

Endoglin Upregulation in the Liver after Bile Duct Ligation in Rats

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Abstract: *Background:* Transforming growth factor beta 1 (TGF- β 1) has been implicated in the stimulation of extracellular matrix synthesis in acute and chronic liver disease. Endoglin (CD-105) is a membrane glycoprotein that binds TGF- β 1 with high affinity. Endoglin is overexpressed in several model of fibrosis. The goal of the present study was to evaluate the effect of bile duct ligation (BDL) on endoglin expression and the effect of endoglin overexpression on liver fibrosis in rats.

Methods: Rat livers were transfected with a vector containing full length human endoglin (h-end) or an empty vector (mock) and 48 hours after were subjected to either BDL or sham operation (SO). Eighteen days after, a blood sample was obtained and bilirubin and serum enzyme activities were measured to assess liver damage. Liver fibrosis was quantified by a computer-assisted image analysis of Sirius red stained livers and by liver hydroxyproline content. Endoglin expression in the liver tissue was assessed by Western blot and immunohistochemistry.

Results: Both immunohistochemistry and Western blot reveals endoglin upregulation after BDL in rats. In addition, these techniques also reveal effective human endoglin transfection in the rat's liver. After BDL, liver fibrosis and plasma levels of enzymes were similar in h-end transfected and mock-transfected rats. Western blots showed higher endoglin expression after BDL in h-end transfected and mock-transfected rats.

Conclusions: The present study provides clear evidence that endoglin is upregulated in the liver of rats with BDL. h-end overexpression did not improve liver fibrosis after BDL in rats.

Keywords: Bile-duct ligation, endoglin, liver cirrhosis, liver fibrosis, transfection, transforming growth factor-beta.

BACKGROUND

Transforming growth factor- β (TGF- β), a regulator of cell growth and differentiation, plays a central role in the response to injury. Active TGF- β binds to specific, high-affinity receptors present on most cells, initiating a signalling cascade that results in many biological effects, including production of cytokines and inflammatory mediators, stimulation of extracellular matrix (ECM) synthesis and inhibition of ECM degradation [1-5]. Two signalling receptors, termed type I and type II, mediate the biologic actions of TGF- β . The extracellular domain of the type II receptor [6] binds the ligand, causing formation of heteromeric complexes incorporating type I and type II receptors [7]. The type II receptor then transphosphorylates the type I receptor, activating its kinase and initiating downstream signalling [7]. Thus, the type II receptor appears to be essential for the biological

activity of TGF- β *in vivo* [7-10]. In several organs, repeated or prolonged injury leads to progressive fibrosis and, ultimately, to the development of excessive, unwanted scarring. The late stage of this process in the liver is termed cirrhosis. TGF- β appears to have a major regulatory role in this process, as shown both in animal models [11-14] and human hepatic injury [15-17]. Similarly, transgenic mice overexpressing TGF- β 1 and adenovirus-mediated gene transfer of TGF- β are characterized by fibrosis in many organs including the liver [18, 19].

Endoglin, also known as CD105, is a 180-kD homodimeric membrane glycoprotein expressed by human endothelial cells [20, 21], macrophages [22], vascular smooth muscle cells [23], mesangial cells [24] and fibroblasts [21]. Endoglin binds TGF- β 1 and TGF- β 3 with high affinity in human endothelial cells [25], in association with the type II receptor [26]. We have reported that endoglin is upregulated in several models of renal fibrosis in rats and mice [27, 28]. Endoglin has been also shown to be upregulated in other fibrotic tissues [29, 30].

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Bile duct ligation (BDL) is an experimental manoeuvre that leads to liver fibrosis, a process likely to be mediated by TGF- β 1 overexpression [31, 32]. The goal of the present study was to evaluate the effect of BDL and the subsequent liver fibrosis on endoglin expression in rats. As we have previously shown that endoglin is able to counteract the profibrotic effects of TGF- β 1 [24], we have assessed if endoglin overexpression had a beneficial effect on liver fibrosis after BDL.

METHODS

Animals

Male Wistar rats weighing initially around 250 g born and raised in the animal facilities of the Universidad de Salamanca were used in the present study. Rats were housed in standard cages in a room controlled for daylight (12 h), temperature (20° C) and humidity (60%) and maintained on a standard rat pellet diet. All experimental protocols were performed according to the guidelines for the ethical treatment of the animals, as specified by the European Union (86/609 and 98/81) and the Ministerio de Agricultura, Pesca y Alimentación de España.

