Liver International 2004: 24: 658–668
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Liver International
DOI: 10.1111/j.1478-3231.2004.0961.x

Basic Studies

Hypoxia potentiates transforming growth factor-β expression of hepatocyte during the cirrhotic condition in rat liver

Jeong W-I, Do S-H, Yun H-S, Song B-J, Kim S-J, Kwak W-J, Yoo S-E, Park H-Y, Jeong K-S. Hypoxia potentiates TGF-β expression of hepatocyte during the cirrhotic condition in rat liver.
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Abstract: *Background/Aims:* Many studies have reported that hypoxia might be associated with angiogenesis and fibrogenesis, and the level of transforming growth factor-β1 (TGF-β1) was increased in fibrotic liver and maximal at cirrhosis. Therefore, we examined the expression of TGF-β1, phosphorylated-Smad2/3 (p-Smad2/3) of the TGF-β immediate down stream signaling system and hypoxic status during hepatic fibrogenesis. *Methods:* Fibrosis of rats was induced by carbon tetrachloride. Collagens were detected with Azan stain. Immunohistochemistry and immunoblotting was used. *Results:* TGF-β1 was mainly produced by hypoxic hepatocytes at cirrhosis although myofibroblasts (MFBs) and macrophages producing TGF-β1 were decreased. Moreover, distribution of p-Smad2/3 in hepatocytes was consistent with those of hypoxic hepatocytes regardless of MFBs. Furthermore, in recovery, most MFBs disappeared, whereas positive reactions of p-Smad2/3 still existed in the hepatocytes of hypoxic areas. Therefore, TGF-β1 expression in hepatocytes might have been associated with hypoxia. *Conclusions:* We put forward the hypothesis that TGF-β1 is mainly produced by MFBs and macrophages at early and middle stages of fibrotic processes, but it is predominantly released by hypoxic hepatocytes in the last fibrotic stage or cirrhosis.

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Key words: cirrhosis – hypoxia – liver – myofibroblast – smad protein – transforming growth factor beta

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Received 15 January 2004,
accepted 01 June 2004

Hepatic fibrosis is a common response to various chronic hepatic injuries and occurs as a consequence of the transformation of hepatic stellate cells (HSCs) into myofibroblasts (MFBs) producing abnormal extracellular matrix, which is mainly induced by transforming growth factor-β (TGF-β), especially TGF-β1 (1–3). TGF-β is synthesized by several mesenchymal liver cells and hepatocytes (4) and generated by infiltrating cells such as lymphocytes, monocytes/macrophages, platelets and activated HSC themselves (5, 6). TGF-β first binds to the TGF-β type II receptor on the cell surface and subsequently recruits the TGF-β type I receptor, thus forming a heteromeric complex between these two types of receptors. Once the type I receptor is phosphorylated by the type II receptor kinase, it phosphorylates Smad2 and/or Smad3, which form hetero-oligomers with Smad4. They translocate from the cytoplasm into the nucleus, where they stimulate or repress gene transcription (7–10). Recently, it has been reported that hypoxia might be associated with angiogenesis and fibrogenesis in experimental biliary cirrhosis (11–13), and that cellular response to hypoxia induced TGF-β2 gene expression and involved signaling via Smad proteins in endothelial cells of human umbilical vein (14, 15).

We recently demonstrated that the number of MFBs is decreased in carbon tetrachloride (CCl_4)-induced rat liver cirrhosis (16). However, many studies have reported that TGF-β1 is being increased in cirrhosis (11, 17–20). Then, how is the expression of TGF-β1 increased in cirrhotic

liver without the increase of MFBs, chief producing cell of TGF-β1? This study was designed to clarify or determine the changes in Smad proteins (phosphorylated-Smad2/3 (p-Smad2/3)) of the TGF-β immediate down stream signaling system, TGF-β1 expression and hypoxia in cirrhotic and recovering rat liver during chronic intoxication with CCl_4.

Material and methods

Experimental designs

Male Wistar rats ($n = 72$) weighing 200–220 g were housed in a room at 22 ± 2 °C and a 12 h light–dark cycle. The animal experiments were performed in accordance with the NIH guidelines for the care and use of laboratory animals. Fibrosis/cirrhosis was induced by intraperitoneal injection of 1.0 ml/kg body weight of 10% CCl_4 in olive oil three times a week for 14 weeks. We divided rats into two groups. These groups are schematically shown in Fig. 1.

Histopathology and immunohistochemistry

Liver pieces were rapidly removed at random and fixed in 10% neutral buffered formalin, processed routinely and embedded in paraffin wax. Sections were cut to 4 μm in thickness. The sections were stained with hematoxylin and eosin and Azan for collagen fibers. To quantify hepatic fibrosis, we used grades 0–4 (16). For immunohistochemistry, sections were deparaffinized in xylene, rehydrated in graded alcohol series, incubated in a solution of 3% H_2O_2 in methanol for 30 min and microwaved at 750 W for 10 min in 10 mmol/l citrate buffer, pH 6.0. Sections were washed with phospate-buffered saline (PBS), and then immunostained with antibodies of α-smooth muscle actin (α-SMA) (Sigma, St Louis, MO), p-Smad2/3 (Santa Cruz Biotechnology, Santa Cruz, CA) and TGF-β1 (The LC (1–30) antibody to mature TGF-β1, gift from NIH). The antigen-antibody complex was visualized by an avidin–biotin–peroxidase complex solution using an ABC kit (Vector Laboratories, Burlingame, CA) with 3,3-diaminobenzidine (Zymed Laboratories Inc., San Francisco, CA). They were then rinsed in distilled water and counterstained with Mayer's hematoxylin or methyl green. For negative control, the primary antibody was replaced by PBS.

