Histol Histopathol (2008) 23: 583-591

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

The anti-fibrotic effect of liver growth factor is associated with decreased intrahepatic levels of matrix metalloproteinases 2 and 9 and transforming growth factor beta 1 in bile duct-ligated rats

Juan J. Díaz-Gil[1], Carmelo García-Monzón[2], Carmen Rúa[3], Paloma Martín-Sanz[4], Rosa M. Cereceda[1], María E. Miquilena-Colina[2], Celia Machín[3], Amalia Fernández-Martínez[4] and Rafael García-Cañero[1]

[1]Department of Experimental Biochemistry, Hospital Puerta de Hierro, Madrid, Spain, [2]Laboratory of Experimental Hepatology, Hospital Santa Cristina, Madrid, Spain, [3]Department of Cellular Biology, Faculty of Biological Sciences, UCM, Madrid, Spain and [4]Centro Investigaciones Biológicas, CSIC, Madrid, Spain

Summary. Liver growth factor (LGF), a mitogen for liver cells, behaves as an anti-fibrotic agent even in extrahepatic sites, but its mechanistic basis is unknown. We aimed to determine the intrahepatic expression pattern of key modulators of liver fibrosis in bile duct-ligated rats (BDL) after injection of LGF. BDL rats received either LGF (4.5 µg/ratXdose, two doses/week, at time 0 or 2 or 5w after operation, depending on the group (BDL+LGF groups, n=20) or saline (BDL+S groups, n=20). Groups were compared in terms of fibrosis (histomorphometry), liver function (aminopyrine breath test), matrix metalloproteinases MMP-2 and MMP-9, transforming growth factor beta 1 (TGF-ß1) and liver endoglin content (Western blotting), and serum tissue inhibitor of metalloproteinases 1 (TIMP-1) levels (ELISA). In BDL+LGF rats, the fibrotic index was significantly lower at 5w, p=0.006, and at 8w, p=0.04, than in BDL+S rats. Liver function values in BDL+LGF rats were higher than those obtained in BDL+S rats (80% at 5w and 79% at 8w, versus 38% and 29%, p<0.01, taking healthy controls as 100%). Notably, in BDL+LGF rats the intrahepatic expression levels of both MMPs were lower at 2w (MMP-2, p=0.03; MMP-9, p=0.05) and 5w (MMP-2, p=0.05, MMP-9, p=0.04). In addition, the hepatic TGF-ß1 level in BDL+LGF rats was lower at 2w (36%, p=0.008), 5w (50%) and 8wk (37%), whereas intrahepatic endoglin expression remained constant in all BDL rats studied. LGF ameliorates liver fibrosis and improves liver function in BDL rats. The LGF-induced anti-fibrotic effect is associated with a decreased hepatic level of MMP-2, MMP-9 and TGF-ß1 in fibrotic rats.

Offprint requests to: Juan J. Díaz-Gil, PhD, Servicio de Bioquímica Experimental, Hospital Universitario Puerta de Hierro, c/ S. Martín de Porres, 4, 28035 Madrid, Spain. e-mail: jdiaz.hpth@salud.madrid.org

Key words: Bile duct-ligated rats, Cirrhosis, Fibrosis, Regeneration

Introduction

Liver cirrhosis is one of the leading causes of death worldwide, chronic infection by hepatitis B and C viruses and alcohol abuse being the most common etiologic factors (Gluud, 2005; D'Amico et al., 2006). Without an effective treatment in the early stages, reversible hepatic fibrosis progresses to irreversible cirrhosis, featured by hepatocellular failure, portal hypertension and an increased risk of developing hepatoma (Bataller and Brenner, 2005). Since the therapeutic options for these complications of cirrhosis are often poorly effective, new approaches to the prevention and treatment of hepatic fibrosis are clearly needed. At the cellular and molecular level, fibrogenesis is mainly characterized by activation of hepatic stellate cells (HSCs) and aberrant activity of transforming growth factor (TGF)-ß1 (Gressner and Weiskirchen, 2006). Based on the current advances in the understanding of cross-talk between HSCs and TGF-ß1, novel therapeutic interventions involving drugs, neutralizing antibodies, soluble receptors and gene therapy have been developed in the attempt to modulate activation of fibrogenic cells and to inhibit excess production of profibrogenic mediators (Liu et al., 2006). Although the initial results are very promising, it is too early to favor any one of these new strategies for the treatment of hepatic fibrosis in humans and, therefore, the search for therapeutic modalities to reverse liver fibrosis continues.

Liver growth factor (LGF) is a hepatic mitogen for liver cells that was purified and characterized by our

group some years ago (Díaz-Gil et al., 1986, 1987, 1988). LGF shows both in vivo and in vitro activity, increasing liver DNA and protein synthesis, as well as hepatocellular mitotic index, with no signs of toxicity or liver degeneration (Díaz Gil et al., 1994a). Concerning the mechanistic action of LGF *in vivo*, we have demonstrated that the mitogenic cascade induced by LGF in rat liver depends, at least in part, on intrahepatic tumor necrosis factor alpha (TNF-ß) stimulation, as anti-TNF-ß antibodies were capable of inhibiting both LGF-mediated liver DNA synthesis and proliferating cell nuclear antigen (PCNA)-positive hepatocytes, identifying portal vein endothelial cells as an important source of TNF-ß in that setting (Díaz-Gil et al., 2003). Interestingly, LGF activity is not restricted to the liver, as we have observed that this growth factor stimulates dopamine terminal sprouting and partially restores motor function in a model of Parkinson's disease in rats (Reimers et al., 2006). Regarding hepatic fibrosis, LGF has been shown to have positive effects on the fibrotic stage, liver function, inflammation and portal hypertension, leading to a significant improvement in survival in different models of cirrhosis in rats (Díaz-Gil et al., 1994b, 1999). Likewise, LGF can exert its antifibrotic action in other tissues. In this respect, we have recently shown that this growth factor reduced carotid artery fibrosis, producing a substantial regeneration of artery wall, lowering blood pressure and improving vascular function in a model of spontaneous hypertensive rats (Somoza et al., 2006).

In order to shed light on the molecular basis of LGF-mediated effects on liver fibrogenesis, we proposed to determine the intrahepatic expression pattern of some important mediators in the fibrogenic process, such as metalloproteinases MMP-2 and MMP-9, TGF-ß1 and its type III receptor, and endoglin, as well as the serum levels of tissue inhibitor of metalloproteinase (TIMP-1), after *in vivo* administration of LGF to bile duct-ligated (BDL) rats, a well-described experimental model of liver cirrhosis (Kountouras et al., 1984), searching for correlations with the outcome of hepatic fibrosis and liver function in this animal model.