Experimental Groups

The animals were distributed in four experimental groups. MOCK-SO: sham operated rats and transfected with transfection vehicle (n= 7). MOCK-BDL: rats with bile duct ligation and transfected with vehicle solution (n= 12). END-SO: rats subjected to a sham operation then transfected with endoglin (n= 7). END-BDL: rats with bile duct ligation and transfected with endoglin (n= 10).

Liver Endoglin Gene Delivery

The pCMV plasmid, derived from the pCMV5 vector, and containing the human pCMV promoter, a universal intron, and downstream polyadenylation signals, has been previously described [33]. The pCMV-EndoL vector, containing the human L-endoglin isoform driven by the cytomegalovirus promoter, was constructed by inserting the 2.3-kb *Eco*RI endoglin cDNA, obtained by digestion of pcEXV-EndoL, into the *Eco*RI digested pCMV vector (Invitrogen) as previously described [34]. Liver transfection with the pCMV-EndoL vector (h-end transfection) containing the human L-endoglin or the pCMV plasmid without endoglin (Mock-transfection) was performed as previously described [35]. In brief, rats were anaesthetised with 2-2-2 tribromoethanol and a small left subcostal incision was performed, the spleen was exposed and punctured with a PE-50 catheter [36]. 20 μ g of plasmid (at a ratio of 1 μ g of DNA/ μ l of TransIT polymer) in a final volume of 1 ml was slowly infused during 3-5 min.

Bile Duct Ligation

After 48 hours, rats were anaesthetised with 2-2-2-tribromoethanol and, under aseptic techniques, subjected to either bile duct ligation (BDL) and excision or sham operation (SO), as previously described [37].

Eighteen days later rats were anaesthetized with 2-2-2-tribromoethanol. After opening the abdomen *via* a midline incision, the abdominal aorta was punctured and 5 ml of blood obtained in heparinized tubes. Plasma and cells were

separated by centrifugation in refrigerated conditions. Perfusion with saline was then performed through the abdominal aorta to remove blood from the body. A section of liver tissue was trimmed down and used for histochemical studies, hydroxyproline determinations and Western blot studies.

Biochemical Analysis

The activities of alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase (EC 3.1.3.1.) and the concentration of total bilirubin in plasma were determined by standard auto-analyser methods on a CIBA CORNING 550 Express. Liver hydroxyproline determinations were performed as previously described by us [38].

Histological Studies

A piece of the liver was trimmed down and fixed by immersion in 4% buffered formalin for 24 hours. After dehydration, pieces were embedded in paraffin. Sections 3 μ m thick were cut, mounted on glass slides and processed either for Masson's Trichrome or immunohistochemistry for light microscopy analyses.

Immunohistochemistry was performed on buffered formalin fixed, paraffin-embedded tissues. Briefly, 3 μ m sections were deparaffined in xylene and rehydrated in graded ethanol before staining with the peroxidase-anti peroxidase method. Endogenous peroxidase was blocked by incubation in 3% hydrogen peroxide.

For the detection of endoglin, a monoclonal mouse anti-human CD105, clone SN6h (1:400 dilution, 1 hour; DAKO CA, USA) was used. Following washes in PBS, the sections were sequentially incubated with the DAKO LSAB+HRP 2 system and finally 3,3'-diaminobenzidine (DAB) was used as chromogen.

For TGF- β 1 a polyclonal rabbit anti-human antiserum (1:50 dilution, 1 hour; Sta. Cruz Biotechnology, Ca, USA) was used. The sections were then rinsed in PBS and the avidin-biotin-complex immunoperoxidase method (rabbit ABC Staining System, Santa Cruz Biotechnology, CA, USA) that uses 3,3'-diaminobenzidine as a substrate were used.

Slides from both techniques were counterstained with hematoxylin, washed in water, dehydrated and mounted with a coverslip using Tissue-Tek (Miles Inc. IN, USA). Negative controls were prepared without primary antibody.