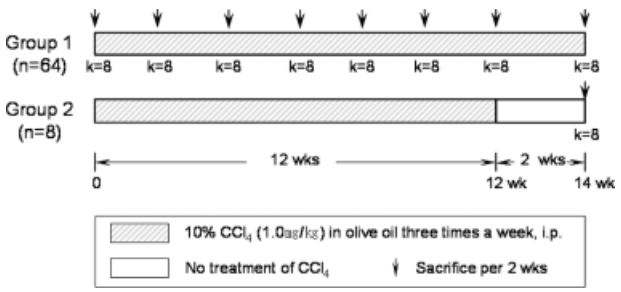

Fig. 1. Experimental design as described in Materials and methods. Group 1 ($n = 64$) is cirrhotic group with CCl_4 treatment for 14 weeks and eight rats are sacrificed at weeks 0, 2, 4, 6, 8, 10, 12 and 14, respectively. Group 2 ($n = 8$) is recovery group with CCl_4 treatment for 12 weeks and is allowed to recover during the last 2 weeks (from 13th to 14th week). These eight rats are sacrificed at week 14. n is the number of rats in each treatment group; k is the number of rats that are killed during each time point.

Hypoxia assay

Nitroimidazole compounds such as pimonidazole are reductively activated at low oxygen concentration and accumulation of the reduced pimonidazole compounds is only dependent on oxygen tension and allows for assessing changes in hepatic tissue oxygenation (21, 22). In brief, intraperitoneal administration of pimonidazole (Hypoxyprobe-1 kit from Dr. James Raleigh, Belmont, MA) at 60 mg/kg of body weight was performed 1 h before killing. Pimonidazole adducts were detected in formalin-fixed paraffin-embedded liver tissue with previously modified methods and directions of Hypoxyprobe-1 kit manual using a monoclonal antibody (22, 23). For negative control, the primary antibody was replaced by PBS.

Immunoblotting

To detect TGF-β1 and α-SMA, snap-frozen liver tissues were homogenized in HEPES-based buffer containing 25 mM HEPES pH 7.5, 300 mM NaCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.5 mM dithiothreitol (DTT), 20 mM β-glycerophosphate, 0.1 mM Na_3VO_4, and protease inhibitor cocktail tablets (Roche, Mannheim, Germany). The lysate was centrifuged at $1200 \times g$ for 10 min at 4 °C to remove solid tissue and debris. Subsequently, the supernatant was centrifuged at $12700 \times g$ for 20 min at 4 °C to obtain soluble cytosolic protein. To detect p-Smad2/3, nuclear extracts were prepared as described by Dennler et al. (24). Briefly, frozen liver tissues were homogenized with ice-cold buffer A (10 mM HEPES-KOH, pH 7.9, 1 mM Na_3VO_4, 0.5 mM DTT, 1.5 mM $MgCl_2$, 10 mM KCl, protease inhibitor cocktail tablets). The lysates were allowed to swell on ice for 30 min and then lysed by 30 strokes of Dounce all glass homogenizer (Kantes Co., Vineland, NJ, USA). Nuclei were collected by centrifugation and resuspended in ice cold buffer C (20 mM HEPES, pH 7.9, 20 mM NaF, 1 mM Na_3VO_4, 1 mM $Na_4P_2O_7$, 0.13 μM okadaic acid, 1 mM EDTA, 1 mM EGTA, 0.4 mM ammonium molybdate, 420 mM

NaCl, 20% glycerol, 1 mM DTT, protease inhibitor cocktail tablets). At this step Phosphatase Inhibitor Mix (Sigma) was added. The nucleus membrane was lysed by 15 strokes of a Dounce all glass homogenizer. The resulting suspension was stirred for 30 min at 4 °C. The clear supernatant was aliquoted and frozen at −80 °C. Protein concentration was determined by the Bradford method (25).

Protein samples (20 μg per lane) were separated by 10% SDS-PAGE. For immunoblotting, proteins were electro-transferred to a Polyvinylidene difluoride (PVDF) membrane (Schleicher & Schuell, Dassel, Germany). Equal protein loading was confirmed by Coomassie blue staining. After blocking with 1.5% bovine serum albumin (BSA) in Tris-buffered saline (TBS), TGF-β1, α-SMA and p-Smad2/3 were detected using rabbit polyclonal antibody against TGF-β1 (1:200) (Santa Cruz Biotechnology), monoclonal α-Smooth Muscle antibody (1:500) (Sigma) and rabbit polyclonal antibody against p-Smad2/3 (1:100) (Santa Cruz Biotechnology), respectively. After washing in TBS, blots were incubated with anti-rabbit and mouse IgG HRP conjugated (Promega, Madison, WI). Specific binding was detected using the Super Signal West Dura Extended Duration Substrate (PIERCE, Rockford, IL) and exposure of the blots to Medical X-ray Film (Kodak, Tokyo, Japan).

Results

Hepatic fibrosis and accumulation of collagen

The grade of hepatic fibrosis changed from grades 0 to 4 (Table 1). At week 0, collagen fibers were normally observed in the areas of portal and central vein (Fig. 2A). Two weeks after CCl_4 administration, collagen fibers increased around the areas of central vein. At weeks 4 and 6, collagen formed bridging fibrosis between central and portal areas or central-to-central areas (Fig. 2B). By weeks 8 and 10, collagen fibers were observed along the peripheral region of the pseudolobules dividing hepatic tissue into different sizes (Fig. 2C). From week 12, areas of the most advanced fibrosis were abundant in collagen fibers and the parenchyma was subdivided into smaller pseudolobules (Fig. 2D). At week 14, remarkable hepatic cirrhosis occurred and an isolated hepatocyte and islands of some hepatocytes were also detected because of dense fibrous tissues (Fig. 2E). In contrast to cirrhotic group, there was a prominent decrease of fibrous septa in the liver of recovery group at week 14. In recovery group, pseudolobules were formed by thin fibrous septa (Fig. 2F). In immunohistochemistry, MFBs positive for α-SMA were mainly observed in the fibrous septa (Fig. 2G). The number of these cells increased during weeks 0–10, but they decreased in the following weeks as described in our previous study (16). By week 14, it looked as if only a few MFBs were present surrounding pseudolobules (Fig. 2H). In recovery group, MFBs were disappeared although thin fibrous septa were still present (Fig. 2I).

