

Partial Hepatectomy–Induced Regeneration Accelerates Reversion of Liver Fibrosis Involving Participation of Hepatic Stellate Cells

JUAN A. SUÁREZ-CUENCA,* VICTORIA CHAGOYA DE SÁNCHEZ,* ALBERTO ARANDA-FRAUSTRO,†
LOURDES SÁNCHEZ-SEVILLA,* LIDIA MARTÍNEZ-PÉREZ,* AND ROLANDO HERNÁNDEZ-MUÑOZ*¹

**Departamento de Biología Celular, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México (UNAM), Mexico 04510, D.F., Mexico; and †Departamento de Patología, Instituto Nacional Cardiología “Ignacio Chávez,” Mexico 14080, D.F., Mexico*

Hepatic fibrosis underlies most types of chronic liver diseases and is characterized by excessive deposition of extracellular matrix (ECM), altered liver architecture, and impaired hepatocyte proliferation; however, the fibrotic liver can still regenerate after partial hepatectomy (PH). Therefore, the present study was aimed at addressing whether a PH-induced regeneration normalizes ECM turnover and the possible involvement of hepatic stellate cells (HSC) during resolution of a pre-established fibrosis. Male Wistar rats were rendered fibrotic by intraperitoneal administration of swine serum for 9 weeks and subjected afterwards to 70% PH or sham-operation. Histological and morphometric analyses were performed, and parameters indicative of cell proliferation, collagen synthesis and degradation, and activation of HSC were determined. Liver collagen content was reduced to 75% after PH in cirrhotic rats when compared with sham-operated cirrhotic rats. The regenerating fibrotic liver oxidized actively free proline and had diminished transcripts for α -1 (I) collagen mRNA, resulting in decreased collagen synthesis. PH also increased collagenase activity, accounted for by higher amounts of pro-MMP-9, MMP-2, and MMP-13, which largely coincided with a lower expression of TIMP-1 and TIMP-2. Therefore, an early decreased collagen synthesis, mild ECM degradation, and active liver regeneration were followed by higher collagenolysis and limited deposition of ECM, probably associated with increased mitochondrial activity. Activated HSC readily increased during liver fibrosis and remained activated after liver regeneration, even during fibrosis resolution. In conclusion, stimulation of liver regeneration

through PH restores the balance in ECM synthesis/degradation, leading to ECM remodeling and to an almost complete resolution of liver fibrosis. As a response to the regenerative stimulus, activated HSC seem to play a controlling role on ECM remodeling during experimental cirrhosis in rats. Therefore, pharmacological approaches for the resolution of liver fibrosis by blocking HSC activation should also evaluate possible effects on liver cell proliferation. *Exp Biol Med* 233:827–839, 2008

Key words: Experimental cirrhosis; partial hepatectomy; collagen metabolism; liver mitochondria; MMPs; TIMPs; hepatic stellate cells

Introduction

Remodeling of hepatic extracellular matrix (ECM) plays a key role in processes such as angiogenesis, inflammation, and regeneration, among others (1), through modulating important cellular functions (2, 3). Fibrous components interact with liver cells, matrix metalloproteinases (MMPs), cytokines, and growth factors, thereby maintaining a homeostatic state (“hepatic ecosystem”) and preserving the liver’s morphology and function (4). Such an equilibrium requires specific liver responses against challenging stimuli that normally lead to cell proliferation and tissue healing (5). However, inadequate responses may occur during chronic damage, resulting in an excessive ECM deposit and formation of a fibrous scar (6, 7), even progressing to advanced fibrosis (cirrhosis) and increasing the risk for hepatocellular carcinoma (8, 9).

Accumulation of ECM proteins is a reversible dynamic process (10), since turnover of ECM components depends on a regulated balance between matrix-degrading enzymes, mainly metalloproteinases (MMPs), and their specific inhibitors (TIMPs). Therefore, hepatic fibrosis frequently results from deficient collagen removal by MMPs (11) and TIMPs overexpression (12). In contrast, fibrosis resolution

¹ To whom correspondence should be addressed at Departamento de Biología Celular, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México (UNAM), Apartado Postal 70-243, México 04510, D.F., México. E-mail: rhernand@ifc.unam.mx

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will depend mainly on increased collagen breakdown and diminished TIMP activities (13).

It is accepted that either fibrotic or cirrhotic livers maintain the ability to regenerate after partial hepatectomy (PH) (14), as well as to modify ECM components (15); however, there are conflicting data regarding the ability of the liver to regenerate after PH in animals and humans with cirrhosis. In carbon tetrachloride-induced cirrhosis in rats and subjected to PH, DNA synthesis and mitotic activity of hepatocytes are decreased (16), showing that the regenerative capacity of the liver after PH is impaired in these animals (17). In fact, it has been reported that liver tissue regenerated after PH in carbon tetrachloride-induced cirrhotic liver is also cirrhotic, despite that slight decreases have been found in tissue collagens and proteoglycans (15). During thioacetamide-induced liver cirrhosis in rats, PH promotes functional restoration of the liver without improving morphological alterations (18), although it has been reported that aspirin and enoxaparin seem to stimulate liver regeneration (19). Similarly, rats rendered cirrhotic by chronic dimethylnitrosamine administration depict severe cirrhosis with poor regenerative response after PH. Nonetheless, the combination of HGF and TbetaTR gene therapy, as well as VEGF therapy, might increase the capacity of hepatectomy to improve fibrosis, hepatic function, and hepatocyte regeneration in these animals (20, 21).

Hence, the structural differences in the regenerated tissues between normal and cirrhotic livers could also indicate different regeneration capacities, which opens the question as to whether an effective regenerative stimulus is capable of ECM remodeling in the cirrhotic liver, since modifications of ECM constituents might regulate fibrogenesis and proliferation of the fibrotic liver (12, 22–25).

The presence of α -smooth muscle actin (α -SMA) positive cells in the fibrotic liver suggests phenotypic modulation in hepatic stellate cells (HSC) and their differentiation towards myofibroblast-like cells. Indeed, the expression of α -SMA may be related to events of the fibrotic process, such as tissue contraction or fibrogenesis *per se* (26). HSC could play a key role in normal ECM deposition during liver regeneration, as well as in promoting fibrosis (27), since a transient activation of HSC occurs during liver regeneration. Moreover, a decreased number of activated HSCs has been considered a condition for fibrosis resolution (28).

The aim of the present study was to evaluate ECM turnover and the role of activated HSC during ECM remodeling in the regenerating fibrotic rat liver. For this, we selected a swine serum-induced liver fibrosis, which resembles human posthepatitis fibrosis (29), accompanied by ECM remodeling and cell proliferative events (30). In fact, this experimental model of very low spontaneous reversibility of liver fibrosis reveals clearly an effective regenerative response after PH. Moreover, the minimal parenchyma necrosis and/or liver functional impairment, even after PH, found in the present model represents some

advantages over other animal models of liver fibrosis, such as those of alcohol- and thioacetamide-induced liver cirrhosis (4, 19, 31), where lower regenerative ability after PH has been reported.

Material and Methods

Materials. [3 H-Methyl]-deoxythymidine (specific activity 2.0 Ci/mmol) and L-[14 C]-proline (specific activity 275 mCi/mmol) were purchased from NEN (Boston, MA). 32 P-Collagen α -1(I) was kindly provided by Dr. Francisco Villarreal (University of California, San Diego, CA).