Morphometric studies were performed on 5 μ m sections by means of a computer-based image analysis system. In brief, sections were stained with Sirius red. Images were captured by a high-resolution videocamera (SONY ccd-iris) connected to a light microscope (Leitz Laborlux S) through a green optical filter (IF 550), using the 20x objective. The evaluation and image analysis procedures were performed with specific software developed on the Visilog 4.1 image analyses program (Fibrosis HR. Master Diagnostica. Granada. Spain) whose application to assess liver fibrosis has been previously described by us [38]. A total of 15 images of each liver slide were captured and processed. For bile duct-ligated groups, two sets of images were captured and analyzed separately: one of periportal and septal areas and another of parenchymal areas. For each image, the values of

fibrosis area were obtained in μm^2 from the morphometric analysis. These values, saved in data ASCII files conveniently labeled, can be exported to any statistical analysis program and calculate several parameters that can express the degree of liver fibrosis in an objective and quantitative way.

Because hepatic fibrosis is a focal phenomenon and because it is possible to observe fields with markedly different degrees of lesion, the results of the image analysis study were expressed as frequency distribution diagrams in order to give more information on data dispersion.

Western Blot

Hepatic cell membranes were obtained by differential centrifugation of homogenized powdered tissue. The protein content was determined by a commercially available variant of the Lowry method (Bio-Rad) using BSA as standard. Protein samples were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to PVDF membrane (Pall-Gelman). Membranes were blocked with blocking buffer and incubated for 90 minutes with three different mouse anti-human endoglin monoclonal anti-bodies: P3D1, that reacts with the N-terminal region of 204 amino acids encoded by exons 1 to 5 [39]. P4A4 that binds to a region of 54 amino acids encoded mostly by exon 7 [39] and TEA1/62.2 [40]. Membranes were washed, incubated for 30 minutes in standard buffer containing horseradish peroxidase-labeled goat anti-mouse IgG (Bio-Rad Laboratories) diluted 1:20000 and washed extensively. The presence of human endoglin dimers, with a characteristic band of 180 kDa, was detected using a chemiluminescence assay (ECL detection reagents, Amersham) and recorded on an x-ray film. As a load control we have used tubulin and α -actin. However, both proteins increased after BDL. Thus, we have chosen to carefully load the same amount of proteins per lane. Intensity of the bands in the film were quantified by densitometry using Scion Image software (Scion Corporation, Frederick, Maryland USA).

Statistical Analysis

One-way analysis of variance was performed using the NCSS program (V 6.0.10). Values from data with a normal distribution were expressed as mean \pm EEM with the Scheffe's correction test employed for multiple comparison. For data not conforming to a normal distribution, values were expressed as median and the Kruskal-Wallis Z value test was used for multiple comparison. $P < 0.05$ or $Z > 1.96$ were considered statistically significant.

RESULTS

Data on body weight, liver weight and survival is shown in Table 1. Body weight increase was higher in non-ligated (MOCK-SO = 91 g; END-SO = 95 g) than in bile duct ligated rats (MOCK-BDL = 71 g; END-BDL = 61 g). Liver weight and hepatosomatic ratio was higher in ligated than in non-ligated rats. BDL rats had a lower survival than sham-operated rats.

Effect of BDL on Endoglin Expression

No endoglin expression in the liver was detectable in MOCK-SO animals by immunohistochemistry using the SN6h anti-human endoglin antibody (Fig. 1A). After BDL (MOCK-BDL rats), endoglin is clearly expressed in non-parenchymal cells. In addition, a clear expression was also observed in fibrosed areas around areas of ductular proliferation (Fig. 1C).

Fig. (2A) shows a representative western blot performed in liver extracts from MOCK-SO and MOCK-BDL rats, and revealed with the P4A4 anti-endoglin antibody, that recognize both human and rat endoglin. Fig. (2B) shows the quantification (in arbitrary units, and corrected by tubulin content of 8 samples per group) in two different blots. We can observe that MOCK-BDL rats had higher endoglin liver content than MOCK-transfected rats. Similar results were obtained by using other two different anti-endoglin antibodies, P3D1 and TEA (data not shown).