Table 1. Hepatic lesions and grade of fibrosis

Week	Lesions	Grade
0	Normal	0
2	Pericentral necrosis and fatty change	1
4	Fibrosis (mild)	2
6	Fibrosis (mild to moderate)	2–3
8	Fibrosis (moderate)	3
10	Fibrosis (severe)	3
12	Fibrosis and cirrhosis	4
14C	Cirrhosis	4
14R	Fibrosis (moderate)	3

Grade 0, none; Grade 1, short collagenous septa extended from central veins; Grade 2, slender septa link the central veins, but lobular architecture is preserved; Grade 3, pseudolobuli are formed by thin septa; Grade 4, parenchyma is subdivided into smaller pseudolobuli by thin septa; C, cirrhotic group with CCl_4 treatment for 14 weeks; R, recovery group with CCl_4 treatment for 12 weeks was allowed to recover during the last 2 weeks (from 13th to 14th week).

Detection of positive cells for the α-SMA, p-smad2/3 and hypoxia

At week 0, MFB positive for α-SMA were observed only in the central veins, portal veins and hepatic arteries (Fig. 3A). Faint positive reactions for p-Smad2/3 were mainly detected within the nuclei of hepatocytes throughout the liver (Fig. 3B) and often in those of Kupffer cells. Hypoxia was restricted to pericentral hepatocytes (Fig. 3C). From weeks 2 to 6, as were expected, distribution of MFBs secreting TGF-β were similar to that of cells positive for p-Smad2/3 and all of these cells were located only in and around fibrous septa (Figs. 2B, 3D and E). Hypoxic hepatocytes were also detected around fibrous septa (Fig. 3F). Most MFBs were detected in the fibrous septa at weeks 8–10, whereas phosphorylation of Smad2/3 and hypoxia were distributed throughout the liver (Fig. 3G–I). Positive reactions for p-Smad2/3 were observed in some hepatocytes, MFBs and endothelial cells (Fig. 3H). Hepatocytes and non-parenchymal cells on the borderline of pseudolobule were especially more positive for p-Smad2/3 and appeared more hypoxic than those of central areas. When cirrhosis occurred from week 12, MFBs were detected predominantly around pseudolobules (Fig. 3J). Predominant intranuclear localization of p-Smad2/3 and its density in the hepatocytes were more pronounced within the

