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Liver growth factor antifibrotic activity *in vivo* is associated with a decrease in activation of hepatic stellate cells

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Summary. The antifibrotic activity of Liver Growth Factor (LGF), a liver mitogen, was previously demonstrated in several models of rat liver fibrosis and even in extrahepatic sites, such as carotid artery in hypertensive rats and rat $CdCl_2$-induced lung fibrosis. In the present study, we have attempted to examine in depth its mechanism of antifibrotic action in bile duct-ligated (BDL) rats, with special emphasis on its activity in fibrogenic liver cells.

BDL rats received either LGF 9 µg/week for 2 or 3 weeks (BDL+LGF, n=20/group) or saline (BDL+S, n=20/group), at times 0, week 2, or week 5 after operation. Groups were compared in terms of liver α-smooth muscle actin (SMA) content (western blotting and immunohistochemistry), hepatic apoptosis, liver desmin content (western blotting), and serum endothelin-1 (ELISA).

LGF produced a marked decrease in liver α-SMA content compared with saline-injected rats, especially evident at longer times (5w and 8w; $p<0.05$), accompanied by a decrease in hepatic α-SMA+ cells. This decrease was not due to the killing of activated hepatic stellate cells (HSC) or myofibroblasts by LGF, since there was a slight decrease in hepatic apoptosis that was more evident at 2w ($p<0.05$). Moreover, LGF did not seem to influence HSC proliferation, as shown by measuring liver desmin content. The antifibrotic activity exerted by LGF seems to be closely related to a modulation of the activation state of fibrogenic liver cells (activated HSC and myofibroblasts) in BDL rats.

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Introduction

We recently demonstrated the antifibrotic activity of Liver Growth Factor (LGF) in bile duct-ligated (BDL) rats (Díaz-Gil et al., 2008), studying its effects at different stages of the fibrotic/cirrhotic process. LGF was able to improve liver function (ABT-k test) and decrease fibrosis, even at stages previously considered to be irreversible (Wensing and Branch, 1990; Zimmermann et al., 1992; Aronson et al., 1993), effects that were accompanied by a reduction of liver MMP-2, MMP-9 and TGF-ß1 content.

LGF is a hepatic mitogen for liver cells that was purified and characterized by our group some years ago (Díaz-Gil et al., 1986b, 1987, 1988). LGF shows both in vivo and in vitro activity, increasing liver DNA and protein synthesis, PCNA-positive cells and 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 hepatic TNF-α secretion by endothelial cells of the portal vein (Díaz-Gil et al., 2003). LGF is also able to stimulate TNF-α secretion in endothelial cells in culture, at a concentration as low as 5 pg/ml, without inflammatory activity (lack of stimulation of ICAM-1 or VCAM-1). Interestingly, LGF activity is not restricted to the liver, as we have observed that LGF was able to stimulate dopamine terminal sprouting and partially restore motor function in a model of Parkinson's disease in rats (Reimers et al., 2006).

Considering the effect of LGF on hepatic fibrosis,

we have shown that injection of LGF reduced fibrosis, increased liver activity and improved key liver functions affected by detoxifying enzymes, portal hypertension and ascites, among others, leading to a significant improvement in survival in different models of cirrhosis in rats (Díaz-Gil et al., 1994b, 1999, 2008). Likewise, LGF can exert its antifibrotic action in other tissues, as we have recently demonstrated in carotid artery fibrosis, where LGF produced a substantial regeneration of artery wall, lowering blood pressure and improving vascular function in a model of spontaneously hypertensive rats (Somoza et al., 2006). Moreover, injection of LGF also decreased lung fibrosis and improved lung function in a model of $CdCl_2$-induced fibrosis in rats (Martínez-Galán et al., 2005).

In an attempt to examine the mechanism of antifibrotic activity of LGF in depth, we studied its effect on the growth and activation of hepatic stellate cells (HSC), which are the key cells in the process of synthesis and accumulation of extracellular matrix (ECM) within the liver. We also studied the serum endothelin-1 concentration as a marker of the fibrogenic process.