Efficiency of Endoglin Transfection

While in MOCK-SO rats endoglin expression by immunohistochemistry was almost undetectable by immunohistochemistry using the SN6h anti-human endoglin antibody (Fig. 1A and insert), rats transfected with human endoglin (END-SO) showed a diffuse expression of endoglin in the liver, mainly in sinusoid endothelial cells (Fig. 1B and insert). The endoglin expression in END-BDL animals (Fig. 1D) was higher than the expression in MOCK-BDL rats (Fig. 1C). In both groups, endoglin expression was strongly detected in sinusoid endothelial cells and in conserved and altered hepatocyte plates.

Western blots studies using the P4A4 antibody also reveals that endoglin expression was higher in END-SO than in MOCK-SO group (Fig. 3). Furthermore, endoglin content was higher in the END-BDL than in MOCK-BDL group (Fig. 3). Similar results were obtained by using other two different anti-endoglin antibodies, P3D1 and TEA (data not shown).

Table 1. Evolution of Body Weight (BL: before Bile Duct Ligation; AL: after Bile Duct Ligation), Liver Weight and Hepatosomatic Ratio (H.R.) at the end of the Experiment and Survival in Bile Duct Ligated and non Ligated Rats

	Body Weight		Liver Weight	H. R.	Survival
	BL	AL			
MOCK-SO	241 \pm 10	332 \pm 12 ^a	12.7 \pm 0.7	0,038 \pm 0,001	100%
MOCK-BDL	250 \pm 8	321 \pm 8 ^a	17.4 \pm 1.1 ^b	0,054 \pm 0,004 ^b	59%
END-SO	234 \pm 2	329 \pm 6 ^a	12.4 \pm 0.4	0,037 \pm 0,001	100%
END-BDL	238 \pm 11	299 \pm 20 ^a	17.5 \pm 1.2 ^b	0,058 \pm 0,008 ^b	50%

Values are means \pm EEM. Statistical significance: ^a: $p < 0.05$ vs BL; ^b: $p < 0.05$ vs SO.