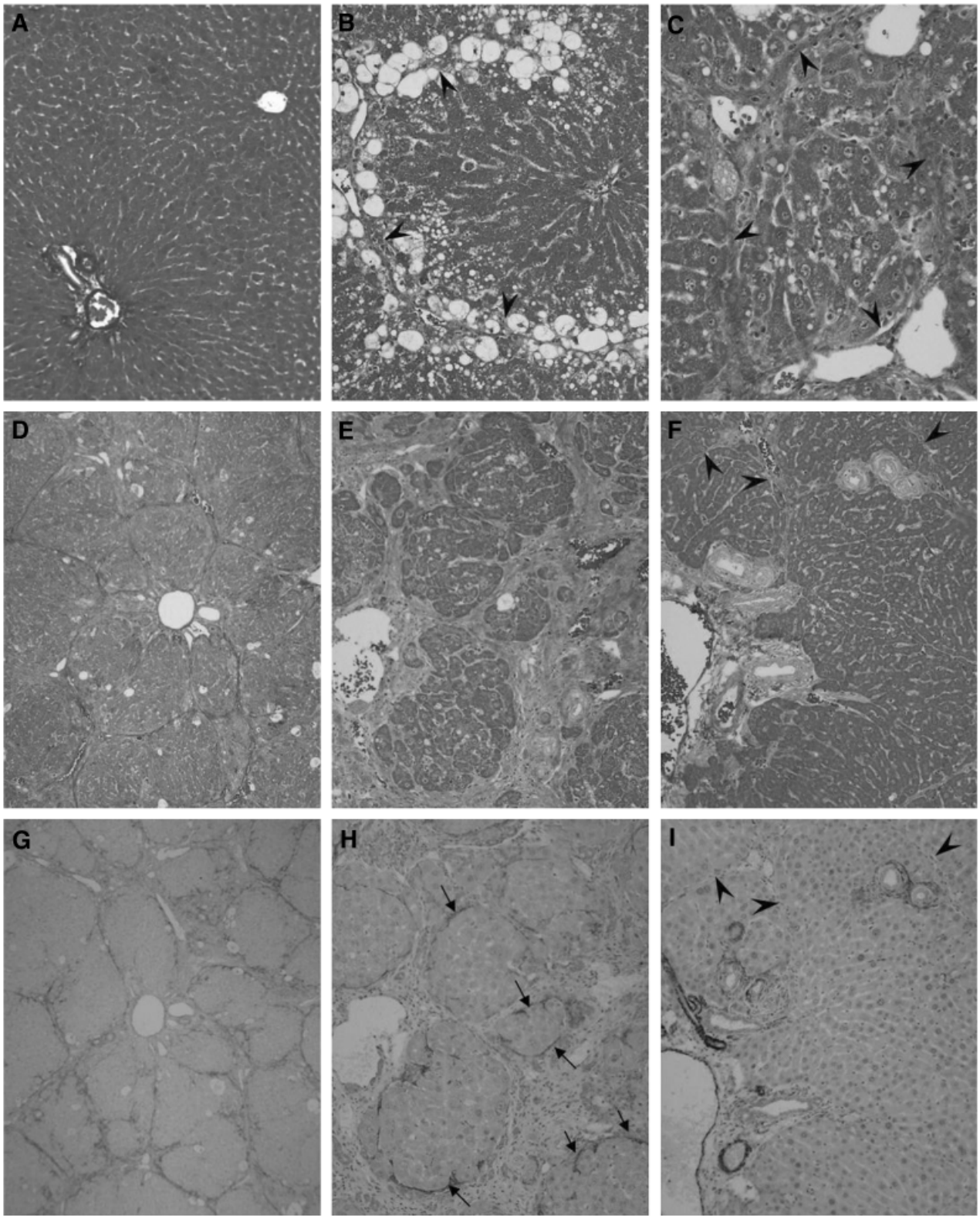

Fig. 2. Hepatic fibrosis and accumulation of collagen in cirrhotic and recovery groups. (A) At week 0, normal collagen fibers in the areas of portal and central vein. (B) Bridging fibrosis (arrowhead) between central veins at week 4. (C) Collagen fibers (arrowhead) along the peripheral region of the pseudolobule dividing hepatic tissue at week 10. (D) Parenchyma subdivided into smaller pseudolobules at week 12. (E) An isolated hepatocyte and islands of some hepatocytes because of dense fibrous tissues in hepatic cirrhosis at week 14. (F) Prominent decrease of collagen and fibrous septa in recovery group (arrowhead). (G) Myofibroblasts (MFBs) expressing α-smooth muscle actin (α-SMA) are mainly observed in the fibrous septa. (H) Marked decrease of MFBs (arrow) at cirrhosis. (I) Disappearance of most MFBs in the fibrous septa (arrowhead) during recovery. Azan stain (A—F); immunostaining of α-SMA with hematoxylin counterstain (G—I). Original magnifications (D and G) ×13; (A, B, E, F, H and I) ×33; C, ×66.

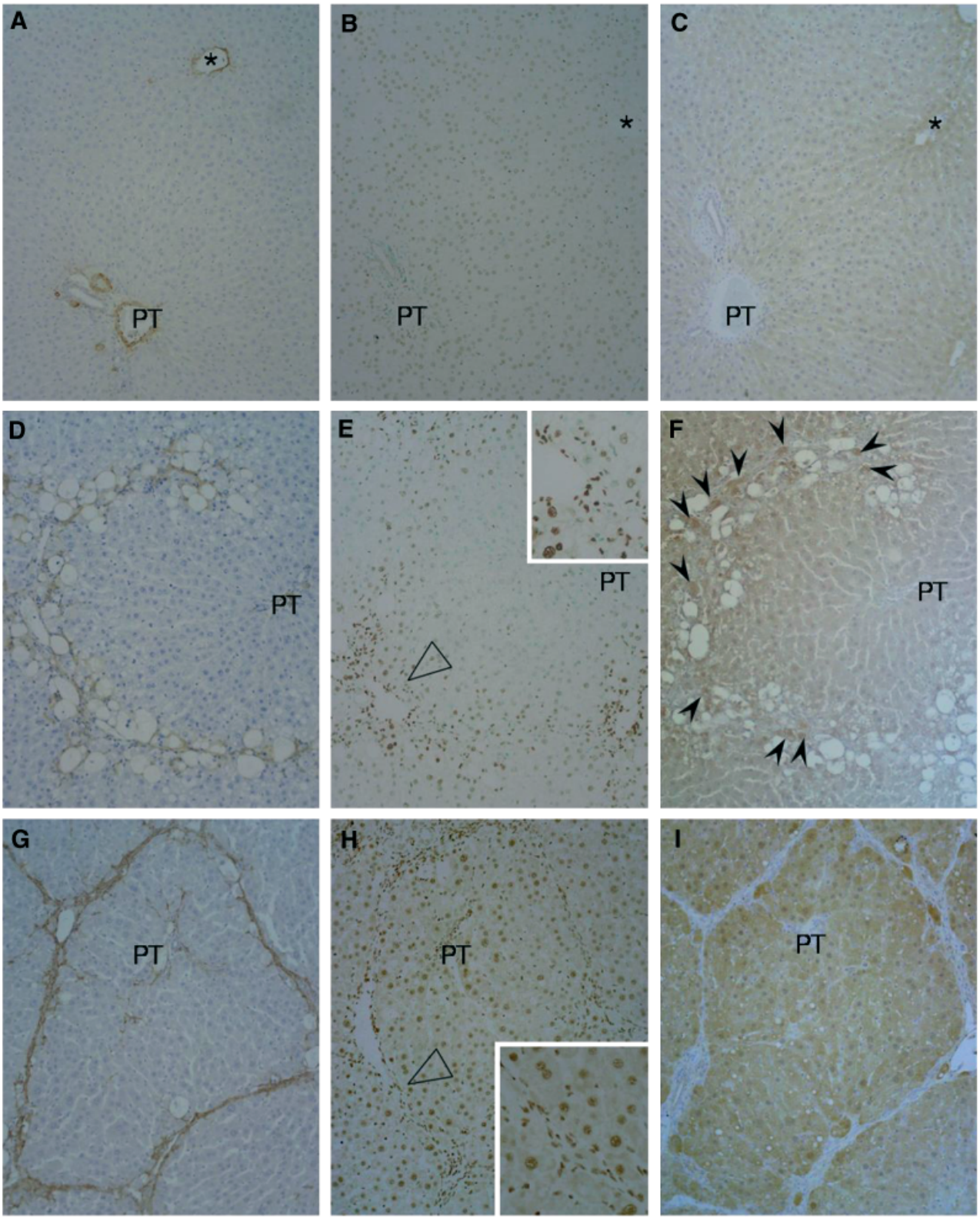

Fig. 3. Positive cells for α-smooth muscle actin (α-SMA), phosphorylated-Smad2/3 (p-Smad2/3) and hypoxia. (A) Few Myofibroblasts (MFBs) at week 0. (B) Faint positive reaction for p-Smad2/3 in the nucleus of hepatocytes throughout the liver at week 0. (C) Some hepatocytes with mild hypoxic areas around central veins at week 0. (D–F) At week 4, distributions of positive cells for α-SMA, p-Smad2/3 and hypoxia were similar to each other that were around fibrous septa (arrowhead). Inset of (E): Magnification of open arrow in (E). (G) MFBs present only in the fibrous septa at week 10. (H, I) At week 10, hepatocytes and nonparenchymal cells on the border line of pseudolobule were more positive of p-Smad2/3 and more hypoxic than those of central areas. Inset of (H): magnification of open arrow in (H). (J–L) Hepatocytes became more hypoxic, positive reactions for phosphorylated-Smad2/3 (p-Smad2/3) are stronger irrespective of MFBs at week 12.

