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Use of Multiple Markers for *In Vivo* Characterization and Quantitation of Hepatic Stellate Cells in Experimental and Human Liver Cirrhosis

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Although there are several known markers for hepatic stellate cells (HSC), few studies have used multiple markers *in vivo* to compare models of experimental cirrhosis and human cirrhosis. The number and phenotype of HSCs was examined using the markers desmin, α-smooth muscle actin (α-SMA), platelet-derived growth factor receptor-β (PDGFR-β), glial fibrillary acidic protein (GFAP) and basic fibroblast growth factor (bFGF). Human cirrhosis was compared to two rat models of cirrhosis: bile duct-ligated (BDL) and carbon tetrachloride (CCl_4)-induced. Expression of antigens on HSCs in lobular areas and fibrous septae were visualized by immunohistochemical staining and quantified by image analysis. In both the BDL and CCl_4 models, there was a significant increase in desmin and PDGFR-β^+ cells in the lobular areas that correlated with the disease progression. Lobular GFAP expression in sham controls was significantly greater than desmin expression, indicating a GFAP$^+$/desmin$^-$ lobular HSC subset. During the development of cirrhosis, a significant down-regulation of lobular GFAP expression occurred. In human cirrhosis, there was significant upregulation of PDGFR-β expression in lobular areas and α-SMA expression in the portal tract, but no GFAP or bFGF expression. In conclusion, similar patterns of expression of multiple HSC markers were observed in biliary and hepatitic cirrhosis, both experimentally and in human. Cirrhosis is associated with an increased number of HSC with expansion of a desmin$^+$/GFAP$^-$ population.

Introduction

It has been well established that hepatic stellate cells (HSCs) play a major role in the process of liver fibrogenesis[1)2)]. HSCs are perisinusoidal cells together with their role in the production of extracellular matrix components (ECM), participate in the storage of vitamin A and in the maintenance of the sinusoidal structure of the hepatic acinus. Once liver injury occurs, HSCs become activated, proliferate and are transformed into cells with the phenotypic and biochemical features of myofibroblasts, and in this activated state they can synthesize ECM components[1)~4)].

Several growth factors are considered to play a role in HSC proliferation and activation. In particular, platelet-derived growth factor (PDGF) has been shown to mediate the proliferation[5)~7)], migration, and ECM synthesis of HSCs via specific receptors[8)~10)]. In normal liver, PDGF and platelet-derived growth factor receptor-β (PDGFR-β) subunit expression is limited to a few mesenchymal cells of the portal tract stroma and vessels[11)].

Basic fibroblast growth factor (bFGF) is another factor which stimulates proliferation of HSCs[12)~14)]. In normal liver, low-level bFGF expression has been detected in the sinusoidal and endothelial cells of major vessels. However, the expression of bFGF has been shown to increase following bile duct ligation and administration of chronic carbon tetrachloride (CCl_4) in rat models[15)]. In contrast to these two mitogens, transforming growth factor-β has a dual function. It inhibits HSC proliferation but provides a major stimulus for ECM production[16)17)]. In the rat, the main marker for quiescent HSCs has been desmin but in humans desmin is not expressed by HSC. The activated HSC phenotype in the rat is characterized by expression of α-smooth muscle actin (SMA)[18)19)]. Other markers of HSCs include glial fibrillary acidic protein (GFAP), which is an intermediate filament generally considered to be specific for cells of astroglial lineage[20)]. Extraglial GFAP has been described in Schwan cells and non-neural tissues[21)]. HSCs in normal rat liver contain GFAP[22)] and GFAP has recently been evaluated as an alternative marker of HSCs[23)].

Although many of the pathways which regulate HSC proliferation and phenotype have been well established *in vitro*[24)], only a few studies on the patterns of expression of multiple HSC markers and potential HSC growth factors *in vivo* during chronic liver injury have been reported. The aim of this study was to quantitate and clarify the localization *in vivo* of the HSC, which express desmin, α-SMA, PDGFR-β, GFAP and bFGF during the evolution of cirrhosis, in experimental models and to compare this with their patterns of expression in established human cirrhosis.

Materials and Methods

Animals

Adult male Sprague Dawley rats, weighing 220 ~270 g, were used in this study. All rats were fed *ad libitum*, and received human care in compliance with the institutional guidelines for the care and use of laboratory animals in research.