Materials and methods

Animals

One hundred and forty-eight male Wistar rats (200-250 g body weight) were housed in Plexiglas cages (Panlab, Madrid, Spain), 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, cirrhosis and, in some cases, peritoneal ascites. BDL rats were randomly assigned to receive two intraperitoneal (IP) doses of 4.5 µg per week of either LGF (BDL+LGF, n=20/group) or saline (BDL+S, n=20/group). 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 the 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).

LGF purification and assessment of activity

LGF was purified from rat serum following the procedure previously reported by our group (Díaz-Gil et al., 1994a). The criteria for purity, absence of other growth factors and/or contaminants in the LGF preparation, were likewise assessed (Díaz-Gil et al., 1986b, 1994a). LGF preparations were lyophilized and kept at +4ºC 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 the DNA (Díaz-Gil et al., 1986b).

Protein extraction and western blot analysis

Serum samples from 20-22 rats were tested for determinations of serum concentrations of certain parameters presented in our preceding paper (Díaz-Gil et al., 2008, Table 1), but western blot and immunodetection experiments were carried out in only 3-4 rats/group (seven different groups). We have determined hydroxyproline content in all BDL and control rats, and we chose 3-4 rats with results very close to the median in each group for western blot and immunodetection experiments.

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-propane-sulfonate (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 13,000 g, 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-α-smooth muscle actin (SMA) (Sigma-Aldrich) and anti-desmin (sc-7559, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). 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 chemiluminiscence (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.

Immunohistochemical analysis of hepatic stellate cells, myofibroblasts, and hepatic apoptosis

Experiments were carried out in healthy rats, as well as in rats injected with either BDL + S (at 2w, 5w and 8w) or BDL+LGF (at 2w, 5w and 8w). Eight-Ìm sections from paraffin blocks were deparaffinated with xylene, and washed sequentially in 100%, 95% and 70% ethanol and PBS. To block endogenous peroxidase activity, they were treated with 0.3% hydrogen peroxide for 15 min in distilled water and subsequently blocked in normal donkey serum (1/50). Sections were incubated with monoclonal anti-α-SMA (mouse IgG2a, Sigma-Aldrich, Clone 1A4; dilution 1/200) overnight at 4°C. Primary antibodies were detected using either biotinylated donkey anti-mouse IgG (H+L) antibodies (1/150, 1.5 h) or the avidin-biotin peroxidase method (Vector Labs). Subsequently, the sections were reacted with 0.06% diaminobenzidine and 0.01% hydrogen peroxide for 10 min. Control sections, in which the primary antibody was omitted, were treated in the same way and showed no immunostaining. Slides were examined by light microscopy, and immunohistochemical findings were evaluated semi-quantitatively (3 rats/group, 40 fields examined/rat).

For the detection of apoptotic liver cells, 8-µm sections from 10% neutral buffered formalin-fixed liver samples were employed. Slides were permeabilized in PBS containing 0.2 mg saponin/ml and 20 µg proteinase K/ml for 20 min at room temperature. After being heated at 56°C in 50% formamide solution (v/v distilled water) at 3% hydrogen peroxide for 5 min and rinsed in PBS, the sections were incubated at room temperature for 30 min with anti-ssDNA mouse monoclonal antibody (dilution 1/100, Biomol International, LP, clone F7-26) and peroxide conjugated anti-mouse IgM. Nuclear counterstaining was performed with hematoxylin and cresyl violet (3 rats/group, 40 fields examined/rat).

Determination of the serum endothelin-1 concentration by ELISA assay

Serum levels of endothelin-1 were measured in all BDL rats included in this study (n=28) by a commercially available quantitative chemiluminescent ELISA (QuantiGlo Human Endothelin-1, R&D systems Inc., Minneapolis, MN), following the manufacturer's instructions. The minimum detectable level of endothelin-1 for this ELISA assay is 0.16 pg/ml. The intra- and inter-assay coefficient of variation for this immunoassay was <4% and <9%, respectively.