Animals and Treatments. Male Wistar rats, with an initial weight of 100–120 g, had free access to lab chow and water in a 12:12-hr light:dark cycle. Swine serum was injected intraperitoneally (ip) following a slightly modified method (29, 32). Briefly, rats were injected with fresh swine serum (3.5 ml/kg body wt) that had been heat-treated and sterilized by filtration (Millipore Corp., Bedford, MA), achieving a total protein amount of 58 mg/ml (29), twice weekly for 9 weeks, and control animals (saline solution) were done in parallel. Thereafter, control (healthy) and cirrhotic animals were subjected to 70% PH, according to Higgins and Anderson (33), or to a sham operation (laparotomy only). Animals were fasted overnight and killed with sodium pentobarbital overdosing at different times after surgery, following the Federal Regulations for Animal Experimentation and Care (Ministry of Agriculture, SAGARPA, Mexico).

Fibrosis Score and Morphometric Analysis.

Thin sections of paraffin-embedded liver tissue were stained with Masson's trichrome stain, and a fibrosis score was evaluated under light microscopy (34). A morphometric analysis was done by digitalizing and analyzing 50–40 $\times$ power fields, calculating stromal and parenchymal areas, as well as the stromal/parenchymal index (Image Pro 5.0; Media Cybernetics, Bethesda, MD).

Parameters Indicative of Liver Regeneration. The rate of liver regeneration was estimated through several approaches: gain of liver mass after PH, determination of thymidine kinase (TK; EC 2.7.1.2) activity in the cytosolic fraction, and estimation of the mitotic index with an optical microscope (Olympus, CH-30; Tokyo, Japan), taking into account the number of mitotic cells present in 10 microscopic fields (40 $\times$ objective). These determinations have been described previously in detail (35).

Collagen Content and Its Turnover. Total liver collagen was extracted and determined by its content of hydroxyproline, as described by Rojkind and González (36). In addition, collagen content was estimated in liver homogenates by Western blot analysis with specific rabbit anti-rat collagen type I polyclonal antibody (Chemicon, La Jolla, CA) diluted at 1:500, and normalized with β -actin, as described below in detail. The free proline pool was determined in perchloric acid-extracts, as previously described (37). Liver proline oxidation and its incorporation

into collagenous proteins (collagen synthesis) were determined *in vitro*, as described in detail elsewhere; briefly, they consisted in incubating 2 g of liver slices (0.5 mm thickness) in the presence of 0.4 mM proline, containing 2 μ Ci of L-(U- 14 C) proline with a specific activity of 275 mCi/mmol (35, 37). Collagen synthesis was also assessed by expression of mRNA for α -1 (I) collagen. Total RNA was extracted from rat liver according to the method of Chomczynski and Sacchi (38), and RNA samples were electrophoresed and transferred to nitrocellulose filters and then hybridized with the 32 P-collagen α -1 (I) probe, as described by Villarreal and Dillmann (39). After film exposure, bands obtained were quantified by image densitometry. A probe for glyceraldehyde phosphate dehydrogenase was used for normalization purposes. Total collagenolytic activity towards endogenous substrates was determined, as previously described, and expressed as nmol of degraded collagen $\cdot$ hr $^{-1}$ $\cdot$ mg $^{-1}$ of liver protein (30, 37); the ratio of collagenase/collagen was calculated also to provide a more reliable picture of collagenase activity under pathologic conditions (30). Additionally, gelatinolytic activity was evaluated by gel zymography, as described by Knittel *et al.* (40). Briefly, 20 μ g of liver protein were mixed with Zymogram Sample Buffer (Bio-Rad, Mexico City, Mexico) and analyzed by 8.5% SDS-PAGE Laemmli gel containing 1 mg/ml gelatin, using standards (Chemicon). Afterwards, gels were stained overnight with Coomassie blue and quantified through image densitometry.

Western Blot Analysis for MMPs/TIMPs. Liver protein levels of MMP13 and of TIMPs (–1 and –2) were analyzed through Western blot assay, essentially as previously described (35). Around 15–40 μ g of liver homogenates were separated in 10% and 15% gels by SDS-PAGE (41). The primary antibodies used were mouse anti-rat MMP-13 monoclonal antibody diluted at 1:300, mouse anti-human TIMP-1 monoclonal antibody diluted at 1:300, and mouse anti-TIMP-2 monoclonal antibody diluted at 1 μ g/ml (all from Chemicon). Mouse anti- β -actin monoclonal antibody (Sigma [St. Louis, MO], diluted at 4 μ g/ml) was additionally tested for normalization. Densitometric analysis was performed using the Collage 2.0 Program in an image photoanalyzer (Fotodyne Inc., foto/Eclipse, New Berlin, WI).

Immunohistochemical Analysis of α -SMA and Electron Microscopy. Deparaffinized and rehydrated (xylol-ethanol) liver samples were subjected to antigen retrieval as previously described (35) and sequentially incubated with peroxidase-blocking solution, monoclonal mouse anti-human alpha smooth muscle actin (DAKO M0851) diluted 1:100, and horseradish peroxidase-labeled polymer containing secondary antibody (DAKO K400711), with intermediate 1 $\times$ PBS (pH 7.6)/0.01% Tween-20 washes. Five independent preparations per group were evaluated and power fields from each slide were morphometrically analyzed to determine the area of α -SMA positive

cells. Electron microscopy to search for activated HSC was performed following standard procedures.

Statistics. All results are expressed as mean $\pm$ SE and the significance of the differences and their interactions was tested by two-way ANOVA and *post-hoc* Newman's test. Statistical significance is indicated as (*) against time zero, or (**) against the sham control group, throughout this study.

Results

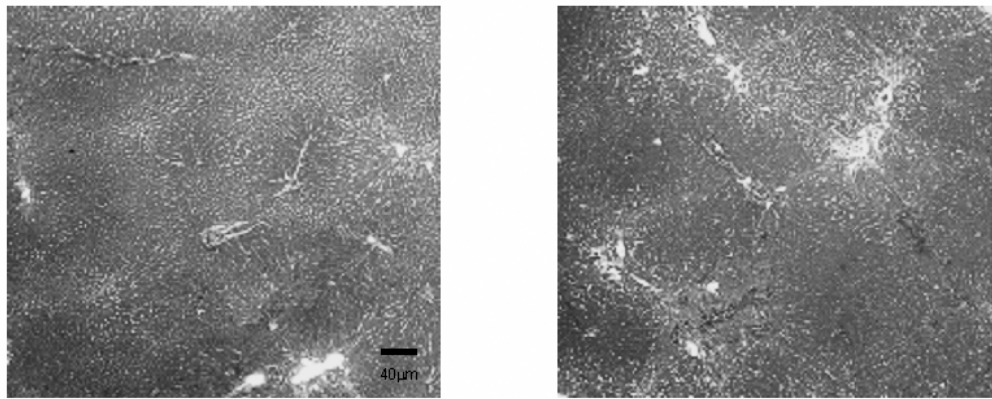
Liver Collagen Content and Morphometric Analysis at the Onset of Fibrosis. Rat liver fibrosis was achieved after 9 weeks of swine serum administration, represented mainly by thin septa linking vascular structures and dividing the whole hepatic parenchyma (Fig. 1A, right). The morphometric analysis revealed increased liver stromal area (Table 1). Accordingly, collagen content was 4-fold enhanced (Fig. 2A), largely coinciding with the detected levels of immunoreactive collagen type I protein (Fig. 2B).

Regeneration of the Fibrotic Liver After PH. Fibrotic livers recovered up to 80% of the original mass within 30 days after PH, when compared with nonfibrotic PH controls (Fig. 3, left). Additionally, liver DNA synthesis (TK activity) was augmented by the 70% PH in both control and liver fibrosis rats, peaking at 24 hrs after surgery (Fig. 3, right top); whereas TK activity remained enhanced up to Day 3 post-PH in controls, cirrhotic animals showed a smaller amount of enzyme activity. Mitotic index was similarly enhanced in both experimental groups, but it was always lower in the fibrotic liver (Fig. 3, right bottom).