One experimental model to induce liver injury and fibrosis involved administration of CCl_4 in 16 rats. All rats were started at a dose of 0.04 ml of CCl_4 (made up to 2 ml with vegetable oil). Animal weight was monitored and the dosage of CCl_4 adjusted accordingly. Liver tissues were harvested 4~5 months following CCl_4 administration. According to this protocol, 6 rats developed septal fibrosis (group I), 6 rats developed early cirrhosis (group II), and 4 rats showed cirrhosis (group III). Six untreated rats served as controls. Another liver cirrhosis model involved bile-duct ligation (BDL) under ether anesthesia in 10 rats; 6 sham-operated animals served as controls. Liver samples were frozen for immuno-histochemical studies.

Human tissues

Normal human liver tissue was obtained from four liver transplantation donors as wedge sections and from the unaffected portion of the liver in five patients requiring liver surgery for hepatic tumors. Liver cirrhosis samples were collected from resected liver specimens at the time of liver transplant. Primary diagnoses were primary biliary cirrhosis (PBC) in 5 and autoimmune chronic active hepatitis (AIH) in 6 patients. Approve to use human tissues was obtained from the institutional committee. Informed consent was obtained from all living patients enrolled in the study.

Immunohistochemical staining of liver sections

Liver tissue was embedded in OCT compound (Miles Inc. Elkhart, IN) and frozen in the vapor phase of liquid nitrogen. Frozen sections, 6-mm thick, were cut onto gelatin-coated slides and air-dried for 1 hour. All incubation and washes were done at room temperature. The sections were fixed in acetone for 10 minutes then air-dried for 1 hour.

