

Association of Transforming Growth Factor- β 1 and α -Smooth Muscle Actin in Experimental Selective Obstructive Cholestasis

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Abstract

Background/Aim: Hepatic bile duct obstruction is a common cause of cholestasis. In cases of persistent obstruction, fibrosis and cirrhosis occur. A detailed analysis of fibrogenesis will allow the treatment for this condition to be refined and outcomes to be improved. We assessed the induction of fibrosis in selective obstructive cholestasis by determining the expression of alpha-smooth muscle actin (α -SMA) and transforming growth factor beta 1 (TGF- β 1).

Materials and Methods: Selective bile duct ligation of two of four hepatic lobes was performed in rats. α -SMA and TGF- β 1 expression was assessed at 30 days by immunohistochemistry, and hepatic fibrosis was quantified. The expression levels of the two markers were graded and compared, while the correlation with fibrosis was tested. Descriptive and inferential statistical tests were performed.

Results: At 30 days, a pre-fibrotic stage was reached. Marker expression was identified in both obstructed and unobstructed parenchyma, with higher expression in the obstructed tissue. TGF- β 1 had a more intense expression, and α -SMA expression was significantly correlated with fibrosis. Ito cells showed the highest level of expression.

Conclusion: Fibrogenesis appears to be initiated in all liver parenchyma early after the induction of cholestasis. α -SMA expression is highly correlated with the degree of fibrosis. A long-term evaluation of the model should be performed for better characterization.

Keywords: Cholestasis, fibrosis, TGF- β 1, α -SMA, liver, experimental selective obstructive cholestasis.

Introduction

Liver fibrogenesis is an important process mediated by complex signaling networks (1, 2). Intra- or extrahepatic obstruction of the bile ducts can subsequently lead to

biliary cirrhosis and the need for liver transplantation. Bile duct stenosis can also affect a transplanted liver, and post-transplant complications are significant (3). Deaths secondary to liver fibrosis and cirrhosis comprise a significant percentage annually, and antifibrotic therapies



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are lacking (4). Elucidation of the evolution of all these changes is important in selecting improved and efficient treatments or even preventing this condition.

Hepatic regeneration depends on several factors. This process can be achieved either through stem cells, which can differentiate into hepatic progenitor cells, or through myofibroblasts. Mesenchymal stem cells are represented in the liver by stellate cells. In hepatic injury, stellate cells are activated, converting into myofibroblast-like cells capable of contraction, proliferation and fibrogenesis (5, 6).

Bile acids, which accumulate in the liver in cholestasis, can induce the apoptosis of hepatocytes and cholangiocytes (7). These molecules can also activate specific intracellular pathways to induce stellate cell proliferation (8).

Transforming growth factor- β 1 (TGF- β 1) is a profibrogenic cytokine that regulates type 1 collagen expression in stellate cells (2, 9). In various animal models, multiple functions of this marker have been described (10-12). Its effect depends on the stage of the disease. This molecule can enhance hepatocyte damage, trigger the transdifferentiation of hepatic stem cells into myofibroblasts, and has a cytostatic or proangiogenic effect on endothelial cells (2, 9). High TGF- β 1 expression was shown to inhibit proliferation of stellate cells, acting as an inducer of apoptosis and directing their conversion to myofibroblasts (13-16).

Another marker that indicates differentiation towards myofibroblasts is α -smooth muscle actin (α -SMA). This molecule is the most consistent marker of activated and myofibroblastic hepatic stellate cells (5, 17-20).

High expression of these two markers indicates the progression of fibrosis, collagen deposition and consequent alteration of the architecture of the liver parenchyma. Experimental study of fibrosis showed that these markers are highly expressed in the liver parenchyma a few days after the induction of fibrosis (13).

We aimed to evaluate the induction of fibrosis in obstructed and unobstructed liver parenchyma after experimental highly selective ligation of the extrahepatic bile ducts. We evaluated the dynamics of these two specific markers of fibrosis at 30 days after induction

of cholestasis and searched for a correlation between TGF- β 1 and α -SMA expression.