Materials and methods

Animals

One hundred and forty eight male Wistar rats, Panlab, Madrid, Spain (220 g initial body weight, see Table 1 for distribution among the different groups), were housed in plexiglas cages, kept at 25°C with a 12-h light/dark cycle, and allowed free access to a standard chow diet and water ad libitum until the time of the study. Animal experiments were done in accordance with the NIH guidelines for the care and use of laboratory animals.

Bile duct ligation and experimental design

Ether-anesthetized rats were subjected to double ligation and section of common bile duct, BDL. Under these conditions, bile flow is reversed and the rats suffer an acute and progressive biliary intoxication, with development of fibrosis and cirrhosis, and in some cases, peritoneal ascites. BDL rats were randomly assigned to receive two intraperitoneal, IP, doses of 4.5 mg per week of either LGF (BDL+LGF, n=20/group) or saline (BDL+S, n=20/group), beginning at time 0 or 2 or 5 weeks later, depending on the group. Therefore, BDL rats received LGF for only 2 weeks, 2w group, or 3 weeks, 5w and 8w groups. All BDL rats were killed at different times (2, 5, and 8 weeks) after ligation in separate groups of 20 animals each. At these time points, serum and liver tissue samples were collected from each rat and processed as appropriate to carry out measurements detailed below. In addition, serum and liver tissue samples were collected from 22 healthy rats for use as control samples, control group, t=0.

Table 1. Values of serum parameters in different groups of rats studied.

	Albumin (g/dl)	ALT (IU/l)	AST (IU/l)	Tprotein (g/dl)	Tbil (mg/100 ml)	AP (IU/l)	GGT (IU/l)	Survival (%)
Control (n=22)	3.18±0.53	53±15	105±26	5.60±0.98	0.07±0.04	240±85	0.27±0.45	
BDL+S, 2wk (n=20)	2.68±0.51	97±31	337±144	5.81±1.33	7.71±2.04	617±231	32±21	82
BDL+LGF, 2wk (n=20)	2.47±0.45	83±20	290±95	5.59±1.0	6.45±1.42	537±186	26±15	95
	p=NS	p=NS	p=NS	p=NS	p=0.03	p=NS	p=NS	p=NS
BDL+S, 5wk (n=20)	2.33±0.34	84±18	244±86	6.12±0.8	8.5±1.5	423±100	17.5±5.5	71
BDL+LGF, 5wk (n=20)	2.06±0.56	108±73	342±137*	7.15±2.0	7.15±2.0	521±182	20.6±7.6	81
	p=NS	p=NS	p=NS	p=NS	p=0.015	p=NS	p=NS	p=NS
BDL+S, 8wk (n=20)	1.94±0.5	127±50	400±134	6.4±1.	7.8±2.9	488±190	21.1±13.9	65
BDL+LGF, 8wk (n=26)	2.15±0.47	92±30	300±95	6.1±1.0	7.5±2.7	417±137	17.3±9.8	76
	p=NS	p<0.05	p<0.05	p=NS	p=NS	p=NS	p=NS	p=NS

ALT: alanine aminotransferase, AST: aspartate aminotransferase, Tprotein: total proteins, Tbil: total bilirubin, AP: alkaline phosphatase, GGT: gamma-glutamyltranspeptidase (GGT), NS: not significant.

LGF purification and assessment of activity

LGF was purified from rat serum following the procedure previously reported by our group (Díaz-Gil et al., 1994b). The criteria for purity, absence of other growth factors and/or contaminants in the LGF preparation, were likewise assessed (Díaz-Gil et al., 1986, 1988a). LGF preparations were lyophilized and kept at 4°C until use, and aliquots were dissolved in saline prior to IP injection. Before using LGF in these experiments, we checked its activity in vivo at several doses, injecting it into normal rats to establish the dose that produced the greatest liver DNA stimulation, as determined by incorporation of ^{3}H-thymidine (New England Nuclear, Dreiech, Germany) into DNA (Díaz-Gil et al., 1986).

Histological evaluation of fibrosis

Liver samples from each rat, including controls, were fixed in 10% buffered formalin, dehydrated in a graded ethanol series, cleared with xylene and embedded in paraffin. The blocks were cut into 8-µm sections, which were stained according to the Van Gieson method. To quantify hepatic fibrosis, we used the Metamorph Imaging System (Universal Imaging Corporation, Downingtown, Pa, USA.). The results were expressed as a ratio of liver tissue area studied (fiber bundle surface area/total area), which was designated as fibrotic index. For a more accurate data analysis, values were obtained in 40 different fields (3 rats/group), and subsequently averaged for each sample.

The development of fibrosis in this model is associated with a considerable increase in liver mass, 80% in the liver to body weight ratio in the first 2w and 95% at 5w, with a substantial increase in the synthesis of the extracellular matrix (ECM) supporting the liver structure. For this reason, we consider morphometric evaluation of liver fibrosis to be a much more exact determination of extracellular fibrosis than a hydroxyproline assay.

Serum biochemical parameters

We determined the following biochemical parameters in serum samples from each rat using a 917-Hitachi Automatic Analyzer (Boehringer-Mannheim, Mannheim, Germany): albumin, alanine amino-transferase (ALT), aspartate aminotransferase (AST), total proteins (Tprot), total bilirubin (Tbil), alkaline phosphatase (AP) and gamma-glutamyltranspeptidase (GGT).

Aminopyrine breath test (ABT)

ABT was carried out following a previously published procedure (Lauterburg and Bircher, 1976). [dimethylamine-^{14}C]Aminopyrine, at a dose of 1 µCi/kg bw (Amersham, UK), was injected IP into rats in 0.15-ml volume, using saline as vehicle. The exhaled $^{14}CO_2$ was collected in 15-min breath samples. A 2-ml aliquot was counted (Wallac 1410 beta counter, Pharmacia-LKB, Stockholm, Sweden). The rate constant for elimination of aminopyrine, ABT-κ, was calculated by means of least squares regression of the logarithm of the counts vs time.