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

Evaluation of fibrogenic liver cells

Figure 1 shows the determination of liver α-SMA content (by western blotting, A) in the different rat groups. Bile duct ligation produced a substantial increase over controls, especially at longer times (0.85-fold at 2w, 8.77-fold at 5w, 5.82-fold at 8w), but rats injected with LGF showed a significant decrease with respect to the values of BDL+S rats at longer times (4.47 versus 8.77-fold at 5w, $p<0.05$; 3.0 versus 5.82-fold, at 8w, $p<0.05$). Assessment of HSC+ cell number was carried out by immunohistochemistry (Fig. 1B). HSC+ cells, nearly undetectable in healthy rats, showed a marked increase after BDL, but LGF caused a partial inhibition of this burst, easily detectable at longer times (57% with respect to BDL+S rats at 5w, $p<0.0001$; 84% with respect to BDL+S rats at 8w). Figure 1C shows a sample of rat liver tissue immunostained with anti-α-SMA (BDL+S, at 5w), and its counterpart (BDL+LGF, at 5w, 1D). Most of the α-SMA+ cells were located around portal tracts.

Measurement of apoptotic liver cells

The decrease in α-SMA+ cells produced by LGF in BDL rats could be due to a LGF-mediated effect that increased activated HSC/myofibroblast cell death by apoptosis; thus, to check this possibility, we performed an apoptosis determination assay. Figure 2A shows the number of apoptotic cells/field in every group analyzed, where a burst of apoptosis was detected in BDL rats 2w after operation, with lower values at later time points. In contrast, in LGF-injected rats, lower values were observed at every point studied, although the decrease was statistically significant only at 2w ($p<0.0001$). Although we did not perform double labeling of SMA and apoptosis, we estimated that about 80% of the apoptosis was detected in α-SMA+ cells. Thus, we concluded that the decrease in α-SMA detected in LGF-injected rats can not be ascribed to an increase in activated HSC/myofibroblast cell killing. Figures 2B and 2C show two examples of liver biopsies of BDL+S rats (B) and BDL+LGF rats (C) at 2w (some apoptotic cells are indicated by white arrows).

Evaluation of HSC number

As the aforementioned decrease in α-SMA+ cells after LGF injection could be explained by an inhibition of HSC growth, we determined liver desmin content