Materials and Methods

Animals. Female Wistar rats weighing 250-300 g were used. All animals were maintained in the animal facility of the Iuliu Hatieganu University of Medicine and Pharmacy, Cluj-Napoca, with a standard laboratory diet and water *ad libitum*.

Surgical protocol. Selective bile duct ligation was performed in 20 Wistar rats using microsurgical techniques. The bile ducts of the median and left lateral lobes were identified and ligated with a bile duct segmental excision performed between ligatures. The right lateral and caudate lobes were left intact. The rats were monitored for 30 days. Then, another laparotomy was performed, and liver biopsies from the ligated and intact lobes were collected. Histological changes were examined by hematoxylin-eosin staining, while TGF- β 1 and α -SMA were immunohistochemically quantified.

The study design was approved by the Ethics Committee of the Iuliu Hatieganu University of Medicine and Pharmacy (no. 81/01.02.2018).

Histology. Liver biopsies were fixed in 10% buffered paraformaldehyde for 24 h. The samples were dehydrated in progressive concentrations of ethanol, cleared in xylene and embedded in paraffin (Thermo Fisher Scientific, Waltham, MA, USA). Electrostatically charged microscopic blades were used. Histological sections were cut at 3 μ m (microTec Laborgeräte GmbH, Walldorf, Germany) and then deparaffinized in toluene for 1 h in a thermostat. Staining was performed using hematoxylin-eosin (H&E) and then examined and photographed using a Leica DM750 binocular microscope with a Leica ICC50 HD camera (Leica Microsystems GmbH, Wetzlar, Germany).

Structural and trabecular hepatocyte architectural changes were evaluated. Kupffer cell hyperplasia, hepatocyte apoptosis and bile duct hyperplasia were

considered characteristic changes of the fibrogenic process. Apoptosis was identified by light microscopy based on the presence of apoptotic bodies (21).

Initially, fibrosis was assessed using a semiquantitative method based on the expression of TGF- β 1 and α -SMA, whose intensity of expression was graded from 0 to 3 as follows: 0, no expression; 1, weak intensity; 2, medium intensity; and 3, high intensity. The evaluation of all samples was carried out by the same pathologist. Then, independent grading was carried out by the pathologist using the Batts–Ludwig score (22) to better characterize the contribution of each marker to the final stage of fibrosis. A comparison between the expression of the two markers was made, and their correlation with fibrosis was assessed.

α -SMA and TGF- β 1 immunohistochemistry. For determination of the expression of the two markers, 3 μ m-thick tissue slides were obtained, deparaffinized and rehydrated. Antigen unmasking was performed by heating the slides in citrate buffer [SignalStain® Citrate Unmasking Solution (10X); Cell Signaling Technology, Danvers, MA, USA] using a microwave. The samples were blocked using 100 μ l of blocking solution (1X Tris Buffered Saline with Tween® 20/5% Normal Goat Serum) for 1 h at room temperature and were then incubated with primary monoclonal antibody to α -SMA [α -Smooth Muscle Actin (1A4), Mouse mAb (IHC Formulated); Cell Signaling Technology] and primary monoclonal antibody to TGF- β 1 (ab92486; Abcam, Waltham, MA, USA) for 24 h at 4°C. A 30-min incubation with a boost detection reagent [SignalStain® Boost IHC Detection Reagent (HRP, Mouse); Cell Signaling Technology] at room temperature was also performed. The negative control was treated only with phosphate-buffered saline. Immunostaining was revealed using 3,3'-diaminobenzidine for 5 min, followed by counterstaining with hematoxylin. The samples were dehydrated with ethanol and mounted (SignalStain® Mounting Medium; Cell Signaling Technology).

The intensity of TGF- β 1 and α -SMA expression was assessed using the semiquantitative method on a scale from 0 to 3, with 0 corresponding to no expression and 3 to high expression, by the same pathologist.