Protein extraction and western blot analysis

Liver tissue samples, 100 mg, from each rat included in the study groups were homogenized in a lysis buffer containing 10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 0.5% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 1 mM ß-mercaptoethanol and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich, St. Louis, MO). In addition, tablets containing a protease inhibitor cocktail (Roche Diagnostics GmbH, Penzberg, Germany) were directly added to the extraction medium. Extracts were vortexed for 45 min at 4°C and, after centrifugation for 20 min at 13,000g, the supernatants were stored at -80°C. For Western blot analysis, whole-cell extracts were boiled for 5 minutes in Laemmli sample buffer with 100 mM DL-dithiothreitol (DTT) (Sigma-Aldrich), and equal amounts of protein (30-100 µg) were separated by 10-12% SDS polyacrylamide gel electrophoresis, and transferred to a polyvinylidene difluoride membrane. Nonspecific binding was blocked by incubation with 5% non-fat dry milk, followed by incubation with the primary antibodies anti-MMP-2 (rabbit polyclonal IgG, Torrey Pines Biolabs, Houston, TX), anti-MMP-9 (rabbit polyclonal IgG, Torrey Pines Biolabs), anti-TGF-ß1 (goat polyclonal IgG, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and anti-endoglin (goat polyclonal IgG, Santa Cruz Biotechnology, Inc.). Anti-rabbit (Sigma-Aldrich) and anti-goat (Santa Cruz Biotechnology, Inc.,) horseradish peroxidase conjugates were used as secondary antibodies where appropriate. Subsequently, blots were developed by enhanced chemiluminescence (Amersham, Arlington Heights, IL). Target protein band densities were normalized by calculating the ratio of ß-actin (Sigma-Aldrich) to the corresponding densities. Different exposure times were employed for each blot to ensure linearity of band intensities. Densitometric analysis was expressed in arbitrary units.

This study was designed to compare LGF effects on certain parameters at given times after injection (final effects), not to assess their variation over the course of the experimental period. For this reason, we chose to use Western blotting instead of the determination of mRNA expression.

Determination of TIMP-1 levels

All serum samples assessed were stored at -80°C and thawed only for use in this laboratory investigation. A commercially available solid-phase sandwich enzyme-

linked immunosorbent assay (ELISA) was employed, according to the manufacturer's instructions, for the quantitative measurement of TIMP-1 (Quantikine, R&D Systems Inc., Minneapolis, MN) in the serum of all rats included in this study. In liver fibrosis, there are parallel increases in TIMP-1 in serum and liver tissue, as demonstrated previously (Mas et al., 2004).

Statistical evaluation

Results were expressed as mean±SD. For statistical analysis of the data, the Mann-Whitney U test for nonparametric and unpaired values or Student's *t*-test was used as appropriate. Results were considered significant when $p<0.05$.

Results

Serum biochemistry values and outcome in rats studied

As shown in Table 1, no significant differences in the serum biochemical profile were observed between the different groups of BDL rats, whether untreated or treated with LGF, at any time point analyzed (with the exception of total bilirubin, lower in BDL+LGF rats, and significantly so at 2w and 5w). However, as expected, ALT, AST, AP and GGT levels were markedly higher, and the serum albumin level was lower in BDL rats than in healthy control rats. Regarding survival of BDL rats, although a trend toward a higher survival rate was observed in LGF-treated rats as compared with untreated

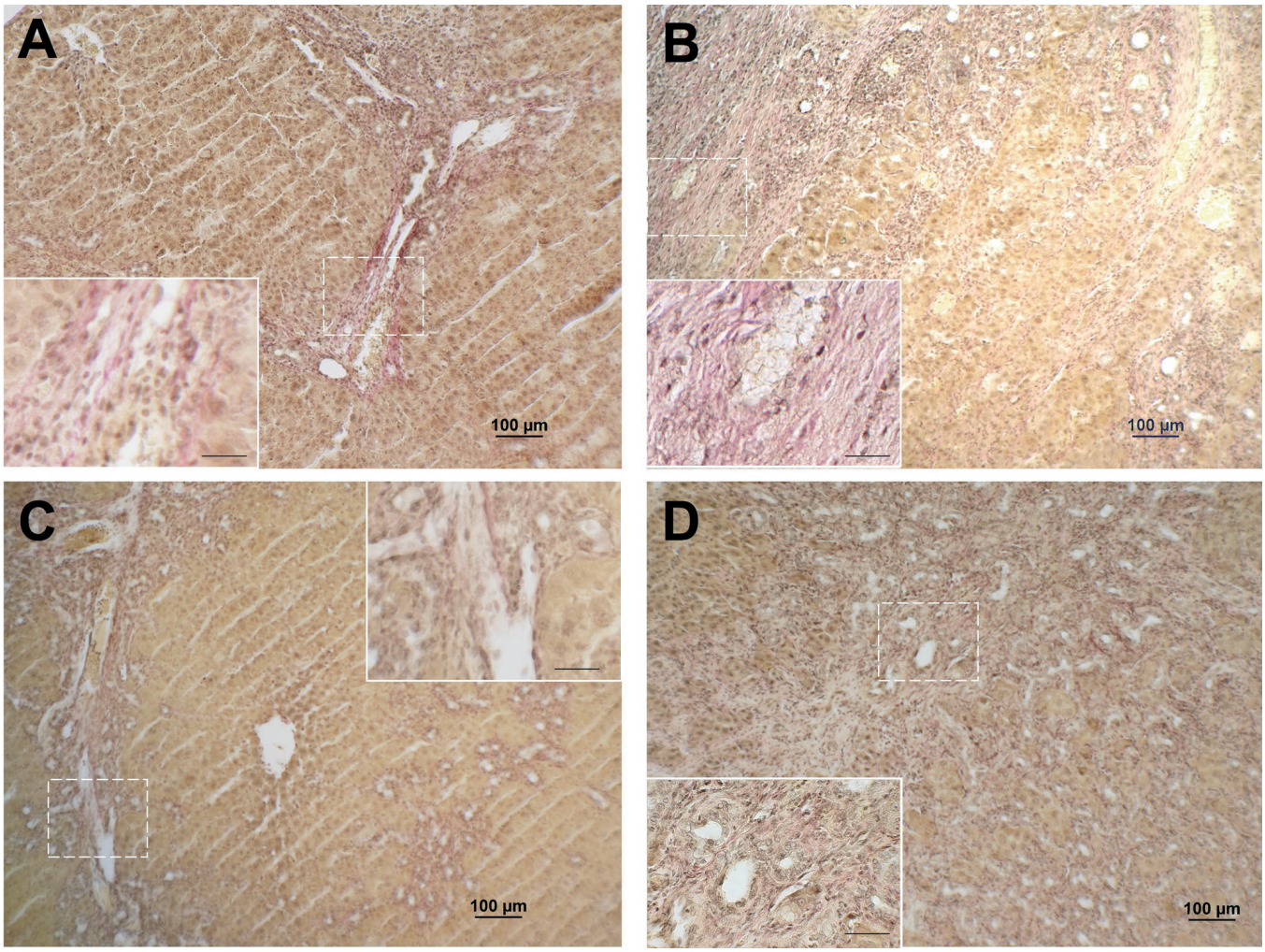

Fig. 1. Microscopic findings in rat livers. **A.** Rat liver after 5 weeks of BDL+S. Abundant fibrosis and loss of cellularity can be observed. Dashed lines indicates an area at higher magnification (insert) in all cases. Black line in inserts indicates 50 µm in all cases. **B.** 8w BDL+S rat liver. Fibrosis and low cellularity were even more pronounced. **C.** Rat liver 5w after BDL with injection of LGF from 2-5w. There is less fibrosis as compared to uninjected rats (**A**), as well as a higher number of hepatocytes. **D.** BDL+LGF rat liver at 8w (injection of LGF from 5-8w), where both effects are even more pronounced with respect to BDL+S rat (**B**), and there is a substantial increase in bile duct proliferation. Van Gieson´s stain.