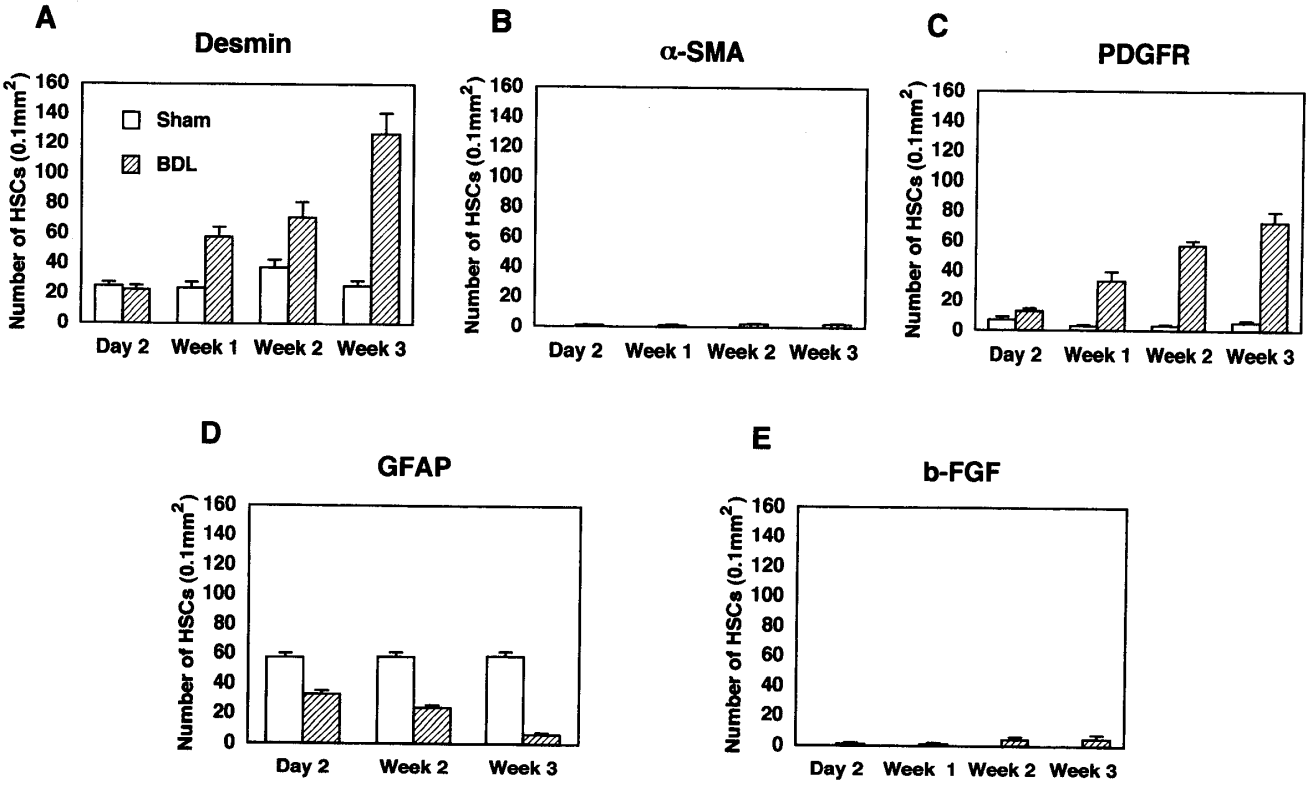

Fig. 1 Number of HSCs positively stained by five markers per 0.1 mm^2 in the lobules of control rats and during cholestasis development
Computer morphometry was used to quantify the optical density of the signal generated by the immunohistochemical staining of HSCs. An optical threshold and filter combination was set to select only the diaminobenzidine.
Vertical bars represent 1 SEM.

Primary antibodies were: mouse monoclonal antibody to bFGF (kindly provided by Dr. John Kenney, Syntex Research, Palo Alto, CA) at a concentration of 20 mg/mL; mouse monoclonal antibody to desmin (D1033; Sigma) at 1: 100 dilution; mouse monoclonal antibody to α-SMA (A 2547; Sigma) at 1: 200 dilution; negative control mouse monoclonal antibody (M7894 ; Sigma) at a concentration of 20 μg/mL; rabbit polyclonal antibody to bovine GFAP (Z0334; Dako, Copenhagen, Denmark) 1: 100 dilution; rabbit polyclonal antibody to PDGFR (SC432; Santa Cruz Biotechnology, Santa Cruz, CA) at 1: 20 dilution, and normal rabbit serum control (Dako) at 1: 500 dilution. All antibodies were diluted in PBS plus 5% normal human serum except where stated otherwise. Primary antibody was added to the sections for 30 minutes, then rinsed and washed in PBS for 5 minutes.

Mouse monoclonal antibodies were detected with rabbit-anti-mouse Ig conjugated with horseradish peroxidase (P0161 ; Dako) at 1: 50 dilution for 30 minutes. Antibody to GFAP was detected with swine antibody to rabbit immunoglobulin (Z0196 ; Dako) at 1: 25 dilution for 15 minutes, washed for 5 minutes in PBS, then incubated with rabbit peroxidase-anti-peroxidase complexes (Z0113 ; Dako) at 1: 50 dilution for 15 minutes. Antibody to PDGFR was detected with swine-anti-rabbit Ig conjugated to biotin (E0431 ; Dako) at 1: 500 in PBS plus 1% BSA for 15 minutes, and the sections were washed for 5 minutes in PBS, then incubated with streptavidin-conjugated to horseradish peroxidase (P0397 ;