Statistical analysis. Descriptive statistics: Assessment of fibrosis was performed using a frequency analysis. The summarization was in terms of the modal value (most frequent score) and empirical distribution. Each liver lobe was treated independently. The assumption for the analysis of the TGF- β 1 and α -SMA indicators was the comparison between a group of ligated lobes and a non-ligated one, both with n=8. The intensity of expression of the two investigated markers based on different cell types was quantified by presenting the number of cells with the highest intensity. To achieve this goal, we carried out a ratio test. The cell types were grouped into two categories: the first category included cells with grades 0 and 1 from the semiquantitative method, while the second category included cells with grades 2 and 3 or a combination thereof. The percentages represent the frequency with which the most intense expression was identified for each of the investigated cell types. Additionally, a general analysis of the stages of fibrosis between ligated and non-ligated lobes was performed, but without the split into α -SMA and TGF- β 1.

Statistical testing: Fibrosis scores were compared between the ligated and non-ligated groups of lobes using a nonparametric Wilcoxon unpaired test to test the differences in the central tendency of the samples. Additionally, the proportion Z score test for two samples was used to compare the ratios of low/high expression of markers between ligated and non-ligated lobes. The reference level of statistical significance was 0.05. The comparison between the ranks of the expression of two markers was performed using the Mann–Whitney *U*-test, while the correlation between the two markers and fibrosis was evaluated using Kendall's tau nonparametric correlation.

Statistical data analysis was performed using R software (R 3.6.3, R Project for Statistical Computing, 2020; <https://www.r-project.org>).

Results

Histological analysis. Analyzing the liver tissue from the obstructed lobes, we observed that the lobular structure and trabecular disposition of the hepatocytes were

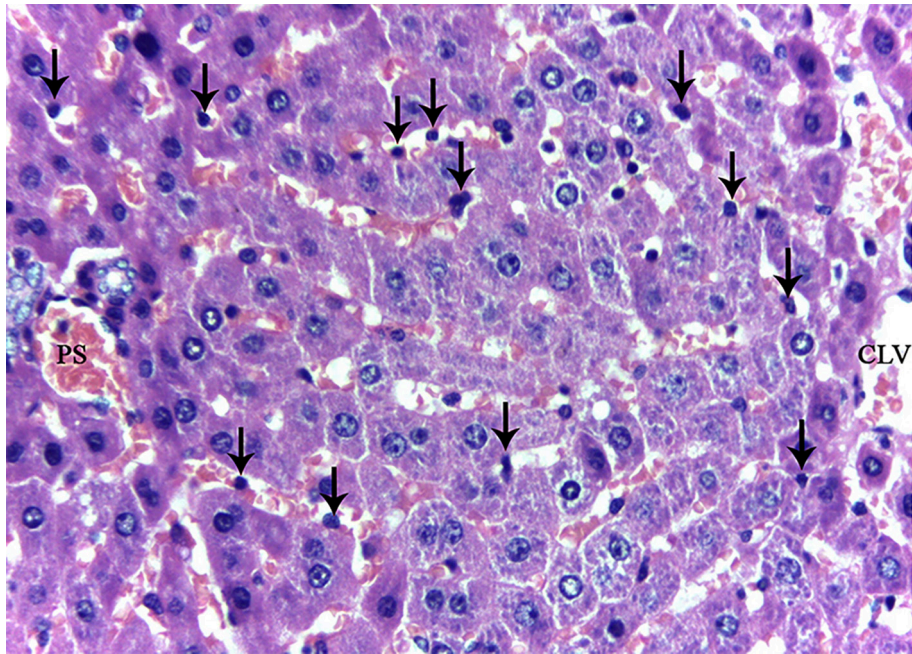


Figure 1. Aspect of the obstructed liver parenchyma. Kupffer cell hyperplasia (arrows) can be seen, with an otherwise preserved liver architecture, with the portal space (PS) and centrilobular vein (CLV) connected by sinusoid capillaries separated by hepatocyte trabeculae. Hematoxylin and eosin, $\times 400$.

preserved. At the sinusoid level, Kupffer cell hyperplasia was identified (Figure 1).