(caption continued in the next page)

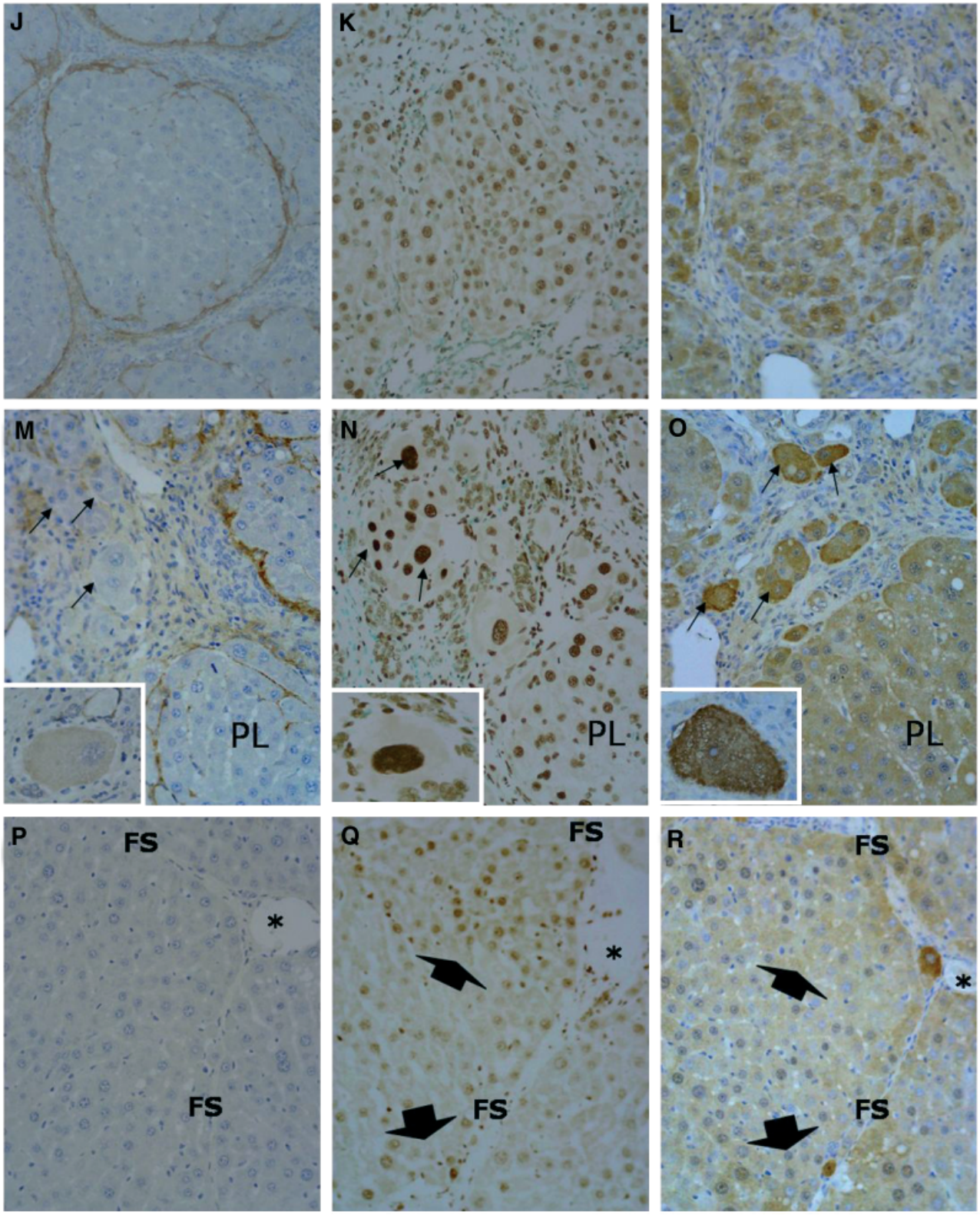

Fig. 3 ***(caption continued from the previous page)***. (M–O) An isolated hepatocyte or some hepatocytes (arrow) enclosed by dense fibrous tissue are the most hypoxic and had the strongest reaction of p-Smad2/3 at week 14. Insets of (M—O): magnification of an isolated hepatocyte at each stain. (P–R) most MFBs disappeared in the parenchyma and fibrous septa, whereas positive reactions of p-Smad2/3 still exist in the hypoxic area (large arrow). Asterisk, central vein; PT, portal triad; FS, fibrous septa, PL, pseudolobule. Immunostaining for α-SMA (A, D, G, J, M, P), p-Smad2/3 (B, E, H, K, N, Q) and hypoxia (C, F, I, L, O, R) with hematoxylin or methyl green counterstain. Original magnifications: (A–I) ×33; (J–R) inset of (E) and (H) ×66; Inset of (M, N and O) ×132.

pseudolobules (Fig. 3K). Hepatocytes in the small pseudolobules were more hypoxic than those in the large pseudolobules (Fig. 3L). At week 14, a hepatocyte or some hepatocytes enclosed by dense connective tissue not containing MFBs were the most hypoxic and had the strongest reaction to p-Smad2/3 antibody (Fig. 3M–O). In recovery group at week 14, most MFBs disappeared in the parenchyma and fibrous septa, whereas positive reaction of p-Smad2/3 still existed in the hypoxic areas (Fig. 3P–R). Therefore, distribution of positive cells for the α-SMA, p-Smad2/3 and hypoxia were identical with each other before pseudolobules were formed at week 8. However, phosphorylated areas of Smad2/3 were consistent with those of hypoxic hepatocyte from weeks 8 to 14. These results suggested that hepatocytes become more hypoxic, phosphorylation of Smad2/3 were stronger irrespective of distribution of MFBs producing TGF-β.