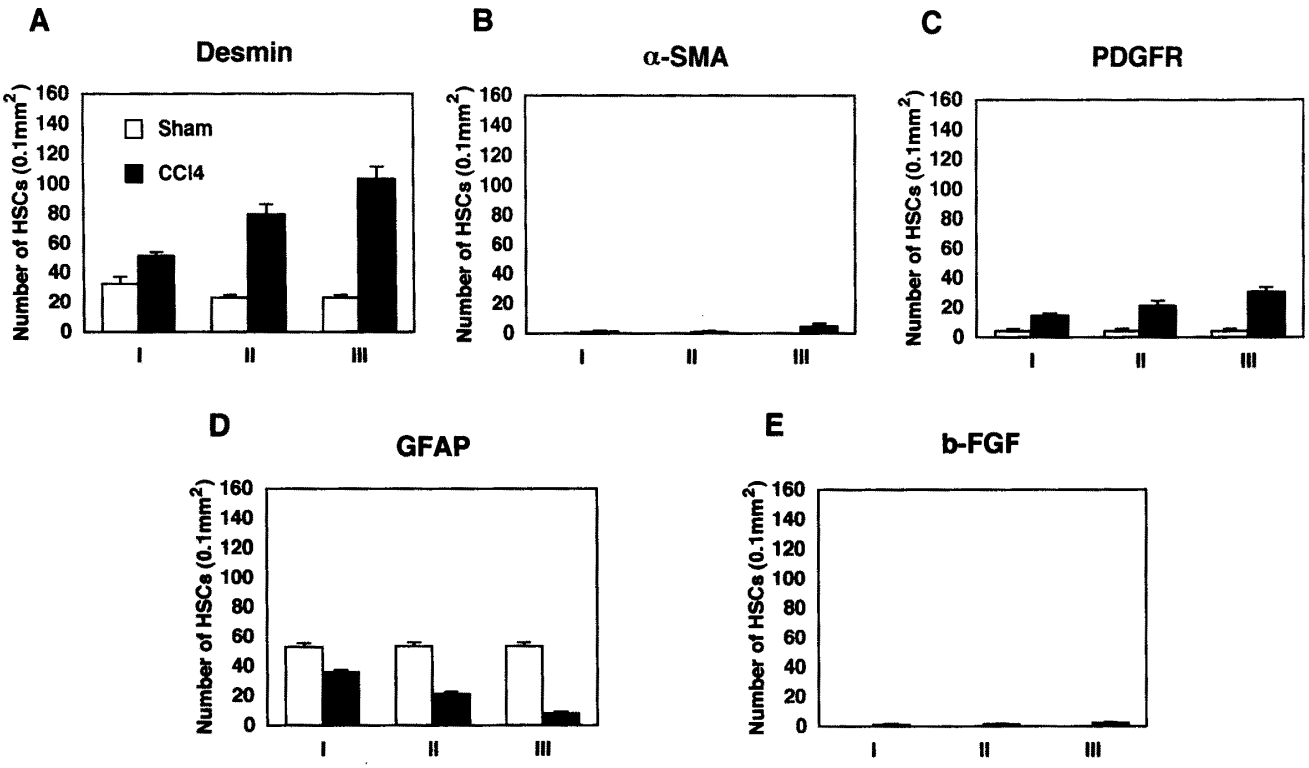

Fig. 2 Number of HSCs positively stained by five markers per 0.1 mm^2 in the lobules of control rats and after CCl_4 administration
Group I: 6 rats with septal fibrosis, Group II: 6 rats with early cirrhosis, Group III: 4 rats with cirrhosis. Vertical bars represent 1 SEM.

Dako) at 1: 250 in PBS plus 1% BSA for 15 minutes.

For staining with biotin-labelled antibodies, endogenous biotin binding activity was blocked, prior to addition of the primary antibody, by addition of 0.1% w/v avidin (Sigma) diluted in PBS plus 1% BSA for 20 minutes. This was followed by washing for 5 minutes in PBS, then 0.01% biotin (Sigma) diluted in PBS plus 1% BSA was added for 20 minutes. All sections were washed for 5 minutes in PBS, then staining was visualized by incubation for 10 minutes with a solution containing 1 mg/ml of diaminobenzidine tetrahydrochloride (Sigma), 0.01% H_2O_2 and 0.3% sodium azide in 0.05 mol/L Tris buffer pH 7.0. The slides were rinsed with water, air-dried and counterstained in Mayer's hematoxylin for 1 minute, then dehydrated and mounted in DePeX (BDH Chemicals, Kilsyth, Australia).

Immunohistochemistry image analysis

The stained cells were counted with the assistance of an image analysis system attached to a light microscope (Chromatic Colour Image Analysis System, Leitz, Sydney, Australia). For HSCs in lobular areas, cells were counted within ten separate randomly selected regions, incorporating a total area of 0.1 mm^2. For portal tracts (fibrous septae), the percentage of the total section area occupied by the positively stained cells was calculated using ten randomly selected regions, incorporating a total area of 2.0 mm^2.

Results

Lobular HSC markers in experimental cirrhosis

In both experimental models, a progressive increase in lobular HSCs, identified by their stellate morphology, extensive long cytoplasmic processes and expression of desmin or PDGFR, was