Many stellate cells were identified adjacent to the hepatocyte trabeculae, characterized by a hyperchromic, condensed nucleus and hyper-eosinophilic cytoplasm. These cells were detached from the trabeculae and highly suggestive of activated stellate Ito cells. Although the structure of the tissue was predominantly normal, these hyper-eosinophilic cells were detected focally not only in the intralobular area but also in the periportal areas, with an isolated distribution.

The unobstructed liver parenchyma showed a normal histological structure.

TGF- $\beta 1$. The immunohistochemical analysis of the obstructed liver lobes showed TGF- $\beta 1$ positivity in sinusoids by Kupffer and other cells, identified as non-hepatocytes and treated as a group, and in hepatic stem cells of the so-called canals of Hering. Background staining was observed in all hepatocytes. The biliary epithelium

was negative, but plasmacytoid cells were identified in large portal spaces, with intense expression of TGF- $\beta 1$. Production of this marker was actively identified not only in hepatocytes from the periportal edge but also in those surrounding the centrilobular venules (Figure 2).

Although in the obstructed parenchyma the architecture was maintained, the identified stellate cells with Ito activation were detached from hepatocyte trabeculae and were positive for TGF- $\beta 1$. The same hyper-eosinophilic cells, as shown by H&E staining, were identified focally inside the lobules.

The unobstructed tissue revealed positivity for TGF- $\beta 1$ in the centrilobular, pericentriolar and mediolobar hepatocytes, partially with focal distribution. At the pericentrolobular level, the vacuolated cells were undergoing apoptosis, with hepatocyte removal in the sinusoidal lumen and strong expression of this marker.

α -SMA. In the obstructed liver lobes, the hyper-eosinophilic hepatocytes expressed α -SMA at a low level. These cells were

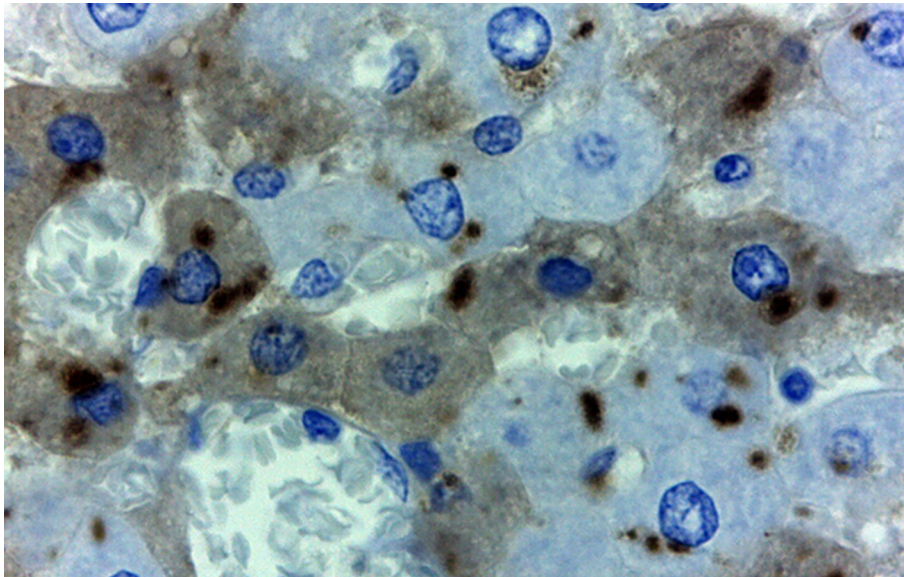


Figure 2. Transforming growth factor- β 1 expression in the obstructed liver. (A) Moderately diffuse cytoplasmic positivity from edge sample and (B) cytoplasmic particles, predominantly localized at the biliary pole of the hepatocytes. Immunohistochemistry, $\times 1,000$.