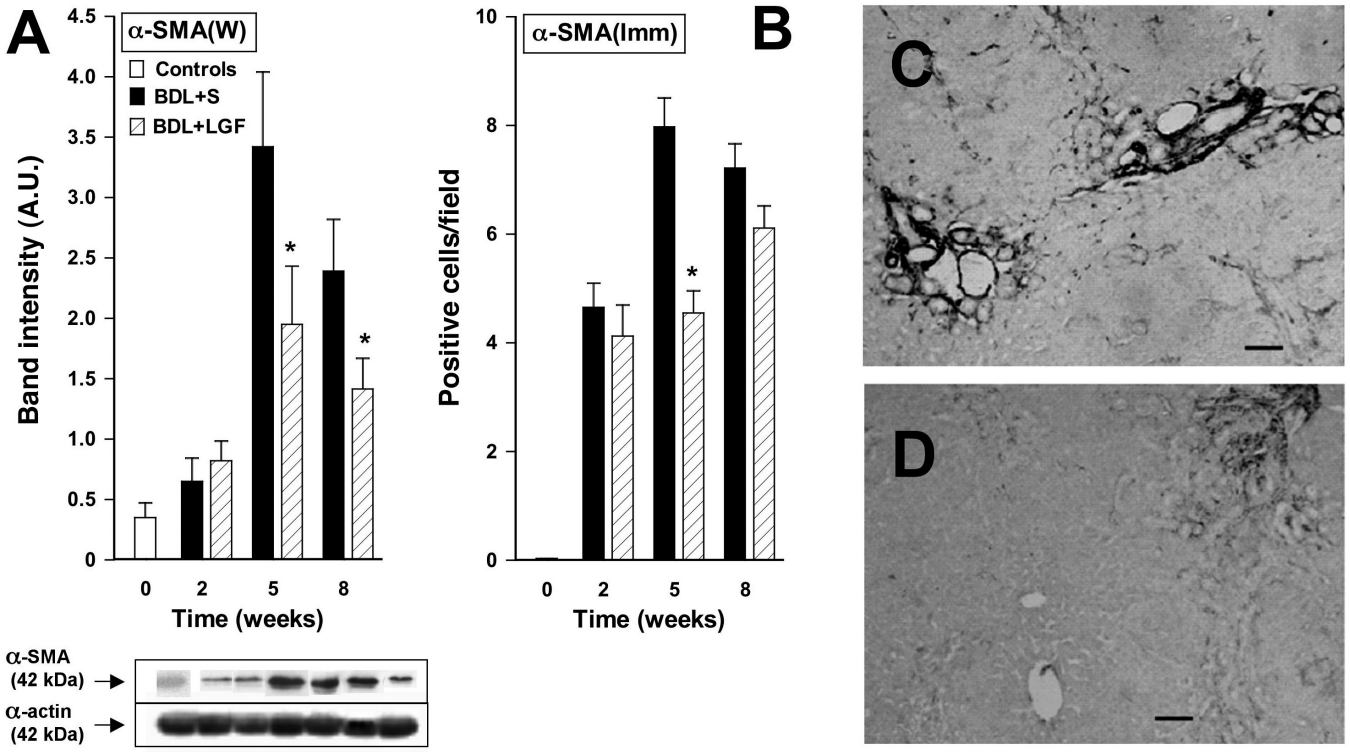

Fig. 1. Rat liverα-SMA content (western blotting, **A)**, number of α-SMA+ cells (immunohistochemistry, **B)** and immunodetection of α-SMA+ cells in different groups of rat livers (**C and D**). **A and B**. White bars show healthy control rats; black bars, BDL+S rats; dashed bars, BDL rats after LGF injection (BDL+LGF). Values are the mean±SD in every case in all figures. n=4 rats/group. *p<0.05 with respect to the corresponding value in BDL+S rats. α-SMA-positive cells were counted in 40 fields in three rats/group. **C.** BDL+S rat liver at 5w. **D.** BDL+LGF rat liver at 5w. Bar scale: 100 μm.