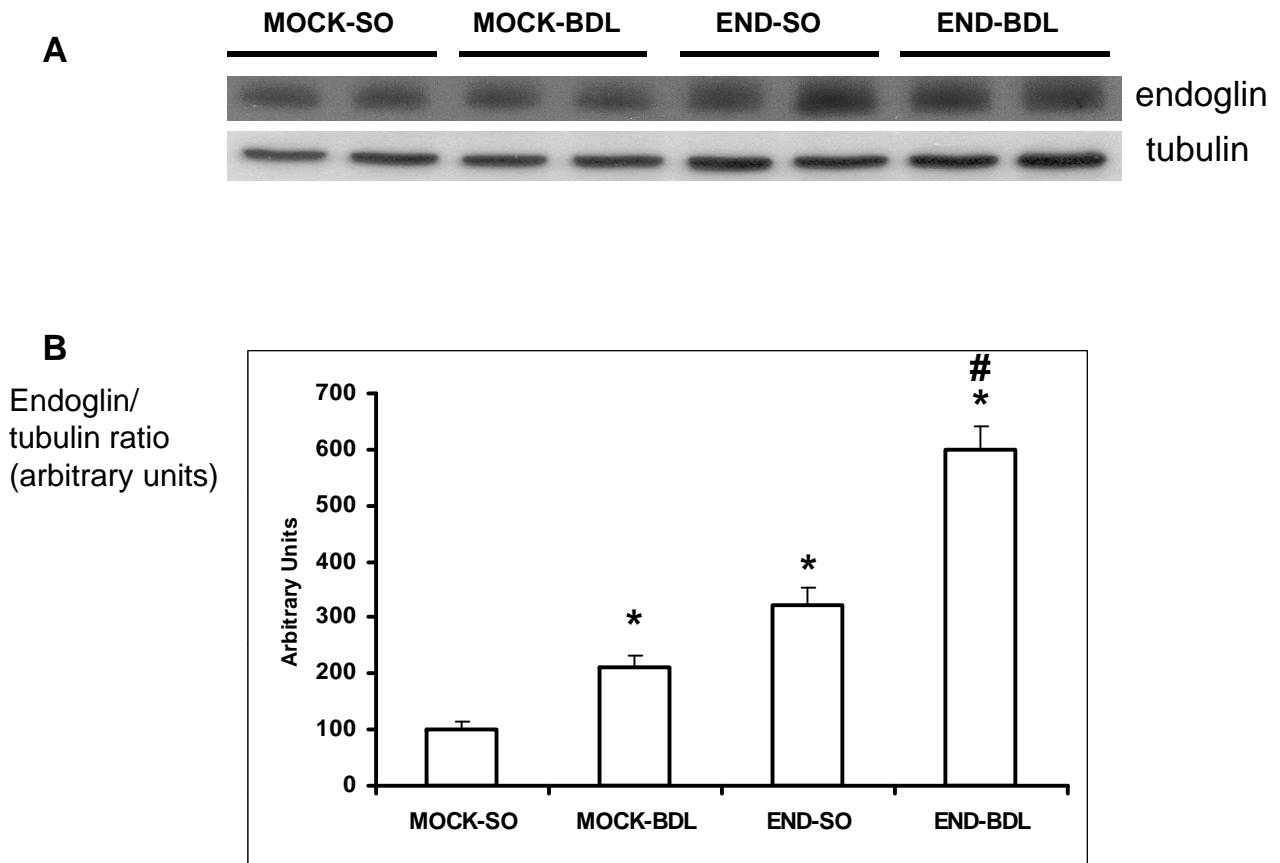


Fig. (3). (A). Representative Western blot analysis of endoglin in livers from the different groups. (B). Bar graphs showing the quantification (mean \pm standard error of the mean) of optical densities of 8 samples per group in two different gels, corrected by tubulin expression. Abbreviations: MOCK-SO: sham operated rats transfected with vehicle solution. END-SO: rats subjected to a sham operation then transfected with endoglin. MOCK-BDL: rats with bile duct ligation transfected with vehicle solution. END-BDL: rats with bile duct ligation transfected with endoglin. * $p < 0.05$ vs MOCK-SO; # $p < 0.05$ vs END-SO.

Effect of Endoglin Transfection in Liver Fibrosis

Liver damage was evaluated by measuring plasma concentration of total bilirubin, alkaline phosphatase, aspartate aminotransferase, and alanine aminotransferase (Fig. 4). No significant differences were observed between END-SO and MOCK-SO groups in plasma concentration of total bilirubin, alkaline phosphatase, aspartate aminotransferase and alanine aminotransferase. Bile duct ligation increased serum bilirubin concentration and the levels of enzyme activities but there were not significant differences between MOCK-BDL and END-BDL groups.

In MOCK-SO and END-SO, TGF- β 1 is expressed in portal extracellular spaces and a tiny expression is detected around sinusoids (Fig. 1E and 1F). In MOCK-BDL group, TGF- β staining was observed in the same places and is extended to fibrotic areas around ducts newly formed (Fig. 1G). END-BDL rats showed a TGF- β 1 staining distribution quite similar to that expressed in mock animals, but in this case it is possible to see TGF- β 1 around hepatocyte plates, mainly where ductular metaplasia is observed (Fig. 1H).

Liver fibrosis has been assessed by different approaches: conventional histological staining, computer assisted image analysis and hydroxyproline content. Light microscopy analysis of slides stained either with hematoxylin and eosin and Masson's trichrome showed that livers from MOCK-SO had a normal pattern with no evident alterations (Fig. 5A).

No alterations in liver structures were observed in END-SO animals (Fig. 5C). Animals from MOCK-BDL group showed proliferation of portal and periportal biliary ductules. In these areas the ducts newly formed had dilated lumens and irregular epitheliums with peripheral intense fibrosis (Fig. 5B). Similar structural changes were observed in END-BDL group (Fig. 5D). However in these animals the loss of plate structure and the diffused hepatocyte, ductular metaplasia with fibrosis were more severe.

Fig. (6A) showed the computer assisted image analysis of Sirius red staining. It is observed that rats subjected to BDL had a markedly increased in fibrotic areas as compared with non-ligated animals, but no significant differences were observed when compared END-BDL-S and MOCK-BDL-S groups between them. No differences are seen when compared MOCK-SO and END-SO, however if compare MOCK-SO and parenchymal zones of ligated animals (MOCK-BDL-P and END-BDL-P) differences are detected. END-SO group has no differences with END-BDL-P group, however, a significant difference is observed when compared with MOCK-BDL-P.