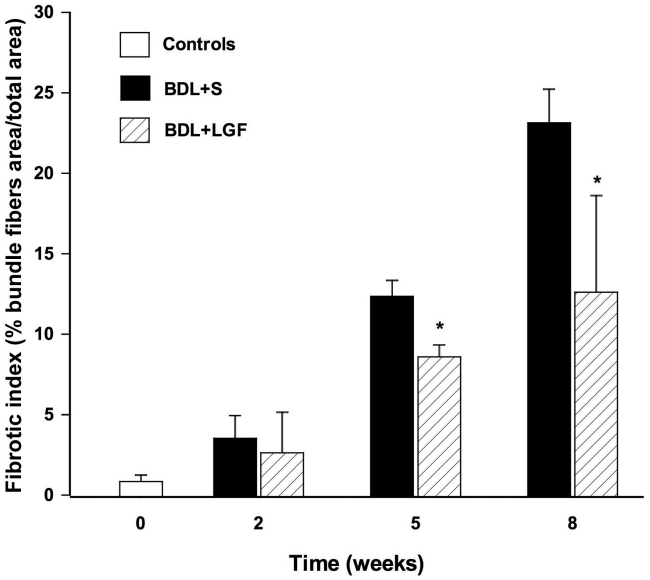

Fig. 2. Measurement of liver fibrosis by morphometric analysis. LGF produced a decrease in fibrosis in every group of BDL rats, but its action was more evident at 5w and 8w, when fibrosis was more abundant. Values expressed as mean±SD. n=3 rats/group. *: $p<0.05$ with respect to the value for BDL+S rats in the same group at every time point.

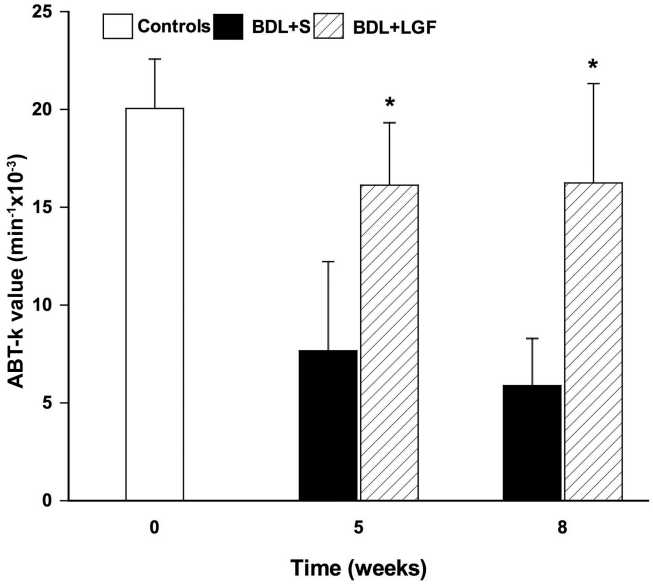

Fig. 3. Liver function by aminopyrine breath test determination. LGF was able to revert liver functionality nearly to that of healthy rats in advanced stages of fibrosis/cirrhosis at 5w and 8w. Values represented mean±SD for all groups analyzed. Bars indicate the same groups as in Fig. 2. n=5 rats/group.

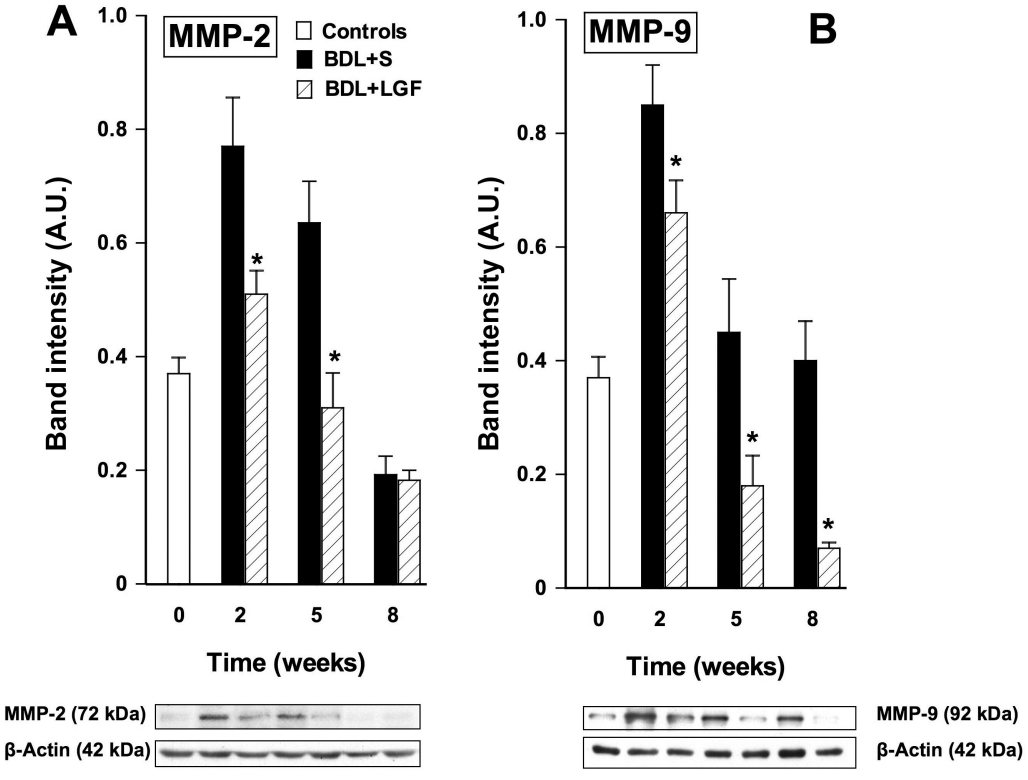

Fig. 4. Liver metalloproteinase content by Western blotting (mean±SD). MMP-2 (**A**), MMP-9 (**B**). LGF action tends to decrease expression of both MMPs in all the periods studied, in parallel with a decrease of fibrosis. Bars indicate the same groups as in Fig. 2. n=4 rats/group.

animals, the differences were not statistically significant.