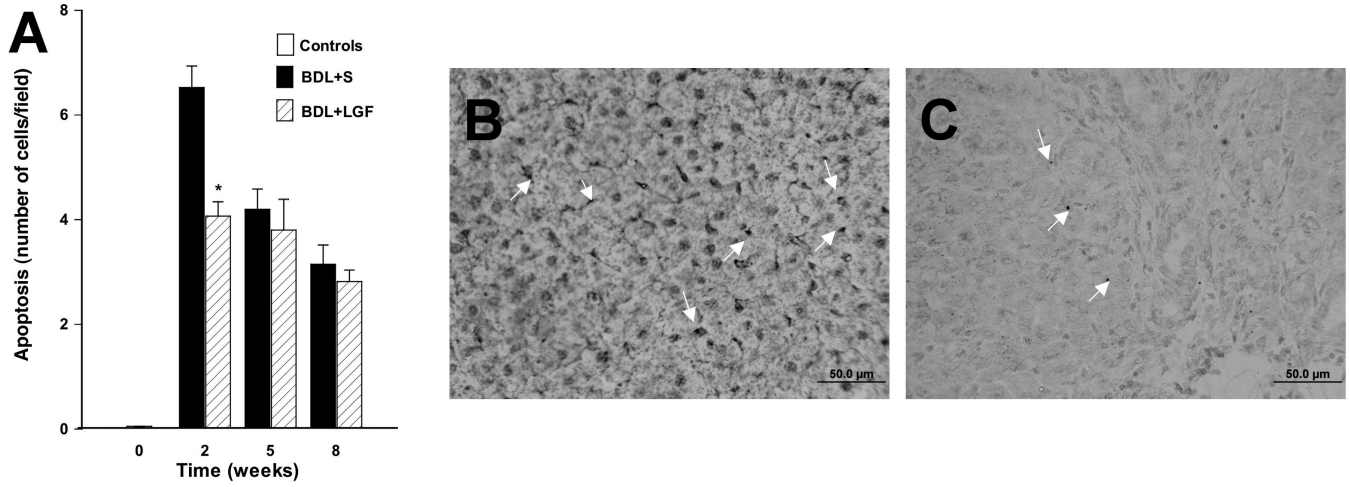

Fig. 2. Number of apoptotic cells in different groups of rats **(A)** and immunodetection of apoptotic cells in rat livers (**B and C**). **A.** Bars indicate the same groups as in Figure 1. Positive cells were counted in 40 fields in three rats/group. **B.** BDL+S rat liver at 2w. **C.** BDL+LGF rat liver at 2w. Some apoptotic cells are indicated by white arrows. Bar scale: 50 μm.

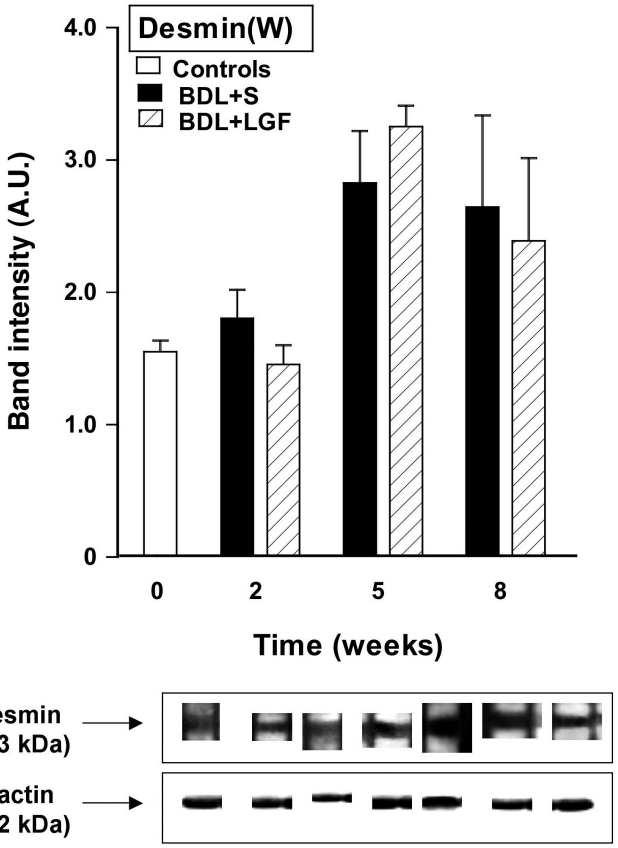

Fig. 3. Rat liver desmin content (western blotting) in different groups of rats. Bars indicate the same groups as in Figure 1.

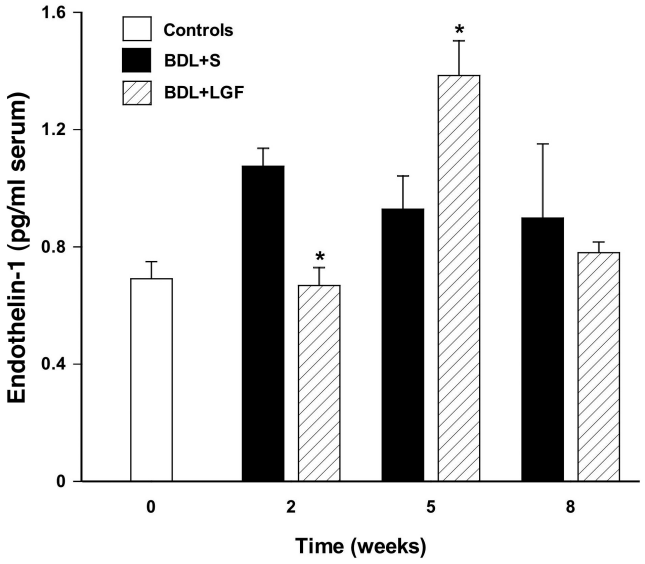

Fig. 4. Rat serum endothelin-1 concentration (ELISA) in the different groups of rats. Bars indicate the same groups as in Figure 1.