Expression of TGF-β1 during hepatic fibrogenesis

Expression of TGF-β1 during hepatic fibrogenesis is shown in Table 2 and Fig. 4. Expression of TGF-β1 was not detected in the normal liver. As fibrosis developed, macrophages were predominantly expressing TGF-β1 in the fibrous septa from weeks 2 to 10 (Fig. 4A–C). However, it was decreased in macrophages when cirrhosis occurred at weeks 12 and 14. On the other hand, during recovery, macrophages expressing TGF-β1 were detected in the regression of fibrous septa (Fig. 4D). Bile ductular epithelial cells mildly expressed TGF-β1 during hepatic injuries (Fig. 4A), but no positive reactions for TGF-β1 were observed in MFBs and HSCs throughout the experiment (Fig. 4). At week 10, a small number of hepatocytes expressing TGF-β1 were detected and located peripherally within pseudolobules (Fig. 4E). However, when cirrhosis occurred, many hepatocytes expressed TGF-β1 within several pseudolobules, and hepatocytes located in the peripheral areas of pseudolobules expressed more TGF-β1 than those of central areas (Fig. 4F and G). Some hepatocytes surrounded by fibrous tissue expressed less TGF-β1 than those within pseudolobules (Fig. 4H), which might be because of degeneration of hepatocyte. In recovering hepatocytes, expression of TGF-β1 was decreased, but some hepatocytes neighboring still existent fibrous septa mildly expressed TGF-β1 (Fig. 4I).

Immunoblot of TGF-β1, p-Smad2/3 and α-SMA

In immunoblotting, the specific immunoreaction using TGF-β1, p-Smad2/3 and α-SMA antibodies were detected in the liver homogenates at weeks 0, 4, 8, 12 and 14 (Fig. 5). A faint single immunoreactive band of TGF-β1 was detected in the homogenate of livers at week 0. As hepatic injuries developed to fibrosis and cirrhosis, a much more prominent single immunoreactive band of TGF-β1 was detected and increased (Fig. 5). However, during recovery, TGF-β1 expression was decreased in rat livers of recovery group that was allowed to recover for 2 weeks (from weeks 13 to 14). Expression of p-Smad2/3 increased or decreased in accordance with changes of TGF-β1, but α-SMA were not coincidence with changes of TGF-β1 as indicated in the immunohistochemical results (Fig. 5), indicating direct evidence of decreasing myofibroblasts during the process of cirrhosis.

Table 2. Expression of TGF-β1 during hepatic fibrogenesis

Week	Expression of TGF-β1 HEP	BDE	MAC	Hepatic lesion/hypoxic status in hepatocyte
0	−	−	−	Normal/none
2–6	−	+	+	Bridging fibrosis/mild hypoxic
8	−	+	++	Pseudolobule/mild–moderate hypoxic
10	− ∼ +	+	+++	Pseudolobule/moderate hypoxic
12	++	+	+	Smaller pseudolobule/severe hypoxic
14C	+++	+	− ∼ +	Smaller pseudolobule/severe hypoxic
14R	+	− ∼ +	++	Decrease of fibrosis/mild hypoxic

TGF-β1, transforming growth factor-β1; HEP, hepatocyte; BDE, bile duct epithelium; MAC, macrophage; C, cirrhotic group with CCl_4 treatment for 14 weeks; R, recovery group with CCl_4 treatment for 12 weeks was allowed to recover during the last 2 weeks (from 13th to 14th week).

Discussion

Until now, it has been considered that MFBs are a major producer of TGF-β, especially TGF-β1 by autocrine stimulation and that TGF-β plays a prominent role in the hepatic fibrogenesis (26, 27). It has also been considered that macrophages can produce TGF-β1 in fibrotic liver (19, 20). There is a prolonged increase of TGF-β expression during hepatic fibrosis in experimental models, such as in CCl_4, diethylnitrosamine, and patients with cirrhosis induced by alcohol or viral hepatitis (11, 17–20). However, we had demonstrated that MFBs and macrophages increased in the development of fibrosis, but decreased when cirrhosis was induced (16). Then, how can the production of TGF-β increase despite of decrease of MFBs and macrophages in cirrhosis? From the above facts, we hypothesized that another major type cell capable of producing TGF-β might be present in the cirrhotic liver. In our results, expression of TGF-β1 was detected and increased mainly in macrophages of damaged and fibrotic areas in proportion to hepatic fibrosis instead of MFBs.