LGF diminished hepatic fibrosis in BDL rats

In BDL+S rats, the degree of hepatic fibrosis ranged from moderate fibrosis at 5w (Fig. 1A) to well-defined cirrhosis at 8w (Fig. 1B), whereas, at week 5, the livers of BDL+LGF rats showed less fibrotic changes (Fig. 1C), which were more evident at 8w, as was a pronounced increase in bile duct proliferation (Fig. 1D). In order to assess the changes observed in liver fibrosis more accurately, we carried out a computer-assisted quantitative analysis of stained liver tissue sections from both groups of BDL rats and from healthy rats. As shown in Fig. 2, the fibrotic index in livers from BDL+S rats increased progressively and significantly over time (3.53±1.45 at 2w, p=0.03; 12.36±0.98 at 5w, p<0.0001; and 23.14±2.08 at 8w, p<0.0001), as compared with the healthy control rats (0.85±0.38). This increase was clearly less marked in BDL rats injected with LGF (2.63±2.52 at 2w, N.S.; 8.59±0.73 at 5w, p=0.001; and 12.61±6.0 at 8w, p=0.04) than in BDL+S rats. It is interesting to note that the LGF effect on hepatic fibrosis in BDL rats was more evident and statistically significant at the advanced stages of the fibrotic process in this experimental model of liver cirrhosis.

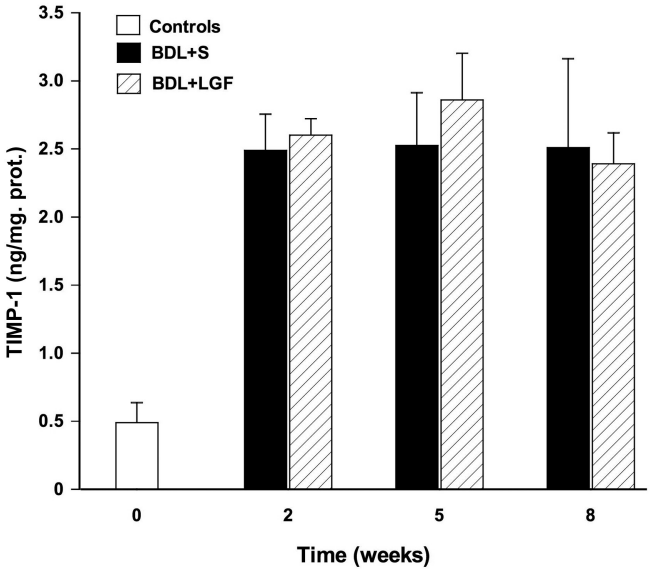

Fig. 5. TIMP-1 rat serum concentration (ELISA) (mean±SD). LGF did not affect TIMP-1 serum levels in any subgroup studied (TIMP-1 serum levels in liver fibrosis increase in parallel with liver content, see Methods for more details). Bars indicate the same groups as in Fig. 2. n=4 rats/group.

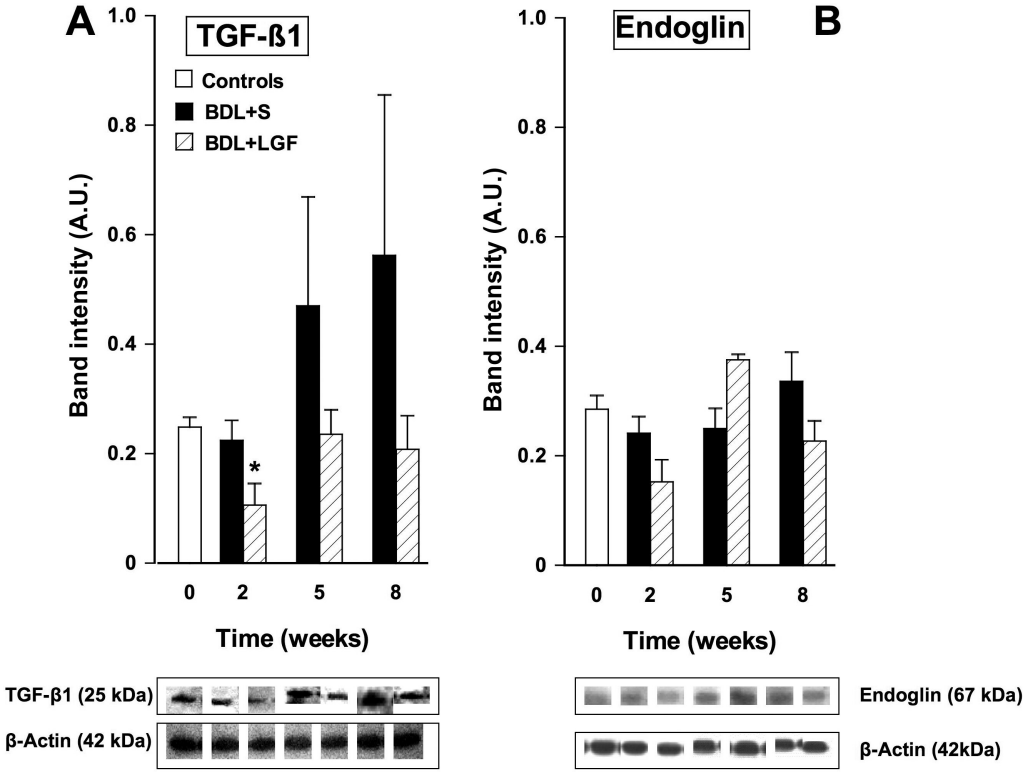

Fig. 6. Liver content of TGF-ß1 **(A)** and endoglin **(B)** by Western blotting (mean±SD). LGF was able to decrease TGF-ß1 levels similar to those of controls in all the periods studied, but did not influence endoglin (a TGF-ß1 receptor). Bars indicate the same groups as in Fig. 2. n=12 rats/group.