(Fig. 3). The western blotting experiments showed an increase at 5w and 8w in BDL+S rats with respect to healthy rats, but the values measured in BDL+LGF rats did not present a significant difference with this technique.

Serum levels of endothelin-1

The serum endothelin-1 concentration (Fig. 4) was clearly stimulated 2w after bile duct ligation (1.07 versus 0.69 pg/ml in healthy rats) and was nearly constant at longer times (0.92 pg/ml at 5w and 0.89 pg/ml at 8w). Interestingly, LGF seemed to exert an inhibition at short times (0.66 versus 1.07 at 2w, $p<0.003$), after which a considerable stimulation (1.38 versus 0.92 at 5w, $p<0.03$) was observed.

Discussion

In a previous publication (Díaz-Gil et al., 2008), we demonstrated the antrifibrotic activity of LGF in BDL rats. In these experiments, the inhibitory effect on the synthesis of certain fibrogenic mediators (MMP-2 and 9 and TGF-ß1) seemed to suggest that LGF modulates the process of HSC activation. Thus, in the present study, we have tried to demonstrate the action of LGF on this key process of activation/transformation of cells secreting substances that contribute to fibrosis.

Two types of cells are involved in the production of fibrosis in the liver: quiescent HSC, which, upon activation by liver injury, change their phenotype into ECM-secreting myofibroblasts, a process termed transdifferentiation (Wu and Zern, 2000). Additionally, liver myofibroblasts, located in periportal and perivenous areas in normal liver, migrate to the site of hepatic injury and undergo an activation process, acquiring a secretory phenotype (Kim et al., 2005). Both activated HSC and myofibroblasts express markedly elevated levels of α-SMA as a characteristic cytoskeletal protein, which is considered a marker of these fibrogenic cells. In this study, BDL rats treated with LGF showed both a significant decrease in liver α-SMA content and a lower number of hepatic α-SMA+ cells as compared with untreated BDL rats (Fig. 1A,B), effects that were especially evident at longer times, 5w and 8w, far beyond the point of irreversible liver damage, proposed to be 4w by other authors (Wensing and Branch, 1990; Zimmermann et al., 1992; Aronson et al., 1993). These findings strongly suggest that LGF could inhibit the HSC activation process.

Several substances, such as hepatocyte growth factor (HGF), have been reported to have antifibrotic effects. This protein increases apoptosis of liver myofibroblasts (Kim et al., 2005) and blocks transition of bile duct epithelial cells to mesenchymal cells (Xia et al., 2006). Similarly, a semisynthetic analogue of fumagillin, TNP-

470, inhibits HSC proliferation and activation (Wang et al., 2000). Searching for an explanation for the antifibrotic effect of LGF, we measured apoptosis (Figure 2) in BDL rats, injected with or without LGF. In LGF-treated animals, we observed a decrease in apoptotic cells that was especially evident at 2w (about 80% of apoptotic cells were α-SMA+), so we concluded that this effect of LGF was not due to an induction of either HSC or myofibroblast cell killing by apoptosis, and that a partial inhibition of HSC and myofibroblast activation mediated by LGF would be the most logical explanation. This notion is supported by our previous demonstration that LGF induces production of hepatic TNF-α (Díaz-Gil et al., 2003), a cytokine that could be partly responsible for the aforementioned LGF-mediated effect in BDL rats, as TNF-α inhibits both apoptosis and proliferation of activated rat HSC (Osawa et al., 2005). Since another possible explanation for the low number of hepatic α-SMA+ cells could be an inhibition of HSC proliferation, we determined the liver desmin content as a marker of HSC. As Figure 3 did not show a difference between LGF-injected and uninjected BDL rats at any point, we concluded that LGF did not seem to influence HSC growth rate. The hypothesis of a possible LGF-mediated decrease in myofibroblast proliferation has not been explored in this study.

