp-13 and mmp-2, are elevated. As proliferation of hepatocytes is completed within 21 days, while mmp-13 levels have not yet fully returned to control levels, mmp-13 (corresponding to human mmp-1) may play a subordinate role in regulating hepatocyte proliferation. This agrees with the absence of any interrelationship between mmp-13 and proliferation during regeneration after partial hepatectomy in rats (Rudolph *et al.* 1999). In contrast, mmp-2 levels return to control levels in livers of 21d-off mice, supporting a possible role of gelatinase A (mmp-2) in priming hepatocyte proliferation.

The most important finding of this study is the rapid decrease of timp-1 expression only 2 days after switching off transgenic TGF- β 1 expression in the mice. This is in contrast to findings obtained during spontaneous resolution of rat liver fibrosis showing a slow decline of timp-1 from 0 to 28 days after withdrawal of the fibrotic stimulus, CCl₄. In this study, approximately 70% of timp-1 mRNA remained after 3 days (Iredale *et al.* 1998). The low levels of timp-1 mRNA in our 2–3d-off mice seems a prerequisite for degrading extracellular matrix and consequently for release of growth factors like HGF, shown to be sequestered within extracellular matrix (Masumoto & Yamamoto 1991, Schuppan *et al.* 1998). Recently published data showing that genetic alterations in *timp-1* lead to shifts in the timing of hepatocyte cell cycle progression, by impacting on HGF bioactivity, confirms this suggestion (Mohammed *et al.* 2005). However, intrahepatic HGF levels in the different off-states remain to be elucidated. Our findings suggest anti-timp-1 strategies such as antibodies or aptamer technology against timp-1 (Nimjee *et al.* 2005) might be promising therapeutic approaches. Such properties might improve the desirable features of TGF- β 1, matrix degrading processes, and might repress undesirable features such as the inhibition of matrix degrading processes. This might also support rapid removal of activated hepatic stellate cells, the main producers of deposited matrix in liver tissue, because timp-1 has been shown to independently prevent apoptosis in this cell type (Murphy *et al.* 2002).

The early decay of timp-1 and the persistently high mmp-2 and -13 expression may explain the failure to develop cirrhosis in the mice of our model, even after very long time periods of TGF- β 1 expression (more than 30 cycles of interval expression over a time of 8–12 months, data not shown). The mandatory switch-off period between two switch-on cycles takes 2–3 days. Because timp-1 expression is decreased already during this period, while high levels of collagenase and gelatinase A provide an elevated proteolytic potential, at least a part of the newly produced extracellular matrix may be degraded during each cycle. Morphometric analysis of collagen deposition in liver sections at the four different time points (on, 2–3d-off, 6d-off and 21d-off) shows a rapid decline of deposited collagen in 2–3d-off mice compared with controls. The deposited collagen disappears, particularly in the perisinusoidal parenchyma. Perivascular-deposited collagen is even slightly elevated in 6d-off and 21d-off mice, although an inspection of liver slides of 21d-off mice indicates normal histology. Whether this slight elevation dissolves at all remains to be elucidated. Interestingly, mmp-2 immunoreactivity occurs transiently in small cells of unknown origin forming in, or invading the area around, the major blood vessels. The additional weak staining of several hepatocytes with anti-mmp-2 antibody seen in 2–3d-off mice points to an extraordinary role of this matrix metalloproteinase in triggering the hepatocytes from apoptosis to proliferation.

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