Effect of LGF on liver function

We investigated whether LGF was capable of improving liver function in BDL rats. For this purpose, we determined the liver detoxification capacity (ABT-k) in healthy rats (control group), in BDL rats injected with saline (BDL+S group), and in BDL rats injected with LGF (BDL+LGF group) 5 and 8 weeks after bile duct ligation (Fig. 3). Under our experimental conditions, BDL+S rats showed a significant and marked decrease in ABT-k values as compared with healthy rats at each time point studied (62% at 5w, $p<0.001$; 71% decrease at 8w, $p<0.0001$). In contrast, BDL+LGF rats had a mild but insignificant deterioration of liver function with respect to healthy rats (20% at 5w, $p=0.06$; 21% at 8w, $p=0.17$). Notably, ABT-k values in BDL+LGF rats were significantly higher than those obtained in untreated BDL rats ($p=0.09$ at 5w and $p=0.003$ at 8w, respectively).

LGF depressed total intrahepatic MMP-2 and MMP-9 content in BDL rats

A statistically significant increase in MMP-2 expression was observed in the livers of BDL+S rats at 2w (2.08-fold, $p=0.004$) and 5w (1.7-fold, $p=0.01$) as compared with healthy rats (Fig. 4A), whereas, at 8w, the intrahepatic expression of MMP-2 was lower (0.56-fold, $p=0.002$). Moreover, at 2w, the MMP-9 protein levels were significantly higher in the livers of BDL+S (2.35-fold, $p=0.001$) than in control rats, whereas the intrahepatic MMP-9 content at 5w (1.21-fold) and at 8w (1.08-fold) was similar to that found in healthy rats (Fig. 4B). In contrast, in BDL+LGF rats, the intrahepatic expression of both MMPs was significantly lower at 2 weeks (MMP-2: 0.67-fold, $p=0.03$; MMP-9: 0.75-fold, $p=0.05$) and at 5 weeks (MMP-2: 0.49-fold, $p=0.01$; MMP-9: 0.4-fold, $p=0.04$) than in untreated BDL rats, reaching levels similar to or even lower than those detected in control rats.

LGF did not influence serum TIMP-1 levels in BDL rats

Serum levels of TIMP-1 were significantly elevated in BDL+S rats at each time point studied (2.49±0.26 ng/mg protein at 2w; 2.50±0.37 at 5w and 2.51±0.65 at 8w), remaining constant over time, as compared to healthy rats (0.49±0.14 ng/mg protein, $p=0.02$) (Fig. 5). However, no significant differences in serum TIMP-1 concentrations were observed in BDL+LGF rats at any time point analyzed (2.49±0.26 ng/mg protein at 2w; 2.86±0.33 at 5w and 2.39±0.23 at 8w) as compared with BDL+S rats.

LGF decreased intrahepatic TGF-ß1 levels but not those of endoglin in BDL rats

The intrahepatic TGF-ß1 content (active form) increased in BDL+S rats at 5 weeks (1.89-fold) and at 8w (2.26-fold), as compared with healthy rats. Interestingly, the intrahepatic level of TGF-ß1 in BDL+LGF rats was lower at 2w (0.36-fold, $p=0.008$), at 5w (0.5-fold) and 8w (0.37-fold) than in BDL+S rats, reaching levels similar to those found in control rats (Fig. 6A). Notably, the beneficial decrease in the amount of TGF-ß1 in the livers of BDL rats produced by LGF was observed at all time points studied, even at later stages of the fibrotic process, when cirrhosis was already established. Since endoglin is a TGF-ß1 binding protein, we examined the expression pattern of endoglin in BDL rats. Western blot analysis revealed no significant differences in the intrahepatic protein levels of endoglin in either BDL+S or BDL+LGF rats with respect to the control group (Fig. 6B).

Discussion

The results of the present study provide evidence that LGF decreases hepatic fibrosis and improves liver function in rats with severe liver disease after BDL. Previous studies have shown that this experimental model of liver injury is characterized by progressive liver failure, portal hypertension and fibrosis (Franco et al., 1979; Wensing and Branch, 1990; Aronson et al., 1993), which is considered irreversible 4 to 8 weeks after BDL (Zimmermann et al., 1992). Taking this fact into account, the antifibrotic action of LGF in this animal model was particularly remarkable, as it was more evident at advanced stages of hepatic fibrosis (5-8 weeks after BDL), even when cirrhosis was already established. Different drugs, such as doxorubicin (Greupink et al., 2006), α-interferon (Muriel, 1996), pirfenidone (García et al., 2002) and rapamycin (Biecker et al., 2005), have been reported to ameliorate liver fibrosis in BDL rats. However, their effects were assessed within a few weeks of BDL, when hepatic fibrosis is still reversible. Therefore, whether these drugs could also be beneficial at later fibrotic stages, as was LGF in the present study, is a question that remains to be addressed. Moreover, Xia et al have recently reported that daily intravenous administration of hepatocyte growth factor (HGF) expression plasmids attenuated hepatic biliary fibrosis in BDL mice (Xia et al., 2006). In contrast, in this study, we have shown that LGF ameliorated liver fibrosis in BDL rats after two 4.5-mg doses administered weekly by IP injection, suggesting a long-lasting pharmacological action of this growth factor.

We proposed to explore potential mechanisms underlying the antifibrotic action of LGF. Hepatic fibrogenesis is a process in which production of the ECM surpasses its degradation. Hepatic stellate cells (HSC) are the primary source of both ECM proteins and the enzymes that degrade them (Olaso and Friedman, 1998), collectively referred to as matrix metallo-proteinases (MMPs). These enzymes are secreted by cells into the extracellular space as proenzymes, which are then activated by a number of specific cleavage

mechanisms. The active enzymes are, in turn, inhibited by a family of tissue inhibitors of metalloproteinases, TIMPs (Roeb et al., 1997). Compelling evidence has documented the association of MMPs with liver fibrosis. For example, in rat hepatic fibrosis induced by bile duct ligation, the activities of MMP-2 and MMP-9 increased 2 days after ligation, reached maximal levels at day 10, and remained high for weeks, suggesting that sustained tissue damage and inflammation due to chronic cholestasis may induce MMPs (Woessner, 1991; Kossahowska et al., 1998; Arthur, 2000). Likewise, in this study, we showed that concentrations of both MMP2 and MMP-9 markedly increased in the liver of BDL rats, reached maximal levels at week 2, and decreased progressively to reach normal levels at week 8. It is noteworthy that LGF administration to BDL rats induced a significant reduction of the intrahepatic levels of both MMPs at each time point studied, with levels at week 5 even lower than those observed in normal liver (Fig. 4). MMPs are detected at the early stage of HSC activation, whereas collagenases are down-regulated in fully activated HSC and the expression of TIMPs is increased (Vyas et al., 1995). Thus, it is reasonable to speculate that LGF might exert an inhibitory effect on MMP-2 and MMP-9 synthesis by early activated HSC, a hypothesis that we are presently trying to elucidate. In addition, and in marked contrast with the findings for MMPs, LGF did not modify the increased serum TIMP-1 levels found in BDL rats, indicating that LGF appears to exert a selective effect on MMP-2 and MMP-9 synthesis, probably at early stages of liver fibrogenesis, without affecting its inhibitor, TIMP-1. These combined effects modify the MMP/TIMP ratio, and could be a factor that limits the further degradation of the fibrotic ECM.