Endothelin-1 is a vasoconstrictor overproduced by both sinusoidal endothelium and HSC after bile duct ligation. Although some authors have identified growth inhibitory properties of endothelin-1 in human myofibroblasts in vitro (Mallat et al., 1995), its potent effects on HSC activation and α-SMA expression have been well defined (Rockey et al., 1998). Taking all these effects into account, it seems that its stimulation right after bile duct ligation could be related to HSC activation/proliferation, and that its action after longer times could be related to its activity as a vasoconstrictor, in relation to the development of portal hypertension (Tièche et al., 2001). In this study, LGF-treated rats had significantly lower serum endothelin-1 levels than untreated rats, but only at early stages after bile duct ligation (2w), showing higher levels at 5w (Fig. 4). These data suggest that the low serum endothelin-1 concentrations observed at early stages after bile duct ligation might be a consequence of LGF-induced inhibition of HSC proliferation.

On the other hand, BDL produces a very significant increase in serum LGF concentration a few minutes after surgery that persists throughout the entire experimental period. This LGF in serum is partially responsible for the enormous increase in liver weight of rats after BDL (from a ratio of liver weight/bw of 3.56% in controls to 6.91% in 5w-rats), in spite of high amounts of endogenous toxins after reversal of bile flow. This endogenous LGF is partially inhibited, possibly bound to an unknown inhibitor of low molecular weight that we discarded in the process of LGF purification. We hypothesized about its existence some years ago (Díaz-Gil et al., 1986a). In this context, the injected LGF, although quantitatively lower than the endogenous LGF, had a pronounced effect on regeneration and fibrosis. To date, we are unable to determine the influence of the injected LGF on endogenous LGF in quantitative terms.

After BDL, TNF-α concentrations in serum and tissue increase dramatically, and remain very high as long as the reversed bile flow persists (Luo et al., 2004; Fernández-Martínez et al., 2006; Cindoruk et al., 2007). On the other hand, the increase in TNF-α expression caused by LGF is only detected locally (in endothelial cells located around the portal vein) in liver tissue, there being no detectable increase in normal rat serum (Díaz-Gil et al., 2003). In this context, we consider that an experiment to study the increase in TNF-α (either the tissue expression or the serum concentration) induced by LGF in BDL rats would not add any relevant information to our results.

An interesting point is the cell type on which LGF exerts its action. LGF injection induces significant DNA and liver cell regeneration, but the only type of liver cell studied in quantitative terms was the hepatocyte (see above references). To achieve a balanced regeneration of the injured liver, we think that LGF must have an influence on the proliferation rate of other liver cell types. However, with the exception of the observed stimulation of endothelial cells induced by LGF (Díaz-Gil et al., 2003), as well as its inhibition of HSC and its aforementioned antifibrotic effect, we have not studied this point in particular.

Concerning extrahepatic sites, we observed a substantial angiogenic activity of LGF in testis, where it increases VEGF2 (Martín-Hidalgo et al., 2007), and subcutaneous action in mice (data not published). On the other hand, LGF induces proliferation of microglia and outgrowth of tyrosine hydroxylase-terminals in a model of Parkinson's disease (Reimers et al., 2006). Likewise, LGF stimulates proliferation of smooth muscle cells in carotid wall of hypertensive rats (Somoza et al., 2006). The actions of LGF on different cell types are really very complex, and the objective for the near future should be to establish a more complete perspective that will provide a comprehensive explanation.

In conclusion, the antifibrotic effect of LGF in BDL rats seems to be exerted at two different levels, one of which involves the modulation of the activation state of HSC and myofibroblasts studied in this work; the inhibition of TGF-ß was demonstrated in a preceding study (Díaz-Gil et al., 2008). The molecular bases of these LGF-mediated effects are currently under investigation.

The use of LGF as a pleiotropic factor for cell and tissue regeneration is presently under international patent application.

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