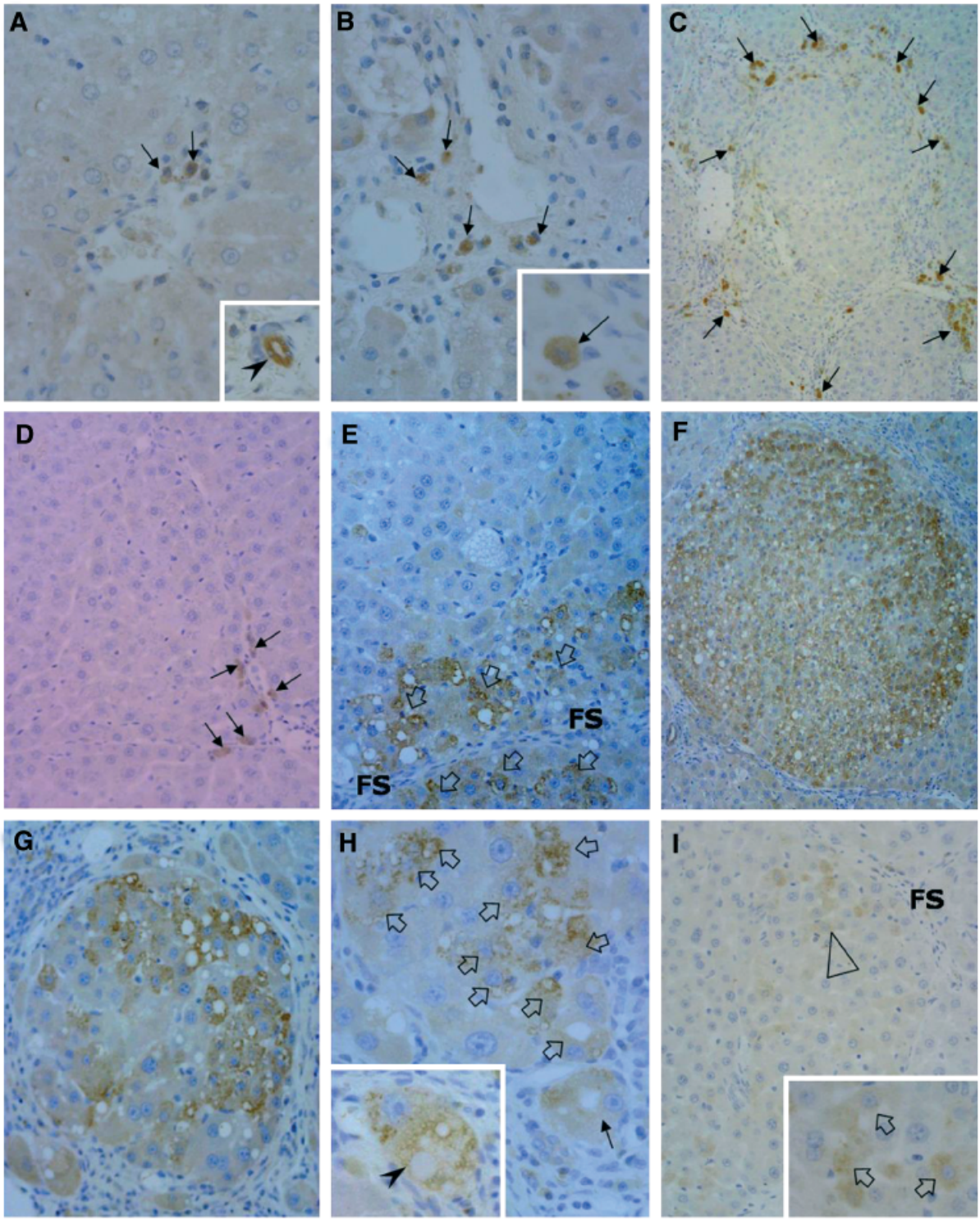

Fig. 4. Transforming growth factor-β1 (TGF-β1) expression during hepatic fibrogenesis. (A) Macrophages (arrow) expressing TGF-β1 around damaged central veins at week 2. Inset of A: bile ducts (arrowhead) expressing TGF-β1 in portal triad at week 2. (B) Macrophages (arrow) expressing TGF-β1 in the fibrous septa at week 6. Inset of B: magnification of macrophage (arrow) expressing TGF-β1. (C) macrophages (arrow) expressing TGF-β1 increased in the fibrous tissue of pseudolobule at week 10. (D) At week 14, macrophages (arrow) expressing TGF-β1 in the regression of fibrous septa during recovery. (E) Some hepatocytes (open arrow) neighboring fibrous septa express TGF-β1 at week 10. (F, G) Many hepatocytes within pseudolobules express TGF-β1 at weeks 12 (F) and 14 (G), respectively. (H) A hepatocyte (arrow) surrounded by fibrous septa express less TGF-β1 than hepatocytes (open arrow) within pseudolobule at week 14. Inset of (H): a hepatocyte (arrowhead) highly expressing TGF-β1. (I) In recovering hepatocytes, expression of TGF-β1 is decreased, but some hepatocytes (open arrowhead) neighboring still existent fibrous septa mildly express TGF-β1. Inset of (I): magnification of hepatocyte (open arrow) expressing TGF-β1 of areas indicated by open arrowhead. FS, fibrous septa. Immunostaining for TGF-β1 with hematoxylin counterstain. Original magnifications, (C, F) ×33; (D, E, G, I) ×66; (A, B, H) inset of (A, H, I) ×132; Inset of (B) ×330.

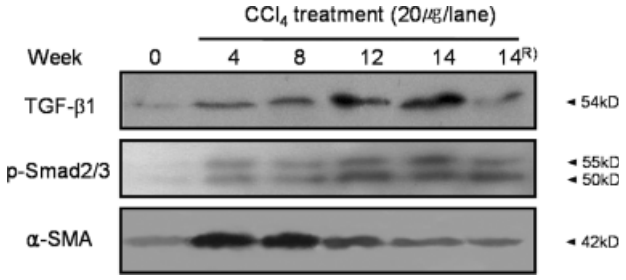

Fig. 5. Changes of transforming growth factor-β1 (TGF-β1), phosphorylated-Smad2/3 (p-Smad2/3) and α-smooth muscle actin (α-SMA) in the CCl_4-induced rat liver cirrhosis. The expressions of TGF-β1, p-Smad2/3 and α-SMA are evaluated by immunoblot at weeks 0, 4, 8, 12 and 14, respectively. As hepatic injuries developed to fibrosis and cirrhosis, a much more prominent band of TGF-β1 appeared. However, it is decreased in the rat liver of recovery group at week 14. Expression of p-Smad2/3 increased or decreased in accordance with changes of TGF-β1, but α-SMA were not coincidence with changes of TGF-β1. R. recovery group with CCl_4 treatment for 12 weeks was allowed to recover during the last 2 weeks (from 13th to 14th week).