Modifications of Deposited ECM During PH-Induced Regeneration of Fibrotic Liver. Sham-operated animals showed a very slow and slight reversibility of fibrosis (Fig. 1B, micrographs 1–4), achieving only 20% reduction of the calculated stroma/parenchyma index (30 days postsurgery; Table 1), and 80% of these animals remained in Grade 4 of fibrosis (abundant septa; Table 2). On the contrary, the 70%-PH-fibrotic rats had a significant reduction in liver fibrosis (Fig. 1B, micrographs 5–8), with a 50% and 75% reduction of the fibrotic stroma (7 and 30 days post-PH; Table 1), and 60% of these animals were classified in Grade 2, and none in Grade 4 (Table 2). This PH-induced reduction of the stroma/parenchyma index was mainly attributed to changes in stromal area rather than to those occurring in the parenchyma (Table 1), since in the 70%-PH-fibrotic animals significantly decreased (over 70%) liver collagen content was found (Fig. 2A). In sham-operated fibrotic rats, liver collagen content did not significantly diminish even after 30 days of recovery. The PH also reduced liver collagen in control rats (Fig. 2A), without correlating with the amount of immunoreactive collagen type I (Fig. 2B).

Collagen Synthesis During PH-Induced Regeneration in the Fibrotic Liver. In control rats, 70%-PH significantly increased liver's free proline pool (collagen

A



B

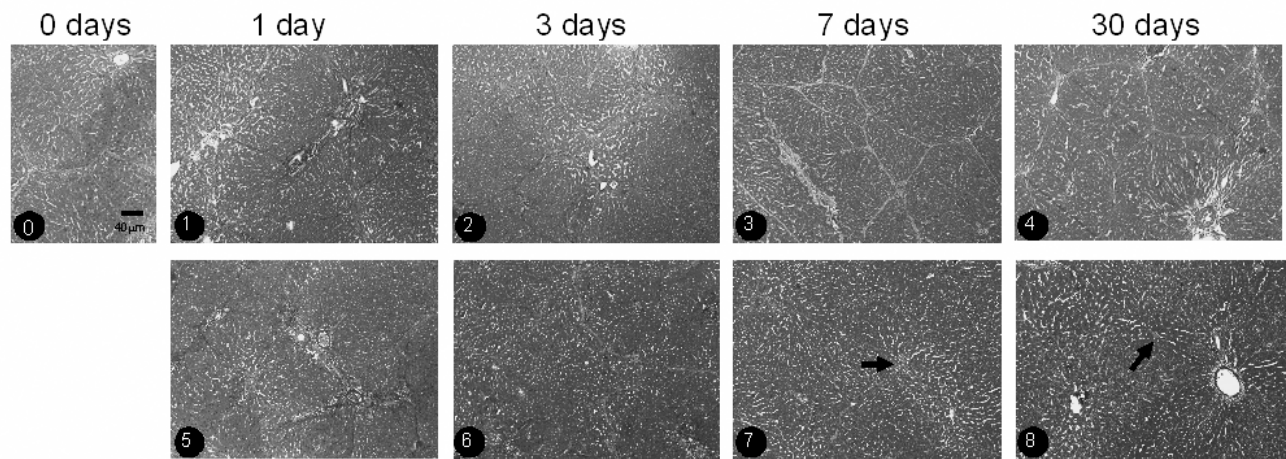


Figure 1. Histological changes of the fibrotic liver after PH. (A) Light microscopy. 25 $\times$ power field images of tissue sections stained with Masson's trichrome, from rat liver non-fibrotic control (left) injected with saline solution, and fibrotic control (right) treated with swine serum. (B) 10 $\times$ power field images from rat fibrotic liver collected at time zero (image 0) and after 1, 3, 7, and 30 days from either sham-operated controls (images 1–4, respectively) or 70% PH (images 5–8, respectively). Arrows indicate scanty fibrotic tissue remaining after 7 and 30 days post-PH.

Table 1. Image Analysis of Liver Tissue after 70% PH^a

Time from 70% PH	Nonfibrotic liver			Fibrotic liver		
	Stromal area	Parenchymal area	Stroma/Parenchyma Index ^b	Stromal area	Parenchymal area	Stroma/Parenchyma Index ^b
–PH						
0 days	0.3 $\pm$ 0.03	29 $\pm$ 0.2	0.10 $\pm$ 0.006	5.0 $\pm$ 0.25	24 $\pm$ 0.3	2.03 $\pm$ 0.15
1 day	0.3 $\pm$ 0.02	29 $\pm$ 0.2	0.10 $\pm$ 0.010	5.1 $\pm$ 0.29	25 $\pm$ 0.3	2.06 $\pm$ 0.14
3 days	0.3 $\pm$ 0.02	29 $\pm$ 0.1	0.09 $\pm$ 0.008	4.9 $\pm$ 0.29	24 $\pm$ 0.2	2.09 $\pm$ 0.14
7 days	0.3 $\pm$ 0.01	29 $\pm$ 0.1	0.09 $\pm$ 0.005	4.4 $\pm$ 0.24	24 $\pm$ 0.3	1.83 $\pm$ 0.11
30 days	0.3 $\pm$ 0.02	28 $\pm$ 0.2	0.10 $\pm$ 0.009	3.9 $\pm$ 0.41*	25 $\pm$ 0.4	1.59 $\pm$ 0.19*
+PH						
0 days	0.3 $\pm$ 0.02	29 $\pm$ 0.2	0.10 $\pm$ 0.007	4.9 $\pm$ 0.22	25 $\pm$ 0.3	2.06 $\pm$ 0.11
1 day	0.1 $\pm$ 0.02*/**	28 $\pm$ 0.3*/**	0.05 $\pm$ 0.006*/**	4.8 $\pm$ 0.23	23 $\pm$ 0.4**	2.04 $\pm$ 0.12
3 days	0.1 $\pm$ 0.01*/**	28 $\pm$ 0.3**	0.05 $\pm$ 0.005*/**	4.7 $\pm$ 0.33	24 $\pm$ 0.5	1.95 $\pm$ 0.16
7 days	0.2 $\pm$ 0.02	28 $\pm$ 0.2*/**	0.09 $\pm$ 0.006	2.3 $\pm$ 0.3*/**	26 $\pm$ 0.5*/**	0.90 $\pm$ 0.12*/**
30 days	0.2 $\pm$ 0.01	28 $\pm$ 0.1*/**	0.09 $\pm$ 0.004	1.2 $\pm$ 0.1*/**	27 $\pm$ 0.3*/**	0.47 $\pm$ 0.04*/**

^a Results are expressed as mean $\pm$ SE of 4 animals per group, analyzed as fifty 40 $\times$ power fields per animal. Statistically significant against time zero (*), and against his control of group (**). –PH = without partial hepatectomy, +PH = with partial hepatectomy.

^b The stroma/parenchyma index = (stromal area/parenchymal area) $\times$ 10¹.