To evaluate the amount of collagen present in liver tissue in a quantitative manner, the hydroxyproline content measured in END-BDL rats was 3.6 higher than in END-SO animals. The hydroxyproline content in MOCK-BDL rats was 3.2-fold higher than in MOCK-SO group (Fig. 6B).

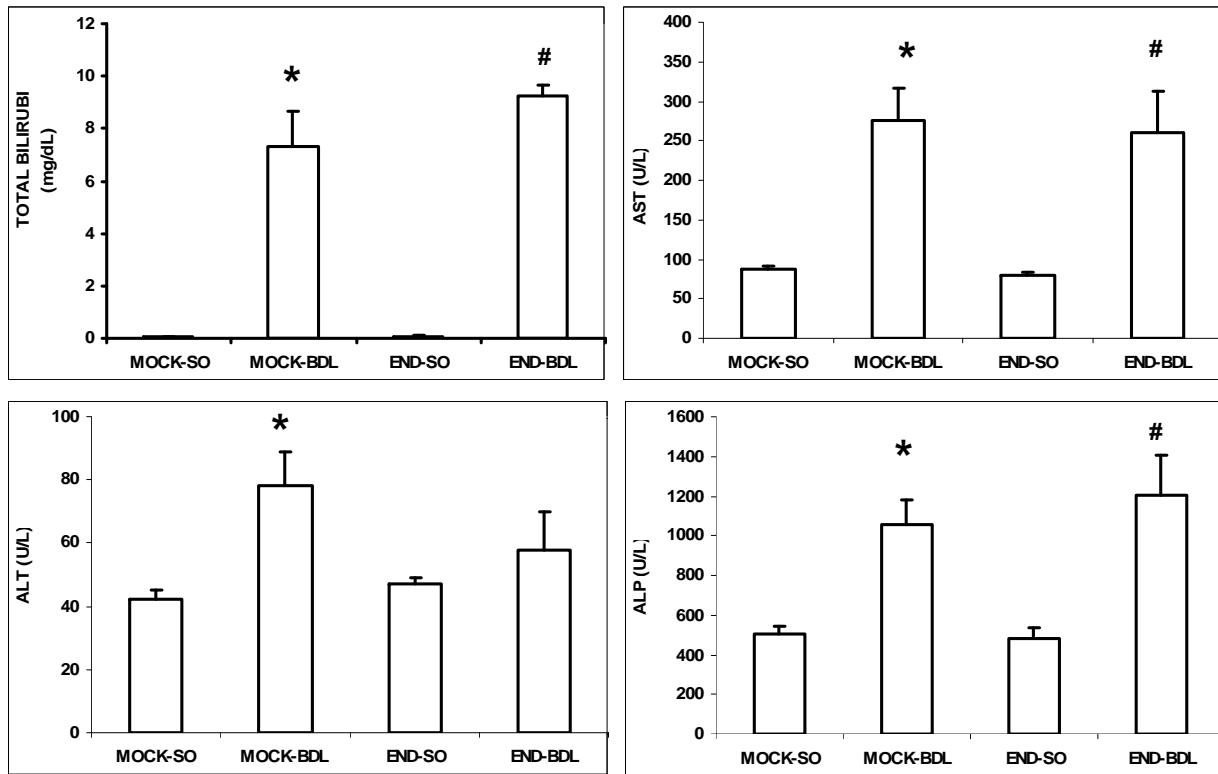


Fig. (4). Plasma levels of total bilirubin and liver enzymes activities. Groups are MOCK-SO: sham operated rats and transfected with vehicle solution (n= 7). MOCK-BDL: rats with bile duct ligation and transfected with vehicle solution (n= 12). END-SO: rats subjected to a sham operation then transfected with endoglin (n= 7). END-BDL: rats with bile duct ligation and transfected with endoglin (n= 10). ALP (alkaline phosphatase), ALT (alanine aminotransferase) and AST (aspartate aminotransferase). Values are means \pm SEM. * $p < 0.05$ vs MOCK-SO rats, # $p < 0.05$ vs END-SO rats.