identified in addition to myofibroblastic transformed Ito cells, which highly expressed α -SMA. The walls of the bile ducts were negative for this marker. At the centrilobular level, in addition to the venous wall, there was also α -SMA positivity of hepatic stellate cells (Figure 3). α -SMA staining presented the same distribution pattern as TGF- β 1, the marker being absent or moderately diffuse in the stellate cells from the periportal space but present in arteriovenous muscular walls of the portal vessels. Hyper-eosinophilic cells with focal intralobular localization expressed a low level of α -SMA.

In the unobstructed liver lobes, α -SMA expression was strictly identified in the endothelium of the portal and centrilobular vessels, with the other elements being negative for this marker.

Assessment of fibrosis. Fibrogenesis was activated in all liver parenchyma, as marker expression was identified in both obstructed and unobstructed parenchyma. The difference was significant, with greater expression in obstructed lobes. The main groups of cells involved in the appearance of fibrosis were Ito cells (93.75% in obstructed vs. 57.25% in unobstructed parenchyma, $p=0.001$) and mediolobular

hepatocytes (43.75% in obstructed vs. 18.75% in unobstructed parenchyma, $p=0.04$) (Table I).

TGF- β 1 was expressed in both ligatured and non-ligatured lobes. A high level of expression was identified only in the ligatured lobes, while a low level of expression was characteristic of the unobstructed parenchyma. The cellular analysis revealed TGF- β 1 expression in all cell types, with a significantly higher expression in Ito cells between the obstructed and unobstructed parenchyma (87.5% in obstructed vs. 37.5% in unobstructed parenchyma, $p=0.001$) (Table II).

α -SMA expression was present in both obstructed and unobstructed hepatic parenchyma. A lower degree of expression of α -SMA was revealed compared with that of TGF- β 1, with no high-level expression.

Cell-level analysis identified Ito cells as having significantly higher α -SMA expression in obstructed compared to unobstructed parenchyma (62.5% vs. 25%, respectively, $p=0.035$) (Table III).

In a comparison of the expression of the two markers, given the same level of independently evaluated fibrosis, α -SMA expression was significantly more frequently