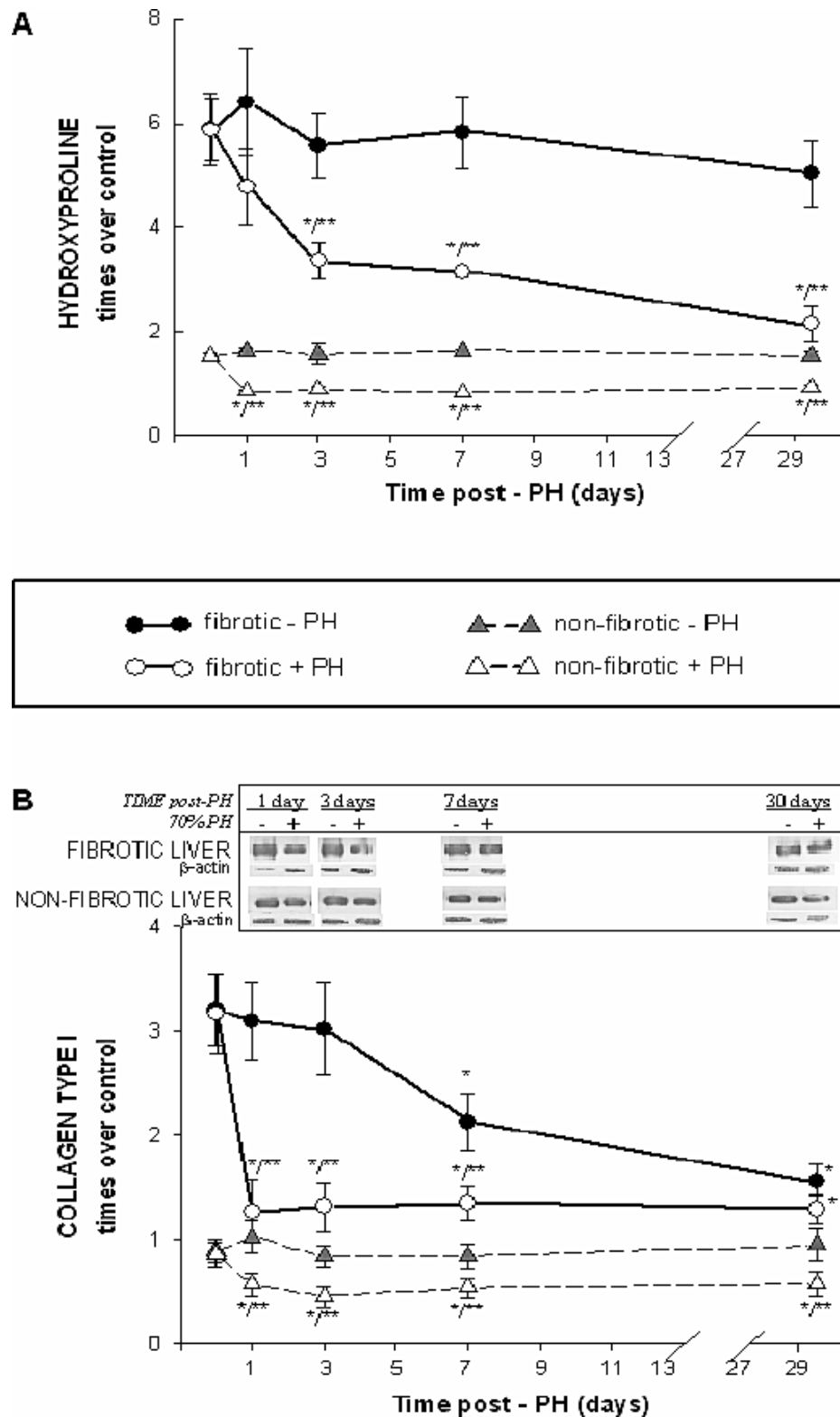


Figure 2. Total collagen reduction in the fibrotic liver after PH. Results are the mean $\pm$ SE of at least five independent observations per experimental group, and are expressed as times over control for panel A, hydroxyproline content ($1.2 \text{ mg} \cdot \text{g}^{-1}$ of liver), and in panel B, Western blot for collagen type I (15,500 arbitrary units, controls). β -Actin was used to normalize final results. Statistical significance: *, $P < 0.025$ against respective time zero control, and **, $P < 0.025$ versus sham-operated controls, at each time point.

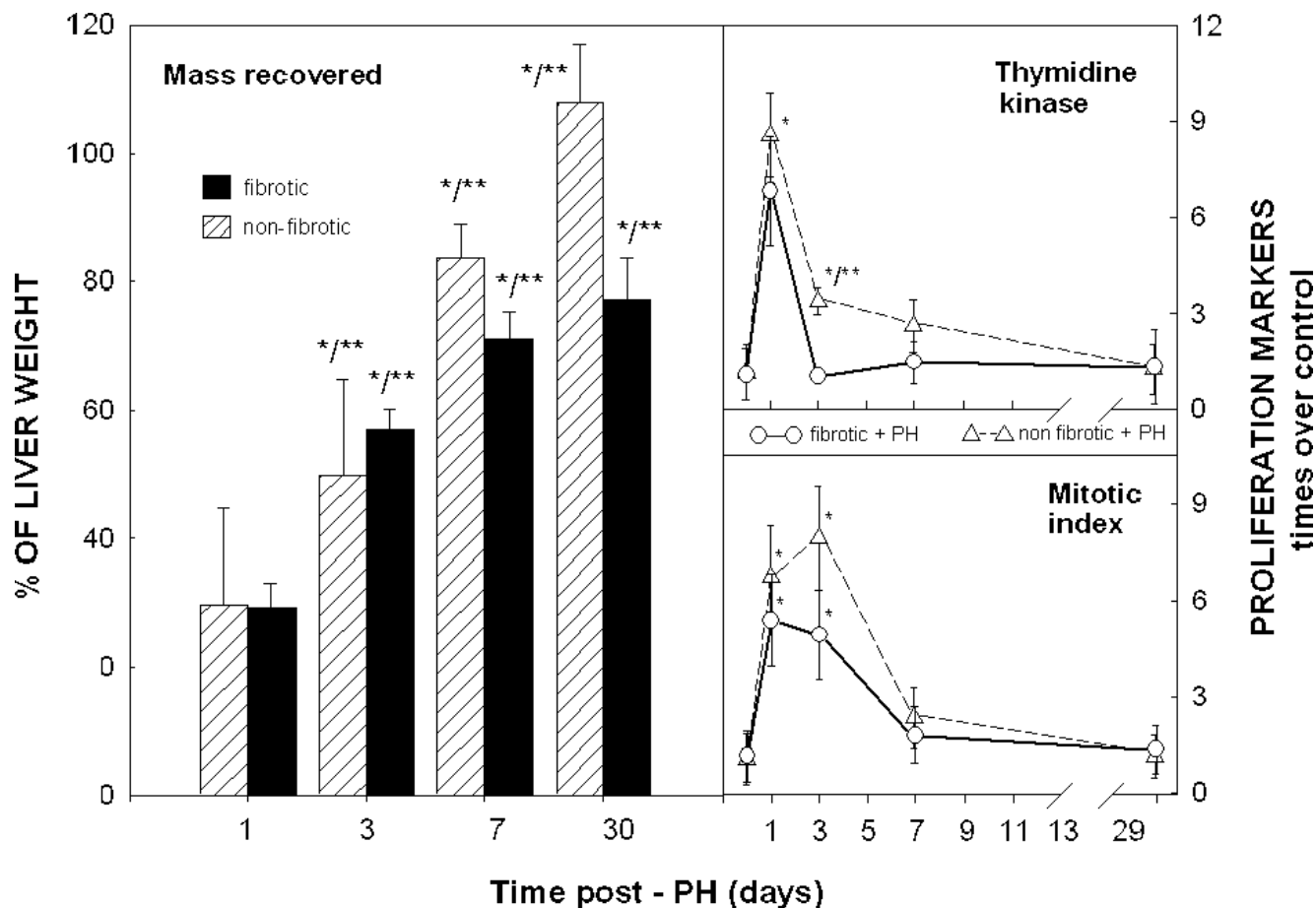


Figure 3. PH-induced cell regeneration in the fibrotic liver. Results are expressed as mean $\pm$ SE for percent of recovery of hepatic mass after PH (left panel), and as times over controls (right panel) for TK activity (top), and mitotic index (bottom). Estimations were performed in fibrotic and non-fibrotic controls after PH. Control values: TK activity, 0.18 ± 0.07 nmols of formed [3 H]-TMP $\cdot$ h $^{-1}$ $\cdot$ mg $^{-1}$ of cytosolic protein; mitotic index of 0.4 ± 0.1 mitotic figures per field in fifty 40 $\times$ -power fields. Statistical significance as in Figure 2.