However, macrophages expressing TGF-β1 predominantly decreased when cirrhosis occurred at week 12. Moreover, MFBs expressing α-SMA decreased at this time although TGF-β1 were increasing at cirrhotic liver (Figs 2, 3 and 5). These results of decreasing number of macrophages and MFBs were consistent with our previous study. In two studies using northern blot analysis and *in situ* hybridization, TGF-β1 mRNA was detected in MFB, macrophages (Kupffer cell) and endothelial cells of CCl_4-treated fibrotic rat liver, but not detected in hepatocytes (19, 20). Surprisingly in contrast to these studies, TGF-β1 was predominantly expressed in hepatocytes within several pseudolobules in advanced fibrotic and cirrhotic liver at weeks 12 and 14. Strong expression of TGF-β1 may be because of the considerable production of TGF-β1 in hepatocytes (Fig. 4F and G). In the recovering liver, some hepatocytes neighboring still existent fibrous septa mildly expressed TGF-β1. However, an unexpected result was that TGF-β1 expression was undetectable in HSCs and MFBs in our study. We can explain why TGF-β1 was not detected in both cells completely: TGF-β1 antibody used in this study was LC (1–30) antibody to mature TGF-β1 which is rabbit polyclonal antibody generated from synthetic peptide with the amino acid sequences of the N-terminal region of mature TGF-β1 (28). Moreover, it works in hepatocyte when they produced mature TGF-β1 (29). Jirtle et al. (29) also reported that the pre [266–278] antibody was stained to nonparenchymal periductular cells and the cells lining the sinusoids while it stained weakly in mature TGF-β1 of hepatocytes. Therefore, in our study, we can hypothesize and speculate as follows: TGF-β1 is mainly produced by MFBs and macrophages at early and middle stages of fibrotic processes, but it is predominantly released by hepatocytes when cirrhosis occurred.

There have been few studies on TGF-β expression of hepatocyte *in vivo* and *in vitro* (4, 19, 29). However, there is no experimentally proven explanation on the mechanism of TGF-β expression in hepatocytes. Recently, it has been suggested that hypoxia might be associated with fibrogenesis in fibrosis and cirrhosis (11–13). Several studies have reported that modulation of oxygen tension was recognized as an important regulator of gene activation of TGF-β in rat brain and human fibroblast and hepatoma cells (30–32). Hepatocytes are known to have a high oxygen consumption rate *in vivo* and *in vitro* and thus will respond most sensitively to changes of oxygen tension in culture (33, 34). Recently, it was reported that cellular response to hypoxia involved signaling via Smad protein in hypoxic endothelial cells by producing TGF-β2 with autocrine regulation (14, 15). Therefore, we analyzed phosphorylation of Smad2/3 and hypoxic status in hepatocytes to study the relationship between hypoxia and intracellular TGF-β signal transduction during fibrogenesis and recovery. In addition, we investigated the relationship between MFBs expressing α-SMA and hepatocytes showing phosphorylation of Smad2/3. In normal rat liver, a very weak phosphorylation of Smad2/3 in the nuclei of hepatocytes and Kupffer cells was detected throughout the liver, indicating that TGF-β might be playing a role in the maintenance of it. Our result was consistent with theories that TGF-β tonically inhibits hepatocyte growth in even intact liver and may play a critical role in the maintenance of consistent liver mass (34). From weeks 2 to 6, we found that distributions of MFBs, hypoxic hepatocytes and p-Smad2/3-expressed hepatocytes were similar to each other. At this time, it was considered that p-Smad2/3 was mainly activated by MFBs and macrophages. However, from the formation of pseudolobule at week 8, most of hepatocytes and nonparenchymal cells expressed p-Smad2/3. Moreover, distribution of hypoxic hepatocytes was only similar to that of p-Smad2/3 expressed hepatocytes, but not to that of MFBs. As hepatocytes within pseudolobules became more hypoxic, the phosphorylations of Smad2/3 on hepatocyte were more intense from weeks 10 to 14. At week 14, most hypoxic hepatocytes showed maximal phosphorylation of Smad2/3 within their nuclei. At this time, an important thing was that MFBs expressing α-SMA were nearly absent around hepatocytes with maximal expression of p-Smad2/3. Then, how is p-Smad2/3 activated in

hepatocytes without activation of HSCs? One possibility is that endogenous TGF-β produced by hypoxic hepatocyte might activate phosphorylation of Smad2/3 in an autocrine manner. Surprisingly, in the recovering liver, we found another important fact that most MFBs disappeared in the fibrous septa, whereas phosphorylation of Smad2/3 still existed in the hepatocytes of hypoxic areas. Above results were supported by expression of TGF-β1 on the hypoxic hepatocytes as described in the results of our study. It is therefore likely that hypoxic hepatocytes in cirrhotic liver predominantly produce endogenous TGF-β instead of HSC and MFB. Moreover, results obtained in our study shed light on an important aspect as to the significance of hypoxia in the TGF-β production of hepatocytes.

In various experimental models, it was shown that therapeutic strategies blocking TGF-β receptor II (TGF-RII) are able to significantly reduce or abolish fibrogenesis (35–37). However, anti-TGF-β therapies have to be carefully considered to specific cell types and physiological stage because TGF-β plays many essential roles in important cellular response. Therefore, it is important to get further insight into the source of TGF-β during hepatic fibrogenesis. It would be even more useful to prevent or improve hypoxic hepatocytes, which produce TGF-β than blocking TGF-βRII. Harvey et al. (38) reported that hepatocytes in cirrhotic livers had normal metabolic capacity but were constrained by a deficit in oxygen supply and that interventions aimed at increasing oxygen supply to the liver might have both short- and long-term therapeutic value in the management of cirrhosis. They also described that the potential therapeutic role in cirrhosis of oxygen supplementation by inhalation was likely to be limited except as support of acute hepatic failure. Froomes et al. (39) showed that the capacity to deliver oxygen by face mask to human patients fell short of the full therapeutic requirement for change in arterial and portal pO_2, as demonstrated in rats (39–41). However, hepatic oxygen supplementation by available techniques should be further investigated as a therapeutic tool for improving liver function in cirrhosis (38).

In conclusion, we put forward the hypothesis that TGF-β is mainly produced by MFBs and macrophages at early and middle stages of fibrotic processes, but it is predominantly released by hypoxic hepatocytes in the last fibrotic stage or cirrhosis. The differential regulation of TGF-β1 production in parenchymal and nonparenchymal cells during fibrogenesis, which was proven in our study, should contribute to understanding the mechanism of hepatic fibrosis and its treatment.

Acknowledgements

The work presented in this paper was supported by a grant (M101KH010001-03K080100514) from the center for biological modulators of the 21st century frontier R&D program, the ministry of science and technology, Korea and the Brain Korea 21 Project in 2004.

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