Among the numerous pro-fibrogenic mediators, TGF-ß1 is the most effective one (Gressner et al., 2002). The hepatic content of TGF-ß1 has been shown to be increased in fibrotic rat and human livers (De Bleser et al., 1997; Clemente et al., 2006), as we observed in our experimental model of BDL rats. Interestingly, we found that LGF substantially abrogated TGF-ß1 up-regulation in the livers of BDL rats, a fact that suggests that the antifibrotic effect of LGF may be exerted by reducing or attenuating the enhanced intrahepatic TGF-ß1 production in BDL rats. In support of this notion, it has already been demonstrated that inhibition of TGF-ß1 prevents progression of liver fibrosis in rats (Nakamura et al., 2000). Since activated HSC are the major source of liver-derived TGF-ß1 after an in vivo fibrogenic injury (Bissel et al., 2001), LGF probably modulates TGF-ß1 production by deregulating the HSC activation process, thus contributing to changes in the fibrogenic response in the liver of BDL rats, a hypothesis that we are trying to verify at the present time. On the other hand, we proposed to investigate the intrahepatic expression level of TGF-ß1 binding proteins, such as endoglin, because endoglin overexpression has been shown to modulate cellular TGF-ß1 responses (Letamendía et al., 1998; Barbara et al., 1999). In our experimental model of BDL rats, the intrahepatic expression pattern of endoglin in fibrotic rats did not differ from that of healthy controls, suggesting that endoglin is not involved in the fibrotic process observed in BDL rats.

In conclusion, our study shows that LGF attenuates liver fibrosis and improves liver function in BDL rats. The LGF-induced antifibrotic effect is associated with a decreased intrahepatic level of MMP-2, MMP-9 and TGF-ß1 in fibrotic rats, suggesting that LGF may exert an inhibitory effect on the synthesis of these fibrogenic mediators by modulating the process of HSC activation. Moreover, the fact that LGF is able to decrease liver fibrosis and improve liver functionality in advanced stages and under the therapeutic conditions described in this work, in addition to its previously demonstrated activity in other models systems (Díaz Gil et al., 1994b, 1999) opens the possibility of considering LGF as a promising molecule to the treatment of fibrosis/cirrhosis in human therapy.

Acknowledgements. We thank the Department of Clinical Biochemistry, Hospital Universitario Puerta de Hierro, Madrid, for technical assistance in the processing of serum samples. We also thank Martha Messman for her English language assistance. This work has been supported by grants to J.J. D-G and to C.G-M from the Instituto de Salud Carlos III (FIS 03/0648, FIS 03/0417, CIBERehd and RTIC G03/015), Spain. A.F-M was supported by a CNIC-Bancaja fellowship.

References

Aronson D.C., Chamuleau R.A.F.M., Fredericks W.M., Gooszen H.G., Hiijmans H.S. and James J. (1993). Reversibility of cholestatic changes following experimental common bile duct obstruction: fact or fantasy? J. Hepatol. 18, 85-95

Arthur M.J.P. (2000). Fibrogenesis. II. Metalloproteinases and their inhibitors in liver fibrosis. Am. J. Physiol. Gastrointest. Liver Physiol. 279, G245-49.

Barbara N.P., Wrana J.L. and Letarte M. (1999). Endoglin is an accesory protein that interacts with the signaling receptor complex of multiple members of the transforming growth factor-ß superfamily. J. Biol. Chem. 274, 584-594.

Bataller R. and Brenner D.A. (2005). Liver fibrosis. J. Clin. Invest.115, 209-218.

Biecker E., De Gottardi A., Neef M., Unternahrer M., Scheider V., Ledermann M., Sagesser H., Show S. and Reichen J. (2005). Long-term treatment of bile duct ligated rats with (sirolimus) rapamycin significantly attenuates liver fibrosis: analysis of the underlying mechanisms. J. Pharmacol. Exp. Ther. 313, 952-961.

Bissell D.M., Roulot D. and George J. (2001). Transforming growth factor ß and the liver. Hepatology 34, 859-67.

Clemente M., Núñez O., Lorente R., Rincón D., Matilla A., Salcedo M., Catalina M.V., Ripio C., Iacono O.L., Banares R., Clemente G. and García-Monzón C. (2006). Increased intrahepatic and circulating levels of endoglin, a TGF-beta1co-receptor, in patients with chronic hepatitis C virus infection: Relationship to histological and serum markers of hepatic fibrosis. J. Viral Hepatol. 13, 625-632.

D´Amico G., Garcia-Tsao G. and Pagliaro L. (2006). Natural history and

prognostic indicators of survival in cirrhosis: a systematic review of 118 studies. J. Hepatol. 44, 217-231

De Bleser P.J., Niki T., Rogiers V. and Geerts A. (1997). Transforming growth factor-ß gene expression in normal and fibrotic rat liver. J. Hepatol. 26, 886-893.

Díaz-Gil J.J., Escartín P., García-Cañero R., Trilla C., Veloso J.J., Sánchez G., Moreno-Caparrós A., Enrique de Salamanca C., Lozano R., Gavilanes J.C. and García-Segura J.M. (1986). Purification of a liver DNA-synthesis promoter from plasma of partially hepatectomized rats. Biochem. J. 235, 49-55.

Díaz-Gil J.J., Gavilanes J.G., Sánchez G., García-Cañero R., García-Segura J.M., Santamaría L., Trilla C. and Escartín P. (1987). Identification of a liver growth factor as an albumin-bilirubin complex. Biochem. J. 243, 443-448.

Díaz-Gil J.J., Sánchez G., Trilla C., Escartín P. (1988). Identification of biliprotein as a liver growth factor. Hepatology 8, 484-486.

Díaz-Gil J.J., Rúa C., Machín C., Cereceda R.M., de Foronda M., Pérez de Diego J., Trilla C. and Escartín P. (1994a). Hepatic growth induced by injection of the liver growth factor into normal rats. Growth Regul. 4, 113-22.