precursor) that correlated with a diminished rate of proline oxidation (Fig. 4A, right). The fibrotic liver also presented an augmented free proline pool (Fig. 4, left), probably due to its decreased oxidation, which was more evident after stopping swine serum administration; PH also enhanced proline availability even though liver proline oxidation was

largely stimulated by PH (Fig. 4, left). Expression of mRNA for α -1 (I) collagen showed a rapid decrease early after discontinuation of swine serum administration (Fig. 4B), but the 70% PH accelerated the reduction of α -1 (I) collagen mRNA transcripts; however, an unexpected increase in expression of α -1 (I) collagen was noted at 30 days post-PH.

Table 2. Histological Assessment of Hepatic Fibrosis^a

Fibrotic score ^b	Fibrotic -PH				Fibrotic +PH			
	1 day	3 days	7 days	30 days	1 day	3 days	7 days	30 days
Grade 1	0/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5 (20)
Grade 2	0/5	0/5	0/5	0/5	0/5	0/5	0/5	3/5 (60)
Grade 3	0/5	0/5	0/5	1/5 (20)	0/5	0/5	2/5 (40)	1/5 (20)
Grade 4	5/5 (100)	5/5 (100)	5/5 (100)	4/5 (80)	5/5 (100)	5/5 (100)	3/5 (60)	0/5
	++++	++++	++++	+++	++++	++++	+++ / ++	-

^a Includes frequency of distribution of animals *n* (%) according to fibrosis score number and evaluation of septa thickness expressed as "+".

^b Fibrotic score (24): Grade 0: no fibrosis. Grade 1: short collagenous septa extended from central veins. Grade 2: slender septa link the central veins, but lobular architecture is preserved. Grade 3: pseudolobuli are formed by thin septa. Grade 4: parenchyma is subdivided into smaller pseudolobuli by thin septa.

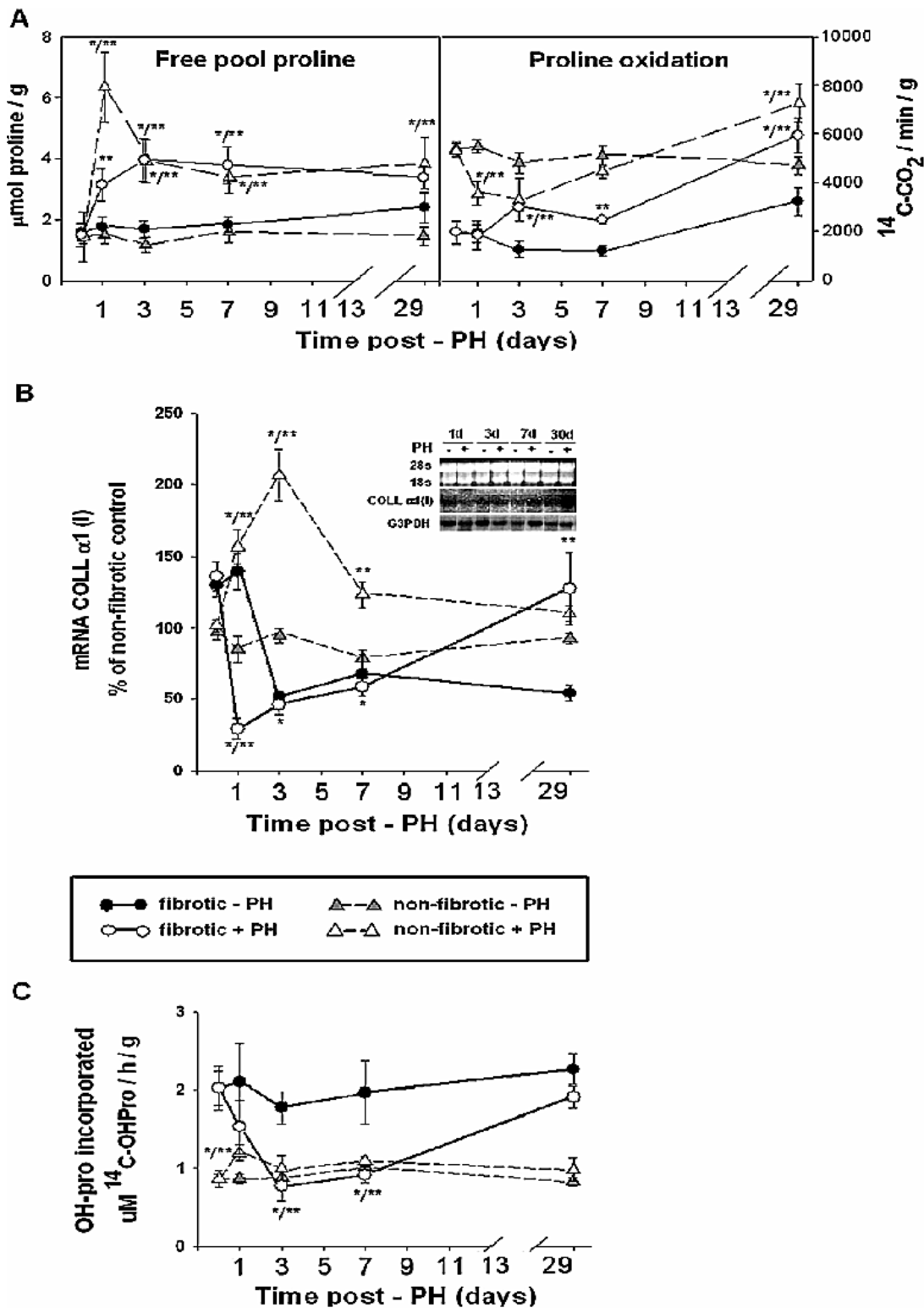


Figure 4. Proline metabolism and rate of collagen synthesis in the regenerating fibrotic liver. Results are expressed as mean $\pm$ SE for $n=5$ animals per experimental group, for panel A, free proline pool (left panel) and its rate of oxidation (right panel). In panel B, expression of mRNA for type I collagen (COLL $\alpha 1$ (I)) determined by Northern blot assay. Here, mRNA for G3PDH was used as standard to normalize the final results, shown as percentage of the non-fibrotic sham control, here considered as 100%. Gel electrophoresis of RNA samples, and bands obtained after probe hybridization in fibrotic livers, either sham or 70% PH, are provided. mRNA from G3PDH was used as standard to normalize the final results, panel C, incorporation of ^{14}C -proline into ^{14}C -hydroxyproline to collagen (synthesis) by liver slices. Symbols for experimental groups, as indicated at the top of panel C. Statistical significance as in Figure 2. OH-pro, hydroxyproline.