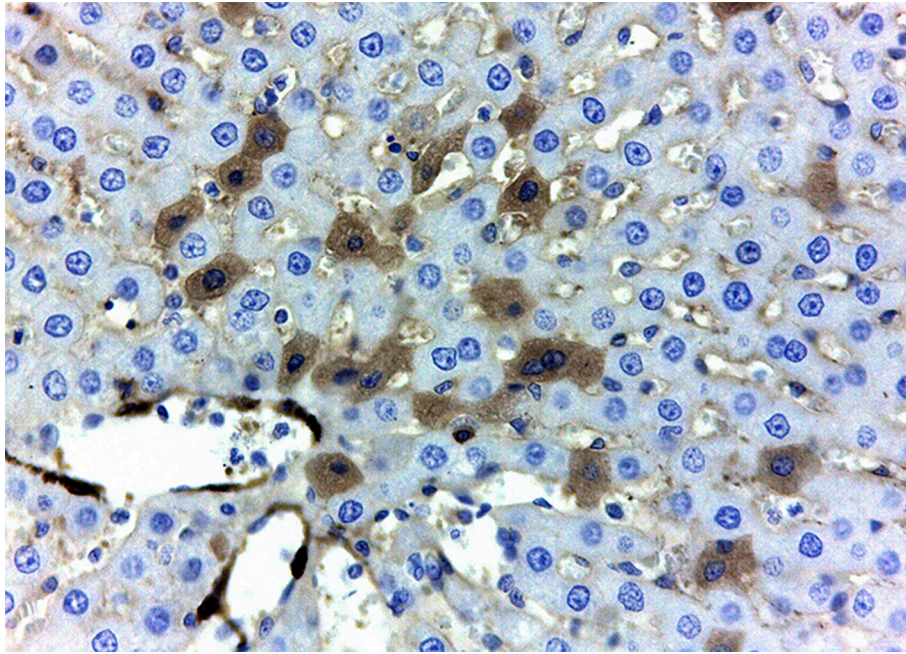


Figure 3. Expression of α -smooth muscle actin in the obstructed liver parenchyma. The expression is identified in pericentrolobular stellate cells (Ito cells involved in fibrotic processes through collagen synthesis). Immunohistochemistry, $\times 1,000$.

identified in the obstructed lobes compared to TGF- β 1, with Ito cells having a higher share of high ranks ($p=0.036$). The same difference was observed in the analysis of unobstructed parenchyma, where not only Ito cells but also Kupffer cells had higher ranks ($p=0.044$ and $p=0.033$, respectively).

The analysis of the correlation between the two markers and fibrosis revealed a moderate positive correlation of α -SMA expression and fibrosis in the obstructed lobes (Kendall's tau=0.673, $p=0.039$) which was statistically significant, while no significant correlation was found between TGF- β 1 expression and fibrosis (Kendall's tau=0.191, $p=0.568$). In the unobstructed lobes, the correlation was weak and not significant for both markers (for TGF- β 1 Kendall's tau=-0.218, $p=0.564$; for α -SMA Kendall's tau=0.333, $p=0.378$).

Discussion

Liver fibrosis is a form of hepatic regeneration in response to chronic cellular injury (1). The choice of a certain path of evolution towards fibrogenic or non-fibrogenic repair

Table I. Quantification of fibrosis based on the expression of the two markers in the obstructed versus unobstructed lobes of rat livers.

Cell type	Fibrosis in lobe		<i>p</i> -Value*
	Obstructed	Unobstructed	
Peri portal hepatocytes	37.5%	18.75%	0.18
Mediolobular hepatocytes	43.75%	18.75%	0.04
Centrolobular hepatocytes	50%	31.25%	0.2
Ito	93.75%	57.25%	0.001
Kupffer	50%	50%	0.9

*Proportion Z-score test for two samples (score 0-1 vs. 2-3). Statistically significant *p* values are shown in bold.

depends on the stimulus type and involves the modulation of numerous factors (1, 23). Bile duct ligation in rats is followed by ductular proliferation and portal fibrosis. Additionally, liver susceptibility to obstruction depends on the age of the animal (24). In addition to a model in adult rats, other experimental models were also successful (21).

Table II. Quantification of transforming growth factor- β 1 (TGF- β 1) expression in the obstructed versus unobstructed lobes of rat livers. The percentages represent the frequency with which the most intense expression was identified for each of the investigated cell types.

Cell type	TGF- β 1 in lobe		p-Value*
	Obstructed	Unobstructed	
Peri portal hepatocytes	37.5%	12.5%	0.87
Mediolobular hepatocytes	37.5%	12.5%	0.87
Centrolobular hepatocytes	50%	37.5%	0.88
Ito	87.5%	37.5%	0.001
Kupffer	37.5%	25%	0.75

*Proportion Z-score test for two samples (score 0-1 vs. 2-3). Statistically significant p values are shown in bold.

In chronic liver disease, extracellular matrix production is disturbed, and in addition to a regenerative process, fibrogenesis is also activated. The result is the appearance of anomalies in hepatic cell-sinusoid interactions, with nodule formation, and these changes are characteristic of cirrhosis (23).

In our experimental model, an advanced stage of fibrosis was not reached at 30 days. However, subtle changes indicating the initiation of fibrogenic repair were present, equivalent to chronic moderate cholestasis. We evaluated fibrosis practically and our findings showed that although fibrogenesis was activated, as indicated by the positivity for the investigated markers, deposition of collagen was not observed.