Díaz-Gil J.J., Rúa C., Machín C., cereceda R.M., Guijarro M.C. and Escartín P. (1994b). Hepatic recovery of dimethylnitrosamine-cirrhotic rats after injection of the liver growth factor. In: Liver carcinogenesis. The molecular pathways. Skouteris G.G. (ed). NATO ASI Series. Vol. 88. Springer-Verlag. Berlin. pp 275-288.

Díaz-Gil J.J., Muñoz J., Albillos A., Rúa C., Machín C., García-Cañero R., Cereceda R.M., Guijarro M.C., Trilla C. and Escartín P. (1999). Improvement of liver fibrosis, functionality and hemodynamics in CCl4-cirrhotic rats alter injection of the liver growth factor. J. Hepatol. 30, 1065-1072.

Díaz-Gil J.J., Majano P.L., López-Cabrera M., Sánchez-López V., Rúa C., Machín C., Trilla C., García-Cañero R. and Moreno-Otero R. (2003). The mitogenic activity of the liver growth factor is mediated by tumor necrosis factor alpha in rat liver. J. Hepatol. 38, 598-604.

Franco D., Gigou M., Szeley A.-M. and Bismuth H. (1979). Portal hypertension after bile duct obstruction. Effect of bile diversion on portal pressure in the rat. Arch. Surg. 114, 1064-1067.

García L., Hernández I., Sandoval A., Salazar A., García J., Vera J., Grijalva G., Muriel P., Margolin S. and Armendáriz-Borunda J. (2002). Pirfenidone effectively reverses experimental liver fibrosis. J. Hepatol. 37, 797-805.

Gluud C. (2005). Mortality from cirrhosis: lack of progress over the last 35 years. Gut 54, 1523-1526.

Gressner A.M., Weiskirchen R., Breitkopf H. and Dooley S. (2002). Roles of TGF-ß in hepatic fibrosis. Front. Biosci. 7, D793-807.

Gressner A.M. and Weiskirchen R. (2006). Modern pathogenetic concepts of liver fibrosis suggest stellate cells and TGF-ß as major players and therapeutic targets. J. Cell. Mol. Med. 10, 76-99.

Greupink R., Bakker H.I., Bouma W., Reker-Smit C., Meijer D.K., Beljaers L. and Poelstra K. (2006). The antiproliferative drug doxorubicin inhibits liver fibrosis in bile duct-ligated rats and can be selectively delivered to hepatic stellate cells in vivo. J. Pharmacol. Exp. Ther. 317, 514-521.

Kossakowska A.E., Edwards D.R., Lee S.S., Urbanski L.S., Stabber A.L., Zhang C.L., Phillips B.W., Zhang Y. and Urbanski S.J. (1998). Altered balance between matrix metalloproteinases and their inhibitors in experimental biliary fibrosis. Am. J. Pathol. 153, 1895-1902.

Kountouras J., Billing B.H. and Scheuer P.J. (1984). Prolonged bile duct ligation obstruction: a new experimental model of cirrhosis in the rat. Br. J. Exp. Pathol. 65, 305-311.

Lauterburg B.H. and Bircher J. (1976). Expiratory measurement of maximal aminopyrine demethylation in vivo: effect of phenobarbital, partial hepatectomy, portacaval shunt and bile duct ligation in the rat. J. Pharmacol. Exp. Ther. 196, 501-509.

Letamendia A., Lastres P., Botella L.M., Roab U., Longa C., Velasco B., Attisane L. and Bernabeu C. (1998). Role of endoglin in cellular responses to transforming growth factor b. A comparative study with betaglycan. J. Biol. Chem. 273, 33011-33019.

Liu X., Hu H. and Yin J.Q. (2006). Therapeutic strategies against TGF-ß signaling pathway in hepatic fibrosis. Liver Int. , 26, 8-22.

Mas M.R., Comert B., Oncu K., Vural S.A., Akay C. and Tasci I. (2004). The effect of taurine treatment on oxidative stress in experimental liver fibrosis. Hepatol. Res. 28, 207-215.

Muriel P. (1996). Alpha-interferon prevents liver collagen deposition and damage induced by prolonged bile duct obstruction in the rat. J. Hepatol. 24, 614-621.

Nakamura T., Sakata R., Ueno T., Sata M. and Ueno H. (2000). Inhibition of transforming growth factor ß prevents progression of liver fibrosis and enhances hepatocyte regeneration in dimethylnitrosamine-treated rats. Hepatology 32, 247-255.

Olaso E. and Friedman S.L. (1998). Molecular mechanisms of hepatic fibrogenesis. J. Hepatol. 29, 836-847.

Reimers D., Herranz A.S., Díaz-Gil J.J., Asensio M.V.T., Paíno C.L., and Bazán E. (2006). Intrastriatal infusión of liver growth factor stimulates dopamine terminal sprouting and partially restores motor function in 6-hydroxydopamine-lesioned rats. J. Histochem. Cytochem. 54, 457-465.

Roeb E., Purucker E., Breuer B., Nguyen H., Heinrich P.C., Rose-John S. And Matern S. (1997). TIMP expression in toxic and cholestatic liver injury in rat. J. Hepatol. 27, 535-544.

Somoza B., Abderrahim F., González J.M., Conde M.V., Arribas S.M., Starcher B., Fernández-Alfonso M.S., Díaz-Gil J.J. and González M.C. (2006). Short term treatment of spontaneously hypertensive rats with liver growth factor reduces carotid artery fibrosis, improves vascular function, and lowers blood pressure. Cardiov. Res. 69, 764-71.

Vyas S.K., Leyland H., Gentry J. and Arthur M.J. (1995). Rat hepatic lipocytes synthesize and secrete transin (stromelysin) in early primary culture. Gastroenterology 109, 889-898.

Wensing G. and Branch R. (1990). Phenobarbital influences the development of sodium retention in liver disease induced by bile duct ligation in the rat. Hepatology 11, 773-778.

Woessner J.F. (1991). Matrix metalloproteinases and their inhibitors in connective tissue remodelling. FASEB J. 5, 2145-2154.

Xia J.-L., Dai C., Michalopoulos G.K. and Liu Y. (2006). Hepatocyte growth factor attenuates liver fibrosis induced by bile duct ligation. Am. J. Pathol. 168, 1500-1512.

Zimmermann H., Reichen J., Zimmermann A., Sagesser H., Thenisch B. and Hoflin F. (1992). Reversibility of secondary biliary fibrosis by biliodigestive anastomosis in the rat. Gastroenterology 103, 579-589.

Accepted November, 23 2007