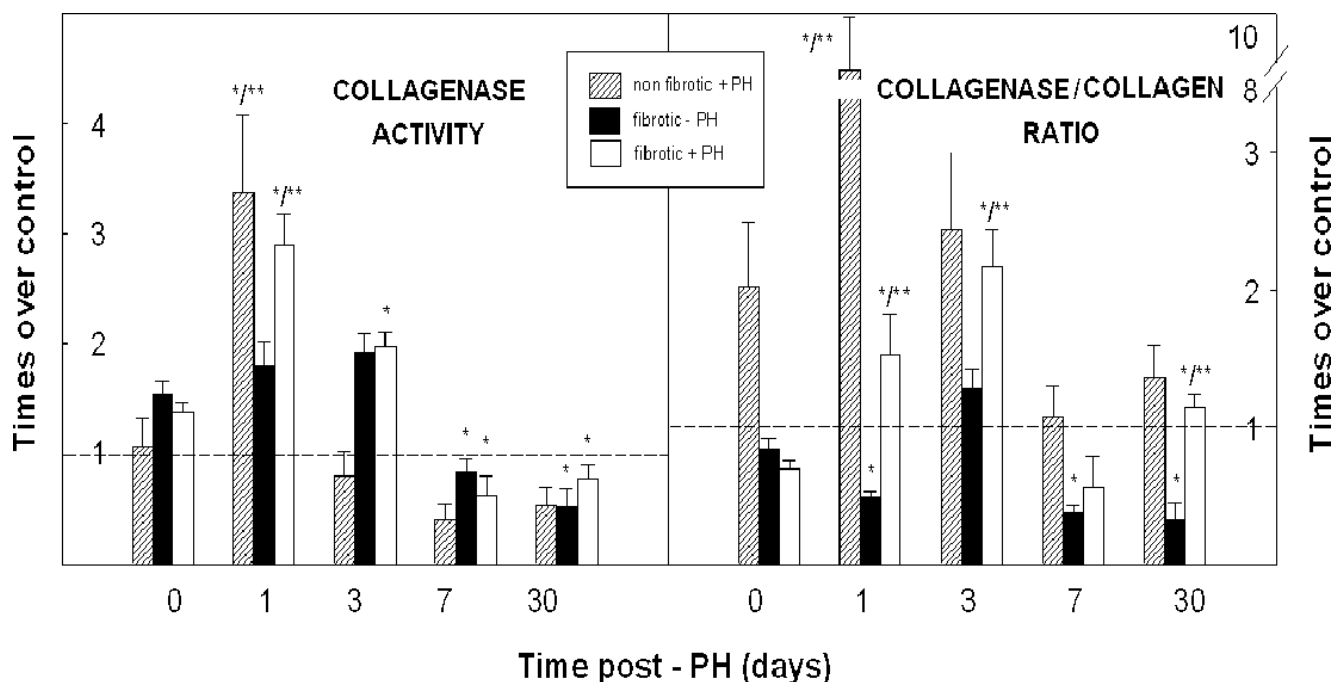


Figure 5. Total collagenase activity during regeneration of the fibrotic liver. Results are the mean $\pm$ SE of 5 independent observations per experimental group, and are expressed as times over controls for collagenase activity (left panel, 0.25 ± 0.03 nmols of degraded collagen $\cdot$ h $^{-1}$ $\cdot$ mg $^{-1}$ of protein, in controls), and for a calculated collagenase/collagen ratio (right panel, 0.05 ± 0.01 , in controls). Symbols at the top and statistical significance are as indicated in Figure 2.

Although reduced transcription of α -1 (I) collagen mRNA was found after sham-operation in fibrotic animals (Fig. 4B), they still had increased collagen synthesis, as indicated by the sustained enhancement of proline incorporation into collagen (Fig. 4C). The 70% PH decreased liver collagen synthesis in cirrhotic rats, but it increased thereafter (30 days), similarly to control fibrotic rats (Fig. 4C), which also coincided with increased collagen type I gene transcription (Fig. 4B).

ECM Degradation Increases During PH-Induced Regeneration of the Fibrotic Liver. At the onset of liver fibrosis, total liver collagenase activity remained without significant modification, but expression of MMP-13, TIMP-1, and TIMP-2 were increased. Early after PH, total liver collagenase activity was increased in fibrotic animals (Fig. 5, left), and the collagenolytic activity (collagenase/collagen ratio) was increased from 0.04 to 0.07 (Fig. 5, right). Stimulated collagenase activity was associated to a persistent increase in the levels of gelatinases, mainly pro-MMP-9 and MMP-2, and lower expression of both TIMPs, as well as accompanied by an unexpected transient fall in MMP-13 expression (Fig. 6). Similar patterns of collagenase activity, gelatinases, MMP-13, and TIMPs were found after 70% PH. Total collagenase activity in sham-operated fibrotic controls continuously decreased after 3 days postsurgery (Fig. 5, left), while expression of MMP-13 and TIMPs was gradually normalized (7 to 30 days post-PH; Fig. 6).

Activated HSC Are Maintained Along PH-

Induced Regeneration of Fibrotic Liver. The amount of α -SMA-expressing cells, presumably activated HSC, was assessed by immunohistochemistry and morphometric analysis (Fig. 7). In the fibrotic liver, activated HSC were mainly distributed in the perivascular area and inside the fibrotic septa (Fig. 7, images A, a). After 3 days of swine serum discontinuation, the control fibrotic group showed a rapid reduction in α -SMA expression, despite the persistence of the fibrotic septa (Fig. 7C, c and Fig. 7D, d). Conversely, there was a sustained expression of α -SMA by activated HSC in the regenerating fibrotic liver, distributed inside the reduced fibrotic septa 7 days after PH (Fig. 7 F, f to H, h), similarly to the findings in healthy animals (data not shown). At Day 30, both control and the regenerating fibrotic liver showed minimal HSC expression (Fig. 7E, e and I, i). Additionally, electron microscopy images from representative fibrotic liver sections after swine serum discontinuation, depicted a high amount of ECM production at the area of interest of fibrous septa, characteristic of activated HSC (Fig. 7, images 1–3).

Discussion

Attaining cirrhosis reversion or resolution through controlling fibrosis progression remains a main goal in the field of hepatogastroenterology. Hence, the present study is an attempt to assess whether the PH-induced regenerative response of the fibrotic liver is able to restore the liver's ECM to normal characteristics, reestablishing the balance between ECM synthesis and degradation, as well as to