Highly selective bile duct ligation can explain these slow, incipient changes, with only two hepatic lobes out of four being obstructed in our model. Another possible explanation is the fact that parenchymal connections, as sources of collateral biliary drainage, were not identified as being interrupted during surgery. In a similar model of selective bile duct ligation, where the bile ducts of three liver lobes were obstructed and the parenchymal connections were also sectioned, progressive development of fibrosis was demonstrated, starting in the first week after surgery (25).

Table III. Quantification of α -smooth muscle actin (SMA) expression in the obstructed versus unobstructed lobes of rat livers. The percentages represent the frequency with which the most intense expression was identified for each of the investigated cell types.

Cell type	α -SMA in lobe		p-Value*
	Obstructed	Unobstructed	
Peri portal hepatocytes	37.5%	25%	0.75
Mediolobular hepatocytes	50%	25%	0.32
Centrolobular hepatocytes	50%	25%	0.32
Ito	62.5%	25%	0.035
Kupffer	62.5%	75%	0.87

*Proportion Z-score test for two samples (score 0-1 vs. 2-3). Statistically significant p values are shown in bold.

Additionally, in rats, interlobar accessory bile channels and rapid bile duct collateral formation are responsible for fast recovery from obstructive cholestasis, as demonstrated by Ni *et al.* (26, 27) using microcholangiography and magnetic resonance studies. These data showed that the stimulus intensity can be modulated *in vivo* dependent on the hepatic interlobar connections and the timing of neof ormation bile ducts.

In our study, partial mechanical obstruction was performed, with evaluation of hepatic changes at 30 days after surgery.

The histological changes, as stated in other studies (28-30), may be prominent in the first week after the procedure, followed by a decrease in the next two weeks and a subsequent increase after the fourth week. This evolution was explained through bile duct collateral neof ormation in the hilum area that plays an incipient role, partially compensating for the suppressed biliary drainage. Later, the process is overcome by the persistence of the stimulus (3). The mechanical obstruction of the bile ducts of only two hepatic lobes represents a weak but constant stimulus, which surpasses the initial inhibition through the neof ormation process and finally manages to trigger fibrogenesis, as illustrated by this research.

The obstructed liver lobe analysis revealed intralobular fibrogenesis. At the cellular level, myofibroblasts are the source of excess extracellular matrix (collagen types I and III) that constitutes pathological fibrous tissues (4, 31). The precursors of myofibroblasts are hepatic stellate cells. Ito cells are the main cell type involved in hepatic fibrogenesis (32, 33), having an important role in hepatic regeneration by mediating endothelial cell and hepatocyte proliferation, and remodeling extracellular matrix elements (12, 34). In hepatic fibrogenesis, these cells transdifferentiate into myofibroblasts with profibrogenic properties *via* cell activation and secrete the matrix that forms the scar tissue from Disse spaces (4, 6, 27).

In H&E staining, the elements that supported the initiation of fibrogenesis were hyperplasia of Kupffer cells and stimulation of hepatocyte apoptosis. These changes are the result of a chronic cholestatic liver stimulus, such as selective bile duct ligation, and apoptosis activated by bile acids accumulated in the liver secondary to obstruction (8). Macrophages enveloping apoptotic bodies produce TGF- β 1 and activate hepatic stellate cells. Through this process, fibrogenic activity of the cells is promoted, with enhanced production of collagen α 1, TGF- β 1 and α -SMA (32, 35). Activated Ito cells secrete TGF- β 1 in response to liver injury (4, 11). This marker also initiates pathways to promote hepatic cell activation (6). In our study, the TGF- β 1 staining method showed that the marker was expressed not only by Kupffer and other sinusoidal cells but also by hepatic stem cells of the canals of Hering, which promote the activation of the stellate cells. Although the biliary epithelium was negative for this marker, in large portal spaces, plasmacytoid cells, intensely expressing TGF- β 1, were identified which are responsible for differentiation of local histiocytes into myofibroblasts and activating fibrogenesis in portal spaces. Additionally, background staining of all hepatocytes was observed, representing activation of TGF- β 1 in all hepatic cells.