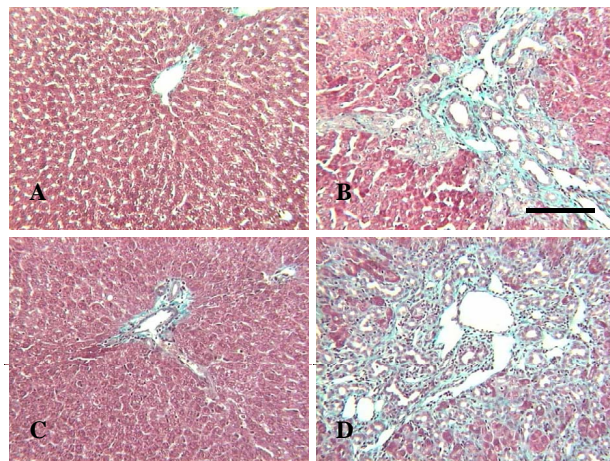


Fig. (5). Light microscopy photomicrographs showing representative images of liver slices stained with Masson's trichrome corresponding to MOCK-SO: sham operated rats and transfected with vehicle solution (A). END-SO: rats subjected to a sham operation and transfected with endoglin (B). MOCK-BDL: rats with bile duct ligation transfected with vehicle solution (C). END-BDL: rats with bile duct ligation transfected with endoglin (D). Bar is 100 μ m.

DISCUSSION

Experimental bile duct ligation (BDL) serves as an excellent model of hepatic fibrosis. In our study, the amount of collagen in liver parenchyma, quantified by liver hydroxyproline concentrations, was increased eighteen days after obstruction. Serum bilirubin and liver enzymes were

also significantly increased. These results are similar to those previously reported for the same model and experimental period [41, 42]. The increase in body weight was in the range previously described by others authors [42-44], and could be related to the marked elevation in liver weight, as indicated by the hepatosomatic ratio. Both collagen accumu-

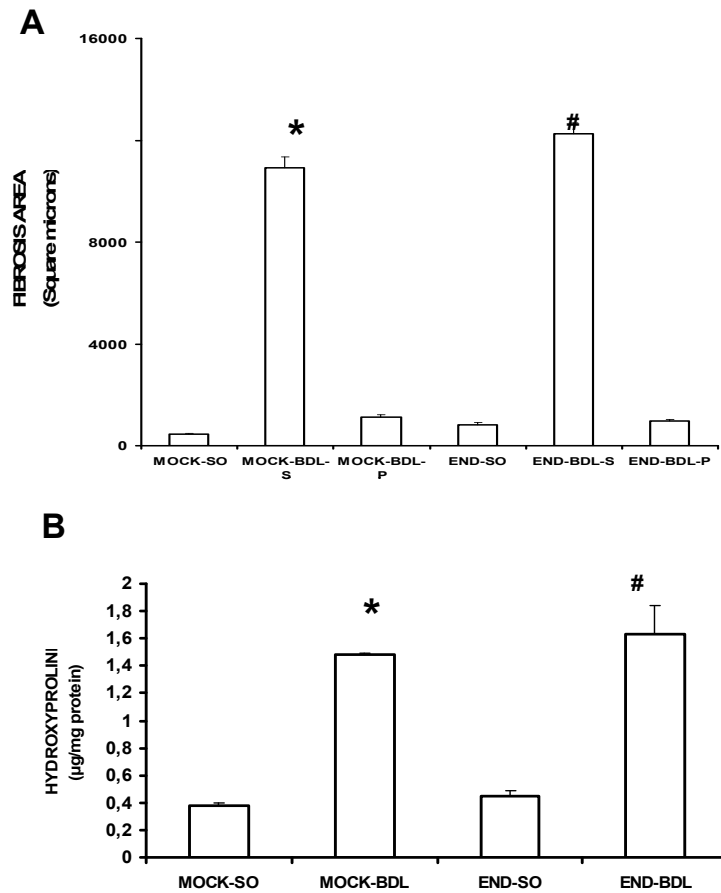


Fig. (6). (A) Morphometrical quantification of liver fibrosis in the septum (S) and parenchyma (P). (B) Hydroxyproline content in the liver of the different experimental groups. Abbreviations: MOCK-SO: sham operated rat transfected with vehicle solution. MOCK-BDL: rats with bile duct ligation and transfected with vehicle solution. END-SO: rats subjected to a sham operation then transfected with endoglin. END-BDL: rats with bile duct ligation and transfected with endoglin. Data are means \pm SEM. * $p < 0.05$ vs MOCK-SO. # $p < 0.05$ vs END-SO.

lation and bile duct proliferation would thus contribute to changes in liver weight.