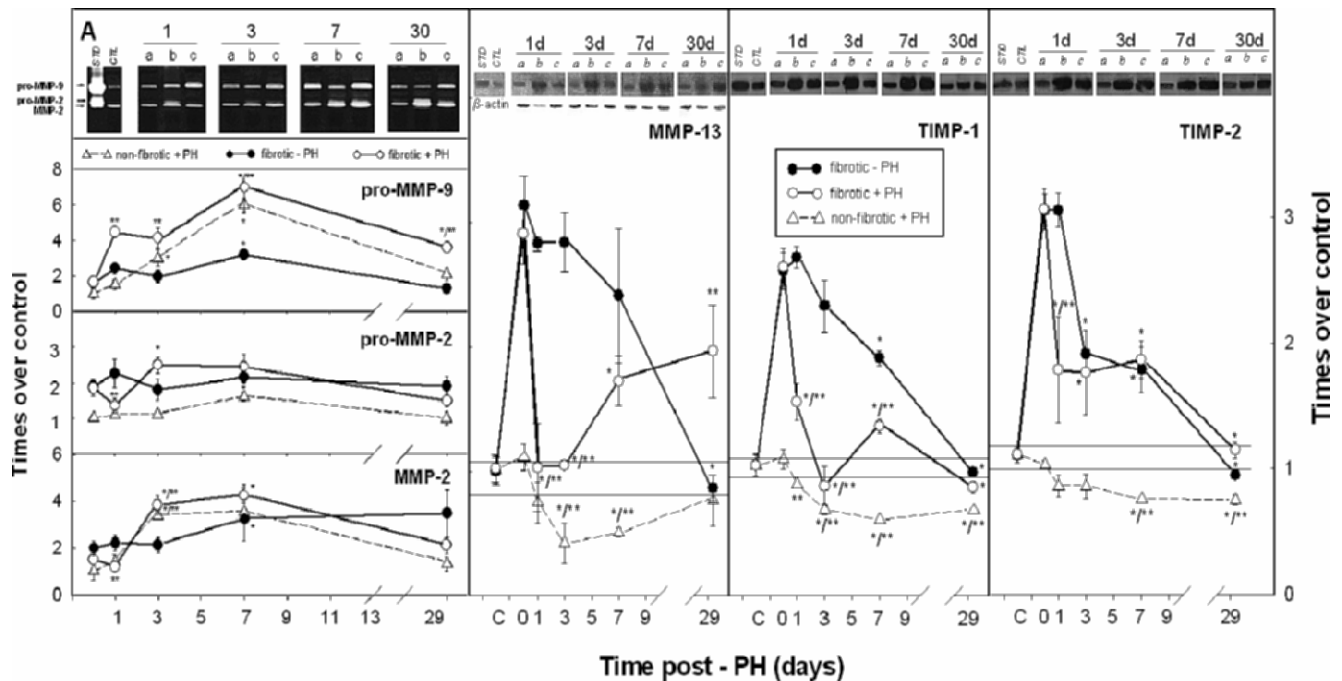


Figure 6. Gelatinase activities, MMP-13, and TIMP protein amount in the fibrotic liver during regeneration. Results are expressed as mean $\pm$ SE of times over the control values ($n = 5$ animals per experimental group), for gel zymography from a time-course of 1, 3, 7, and 30 days after PH (panel A) in (a) non-fibrotic with PH, (b) fibrotic sham, and (c) fibrotic PH animals. CTL = nonfibrotic sham-operated control. Below, densitometric analysis of activity bands corresponding to pro-MMP-9, pro-MMP-2, and MMP-2 in fibrotic sham-operated ($\bullet-\bullet$) and PH-operated animals ($\circ-\circ$), as well as in controls subjected to PH ($\Delta-\Delta$); STD = zymography standard. Western blot analysis for MMP-13, TIMP-1, and TIMP-2 protein expression for the same time course (right panels). STD = positive control, and CTL = nonfibrotic sham control. Densitometric analysis and normalization by β -actin were carried out. Control range as indicated by the horizontal bar. Statistical significance as noted in Figure 2.

explore a putative role of HSC in modulating these processes. For this purpose, a swine serum-induced experimental liver fibrosis was chosen as a suitable model for studying liver regeneration and ECM modifications, since it is characterized by scanty parenchyma necrosis and very slow fibrosis reversibility (14). In this model, PH readily induced a regenerative response in a similar fashion to what occurs in the regenerating normal liver (Fig. 3). Thus, this fibrotic liver preserves an adequate regenerative potential, probably due to the absence of cell necrosis and conserved liver function.

Associated to the aforementioned, there were significant ECM modifications (Fig. 2A and B) that progressively led to an almost complete resolution of the liver fibrosis after PH, which did not occur in sham-operated fibrotic rats, at least during the time period evaluated here. Although the mechanisms underlying reduction of deposited ECM in the proliferating liver are unknown, an increased liver cells/ECM ratio due to enhancement of hepatocyte mass, without concomitant increase of ECM, could occur in the normal liver (3). This was not the case with the present results, since the morphometric analysis and the normalization of our results by proliferative markers and the hepatocyte area determined in imaging analysis revealed an actual reduction of fibrosis that agreed with the actual diminution of the total collagen amount in PH-subjected fibrotic rats (Figs. 1 and 3, Tables 1 and 2). This argues against a reduction of fibrotic

stroma exclusively due to a "diluting" effect exerted by parenchymal proliferation. Then, the PH-induced decrease in ECM comprised the whole remnant organ, rather to be localized in "areas of regeneration."

Having demonstrated that PH elicited a resolution of a swine serum-induced liver fibrosis, the question arises concerning the mechanism(s) involved in ECM remodeling. Liver regeneration affects ECM through mechanisms that include increased collagen synthesis, activation of urokinase, plasmin, and MMPs (42, 43). Mitochondrial oxidation of proline was restored in the fibrotic livers, which agrees with the proposed involvement of mitochondrial function and NAD/NADH redox state in regulating collagen synthesis and breakdown in the cirrhotic rat liver (35, 44), indicating that mitochondrial function is still preserved in the cirrhotic animals and is susceptible to stimulation by PH, as occurs in the normal rat liver (45). In addition, the 70% PH stimulated collagen breakdown, as indicated by the increased total collagenase activity and by the relative collagenolytic activity (collagenase/collagen ratio; Fig. 5). In this context, it is well documented that MMP/TIMP balance is a determinant for ECM turnover and collagen deposition (41). Increased expression of MMPs and TIMPs at the onset of liver fibrosis can play a major role in the dynamic ECM remodeling, and their imbalance would result in excessive ECM deposition (Fig. 6), similar to that found in other fibrotic models (46). Indeed, there is a large body of