This activation of fibrogenesis in portal spaces has an antimitotic effect and stimulates mesenchymal transdifferentiation (36). Thus, necrosis/apoptosis activated by cholestasis will not be regenerated by

proliferation of Hering stem cells, and their division will be blocked by TGF- β 1 (6) through fibrosis.

Additionally, we observed hyper-eosinophilic stellate hepatocytes, isolated or concentrated on bridges, and low expression of α -SMA next to myofibroblastic-transformed Ito cells, which were intensely positive for α -SMA. Their presence might have been the result of the bile acid stimulation of Ito stellate cells, which can transdifferentiate into hepatocytes on one hand (37) but on the other are converted towards myofibroblasts by TGF- β 1 (9, 31).

Ito cells are strongly positive for α -SMA in chronic liver diseases (38, 39). In the fibrous liver of rats, cells expressing α -SMA appear in the perisinusoidal space, fibrous septa and surrounding regenerative nodules and are a good marker for detecting myofibroblast-like cells (40, 41). This molecule represents the most consistent marker for activated and myofibroblastic hepatic stellate cells in the human liver (15). In our study, the positivity of the pericentrolobular stellate cells indicates the activation and transdifferentiation of Ito cells towards myofibroblasts.

This pattern of expression at 30 days was different from that in other experimental studies of fibrosis, which showed rapid evolution and a high expression of α -SMA as early as a few days after the induction of fibrosis (42, 43).

In the analysis of the unobstructed liver lobes, although a normal liver structure was found, we also identified hyper-eosinophilic stellate hepatocytes, as in the obstructed lobes, with a focal deposition. These cells had marked expression of TGF- β 1 but were also positive for α -SMA. Additionally, TGF- β 1 was activated in a focal bridge pattern of distribution, while α -SMA was expressed by the muscular walls of the portal vessels.

A more intense fibrogenic response was obtained in a similar model of selective bile duct ligation, where a higher proportion of liver parenchyma was subjected to bile duct obstruction. Cell differentiation into myofibroblasts was indicated by the increase in α -SMA expression a few days after surgery. The expression, however, normalized 60 days after surgery (10). In unobstructed lobes, delayed

expression of α -SMA (not present at 7 days but increased at 60 days) showed a slower progression of hepatic fibrogenesis. However, fibrotic markers were described in both cholestatic and adjacent liver parenchyma, as described in our model. These results suggested that the activation of the fibrogenic process was promoted in the unobstructed lobes by endocrine or paracrine mechanisms (10). These mechanisms are likely activated early in the process, given the alterations we identified in our analysis.

At 30 days after surgery, we quantified the level of fibrosis. A pre-fibrotic stage was identified, so we focused on the expression of the two markers. A significantly higher degree of expression was expected in the obstructed parenchyma, and high expression of the markers was identified in both types of parenchyma. We also identified cells involved in the process in both types of parenchyma that can be targeted or intercepted during their differentiation pathways. A more thorough analysis of the model should be performed to follow the evolution of changes in the long term.

Conflicts of Interest

The Authors declare no conflict of interest.

Authors' Contributions

Ailioaie Raluca Cristina: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Software; Supervision; Validation; Visualization; Roles/Writing - original draft; Writing - review & editing. Ciuce Catalin: Investigation; Methodology; Visualization. Fagarasan Vlad: Investigation; Methodology; Visualization. Ionescu Calin: Project administration; Supervision; Validation; Visualization. Scurtu Radu Razvan: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Software; Supervision; Validation; Visualization; Roles/Writing - original draft; Writing - review & editing.

Artificial Intelligence (AI) Disclosure

No artificial intelligence (AI) tools, including large language models or machine-learning software, were used in the preparation, analysis, or presentation of this manuscript.

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