This study also detected a marked increase in hepatic endoglin expression after BDL. These results agree with previous studies reporting endoglin overexpression in animal models of organ fibrosis [27, 28]. After BDL, endoglin is clearly expressed in non-parenchymal cells. In addition, a clear expression was also observed in fibrosed areas around areas of ductular proliferation. These data agree with the observation of Medina *et al.*, [45] who have observed in biopsies from patients with primary biliary cirrhosis that endoglin was expressed in sinusoidal endothelial cells, particularly in periportal areas, in association with the inflammatory infiltrates.

The mechanism responsible for the increase in endoglin expression is not clear but could be related to TGF- β 1 overexpression. Animal models of experimental liver fibrosis, such as carbon tetrachloride-induced fibrosis [46], bile duct ligation [31] or schistosomiasis infection [11] have reported increased concentrations of TGF- β 1 very early in the course of fibrogenesis in these animal models. TGF- β 1-induced endoglin expression has been shown in cultured human monocytes and in the U-937 monocyte line [47], and in cultured mesangial cells [24]. Whereas mesangial cell is major profibrogenic cell in the kidney, hepatic stellate cells is the

most profibrogenic cell type in the liver, and endoglin has been reported to be present in hepatic stellate cells, which are a target for TGF- β 1 [48]. Thus, it could be suggested that increased endoglin expression in livers with BDL may occur as a consequence of the increase in TGF- β 1. However, in our study, we detect endoglin mainly in endothelial cells that are the cells with the higher endoglin content, and also respond to TGF- β 1 increasing endoglin expression [49].

About the role of increased endoglin expression in liver fibrosis, previous studies have obtained preliminary evidence that endoglin overexpression can reduce the effects of TGF- β 1 on extracellular matrix synthesis and cell proliferation [34, 47, 50]. Thus, one might expect that the increased expression of endoglin by liver tissue would contribute to a reduction in the amount of TGF- β 1-induced ECM production. Thus, to test this hypothesis, the next purpose of our study has been to assess the effect of endoglin overexpression in the liver on BDL-induced liver fibrosis. For this purpose we have performed specific liver transfection as previously described by us [35] with a plasmid containing full-length human endoglin (h-end) cDNA. The effectiveness of endoglin transfection has been assessed by either Western blot or immunohistochemistry. Western blot using several antibodies against different epitopes of the molecule, demonstrated that endoglin expression was higher in h-end- than in

mock-transfected rats. Immunohistochemistry using an anti-human endoglin antibody revealed almost no stain in mock-transfected. In h-end transfected rats, immunohistochemistry revealed the presence of endoglin in the sinusoidal endothelial cells, thus demonstrating the efficacy of the transfection method.

However, our results demonstrate that an increased endoglin expression do not imply an improvement neither in liver function nor in liver fibrosis after BDL, when compared with mock-transfected rats. In other experimental model of organ fibrosis such as unilateral ureteral obstruction [27] results shown that the absolute level of endoglin is not critical for the renal fibrosis process. This negative result might be explained by the fact that endoglin and TGF- β 1 protein and receptors are not coexpressed in the same cell type. Roulot *et al.*, [32] show a dissociation of the antiproliferative and profibrogenic effects of TGF- β 1 that may be determined at the level of the receptor. The TGF- β 1 type II receptor is down-regulated during liver fibrosis, likely as a direct consequence of increased TGF- β 1 release. Growth-inhibitory effects of TGF- β 1 are reduced commensurately, but profibrogenic effects are maintained. This uncoupling of the effects of TGF- β 1 probably reflects quantitative changes in TGF- β 1 receptor signaling. The down-regulation of the T β RII in smooth muscle cells derived from human atherosclerotic lesions is associated with loss of an antiproliferative response to TGF- β 1 but an overproduction of ECM components [51].

In conclusion, our results shown that BDL induces endoglin overexpression in rat liver. In addition, the specific liver overexpression of endoglin induced by human endoglin overexpression has not beneficial effects on liver fibrosis induced by BDL in rats.

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