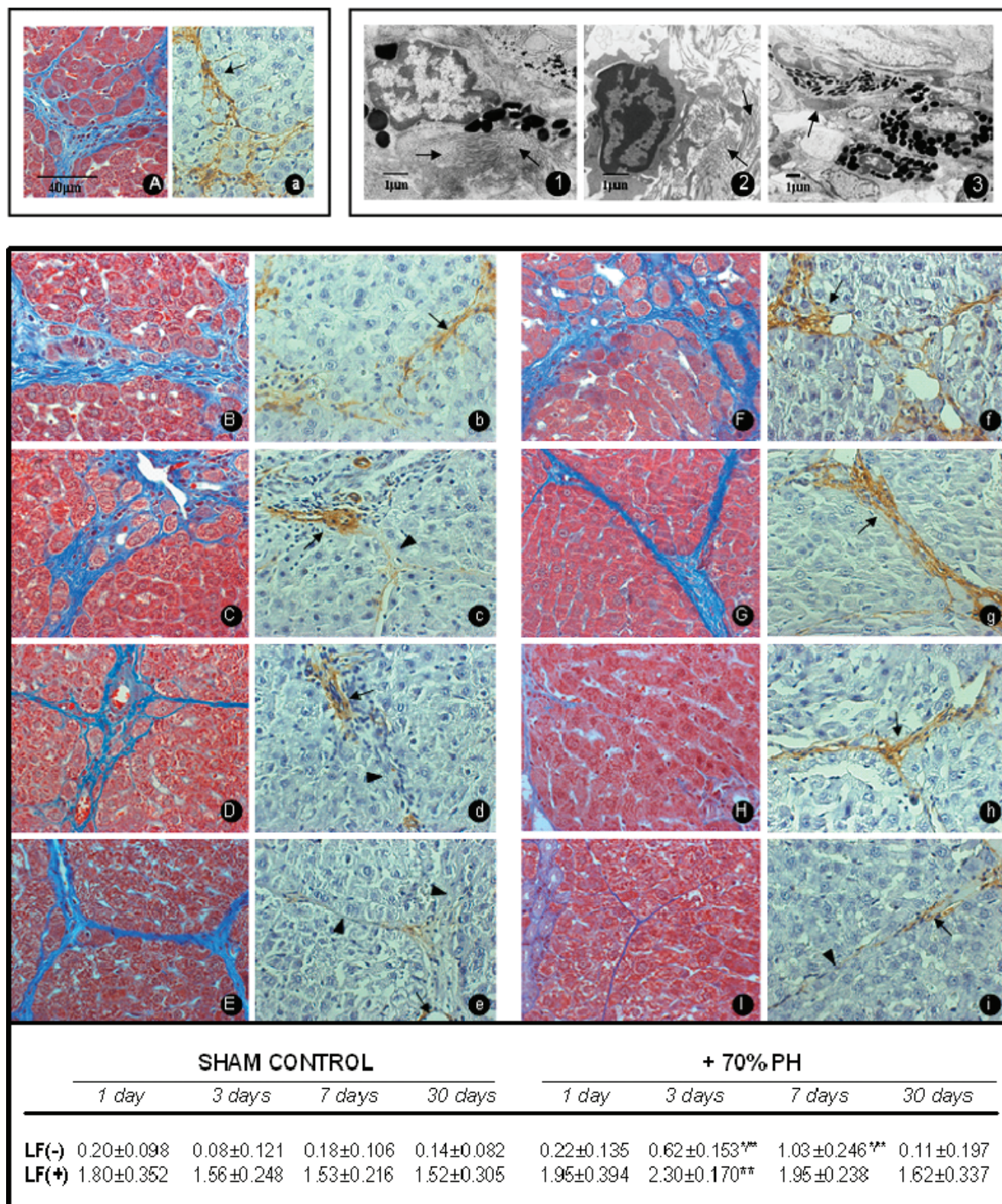


Figure 7. Expression of alpha smooth muscle actin (α -SMA) in activated HSC during regeneration of the fibrotic liver. Immunohistochemical determination of α -SMA-positive cells (activated HSC) is depicted. Representative 40 $\times$ power field samples from fibrotic rat liver at time zero (image a) and after 1, 3, 7, and 30 days from either sham controls (images B–E, respectively) or 70% PH (images F–I, respectively). Masson stain was used to identify the presence of fibrotic tissue at onset of liver fibrosis (image A) and after 1, 3, 7, and 30 days in both, sham-operated (images B–E, respectively) or regenerating fibrotic liver (images F–I, respectively). Arrows indicate positive reaction, including hepatic vascular endothelium, as internal control. Arrowheads indicate amorphous material, corresponding to fibrotic tissue, containing α -SMA negative cells. Electron microscopy at tissue septa level, showing activated HSC (lower lipid drops and higher ECM production) are included (images 1–3).

literature on activated HSC contributing to fibrosis; a critical event in fibrogenesis is activation of resident perisinusoidal cells (HSC). Stellate-cell activation is characterized by many important phenotypes, including enhanced extracellular matrix synthesis and prominent contractility (47). It is accepted that the profibrogenic effect of ethanol metabolites is mediated by the stimulation of a complex network of cytokine actions, which also culminates with activation of HSC. Hence, the development of several animal models of liver fibrosis, as well as isolation and cultivation of hepatic stellate cells, has led to propose that activation of HSC is a key event in the fibrogenic process and many details on this finely tuned mechanism are now available (48, 49).

From here, we propose two possible mechanisms probably mediating PH actions on liver fibrosis: 1) by preparing ECM structure for further collagenase degradation, involving mainly gelatinase activities (50) and 2) by participating in the so-called hepatocyte priming, since ECM remodeling by MMPs could also foster the release of mitogenic cytokines and growth factors (51, 52). Furthermore, the apparent upregulation of MMP-9 and the lack of increase in the activity of MMP-2, elicited by PH in animals with liver fibrosis, seem to indicate the cell types involved in this PH-mediated effect. Indeed, MMP-9 could be expressed mainly by HSCs, whereas MMP-2 could be expressed by Kupffer (macrophage) cells. This whole picture seems to have failed in sham-operated fibrotic rats, where the balance between collagen synthesis and degradation seems to have been insufficient.

The regeneration of the remnant liver in the presence of cirrhosis accelerates immediately after PH, but the extent of regeneration is frequently insufficient. Several features have been claimed to be involved in the diminished regenerative capacity shown by the cirrhotic liver. For instance, PH-induced lipid peroxidation in massive resection of the cirrhotic liver may inhibit activation of mitochondrial respiration, decreasing the energy status (53). In addition, little activation of proliferating cell nuclear antigen (PCNA) has been noted in cirrhotic livers after PH (54), probably related to an early expression of TGF- β 1 (55). Moreover, impaired regeneration of the cirrhotic liver seems to be associated not only with a lower level of proliferating signals but also with a strong decrease in receptors for growth factors (56). Nonetheless, experimental cirrhotic livers could effectively respond to HGF supply after PH, as well as to the injection of recombinant adenoviral vectors encoding VEGF and HGF (57–59). Altogether, evidence indicates that the cirrhotic liver still possesses a variable capacity for responding to proliferative stimulus, but the amount of variability remains unknown.

It is feasible that activated HSC are responsible for ECM remodeling in this model (Fig. 7). The role of HSC during ECM remodeling has been largely documented in experimental models of hepatic fibrosis, regeneration and reversion of fibrosis (3, 42, 60). HSC became activated during liver fibrosis, and remained activated during regeneration of the fibrotic organ, where significant ECM remodeling was observed; whereas they rapidly decreased in sham-operated fibrotic controls. This suggests the possible role of activated HSC in fibrogenesis and ECM reduction in this experimental model. An attractive explanation, although not tested here, is that functional changes in the population of activated HSC or of new clones, showing different features of ECM production, participate during liver regeneration in this experimental model. A sustained or new activation of HSC, occurring during liver regeneration and coinciding with reduction of deposited ECM, disagrees with the attributed role of HSC in fibrogenesis (61), and rather indicates that activated HSC play a major role during PH-induced liver regeneration (62). Indeed, the stimulus for activation may arise from a controlled lipid peroxidation that occurs during rat liver regeneration (63, 64), whereas a decrease in the number of activated HSC may occur by either reversion to a quiescent phenotype or by apoptosis, as well as by interactions with Kupffer cells that are mediated by various cytokines and growth factors (65). It is obvious that the induced liver fibrosis occurred with evident inflammation, and that liver regeneration, induced by PH, could impose additional and characteristic inflammatory events. However, since the outcome was quite different between sham-operated and cirrhotic animals subjected to PH, regarding reversion of liver fibrosis, the response to regeneration in cirrhotic livers seems to be different from that of normal livers. In conclusion, swine serum-induced rat fibrotic liver showed a high proliferative ability after PH, as well as significant variations in ECM content. Although the evidence is still indirect, preservation of mitochondrial function could play a key role in the remodeling of ECM, as evidenced by a recovered balance between collagen synthesis and ECM degradation. Activated HSC are proposed as responsible for regulating such an ECM production/degradation balance, partially controlling liver cell proliferation. Therefore, pharmacological approaches for the resolution of liver fibrosis by blocking HSC activation should also evaluate possible effects on liver cell proliferation. We are not proposing large liver resections as a therapeutic approach for treating cirrhosis, but the present results could have a meaning in the response of the cirrhotic liver in the event of surgical removal of small well circumscribed hepatocellular cancer.

Below the images, area of α -SMA immunoreactive cells was calculated as [(total immunostained area – immunostained vessels and non-specific areas) $\times$ 100] / whole image area. Arbitrary units from software Image-Pro v5.0 were used. LF = Liver fibrosis. Controls at time zero: LF (–) = 0.15 ± 0.085 ; LF (+) = 1.8 ± 0.295 . Results are expressed as mean $\pm$ SE of 5 animals per group, and fifty 40 $\times$ power fields per liver sample. Statistically significant against time zero (*), and against his control of group (**).

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