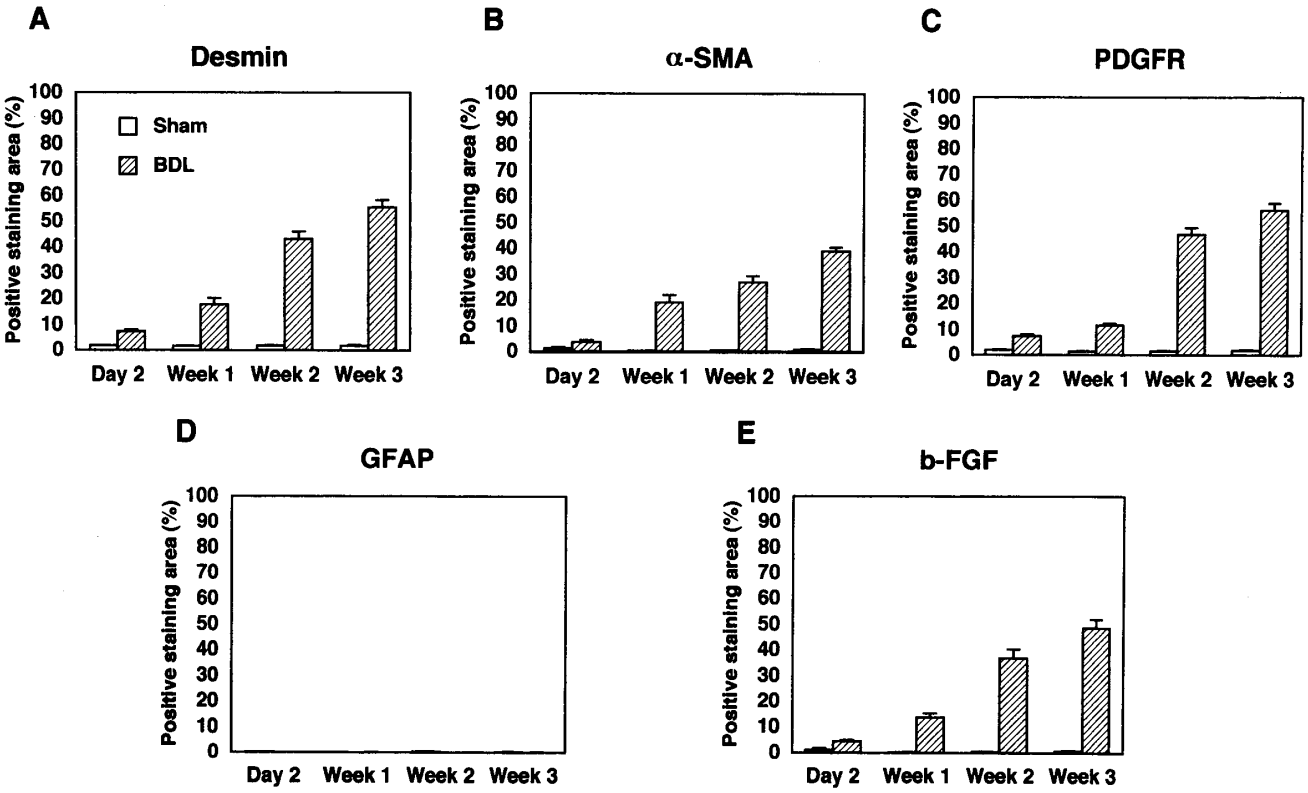

Fig. 3 The positively stained area in the portal tracts is represented by % of the total section area occupied by positively stained cells by five markers in control rats and during cholestasis development
Computer morphometry was used here also. Vertical bars represent 1 SEM.

observed. The number of desmin$^+$ cells in the lobular areas was increased six-fold in the BDL rat model (Fig. 1A) and five-fold in the CCl_4 model, compared to controls (Fig. 2A).

These increases correlated with the disease progression in both models. Lobular PDGFR$^+$ cells were also increased 5.7-fold in the BDL model and two-fold in the CCl_4 model with the progression of cirrhosis in both models (Figs. 1C and 2C). In sham control rats, lobular GFAP expression on HSCs was significantly greater than desmin expression, with approximately 30% more GFAP$^+$ cells than desmin$^+$ cells, indicating majority of lobular HCS is GFAP$^+$/desmin$^-$ subset in control rats. During the evolution to cirrhosis in both rat models, a significant decrease in the number of lobular GFAP$^+$ HSCs (Figs. 1D, 2D and 6B, C), which was inversely proportional to the increase in desmin$^+$ HSCs, was observed (Figs. 1A and 2A).

α-SMA and bFGF in the lobular areas of both cirrhotic liver and sham control animals were only slightly expressed (Figs. 1B, E and 2B, E).

Portal and fibrous septal expression of HSC markers in experimental cirrhosis

In the portal tracts, desmin, α-SMA, PDGFR and bFGF, but not GFAP, were progressively upregulated with the development of cirrhosis in both experimental models (Figs. 3, 4). During disease development, the portal tracts became extended and eventually formed fibrous septae. The extent of staining of these portal tract/fibrous septa areas was much greater in BDL compared to CCl_4. In the BDL model, the expression of desmin, α-SMA, PDGFR and bFGF was strongest in the areas that surrounded proliferating

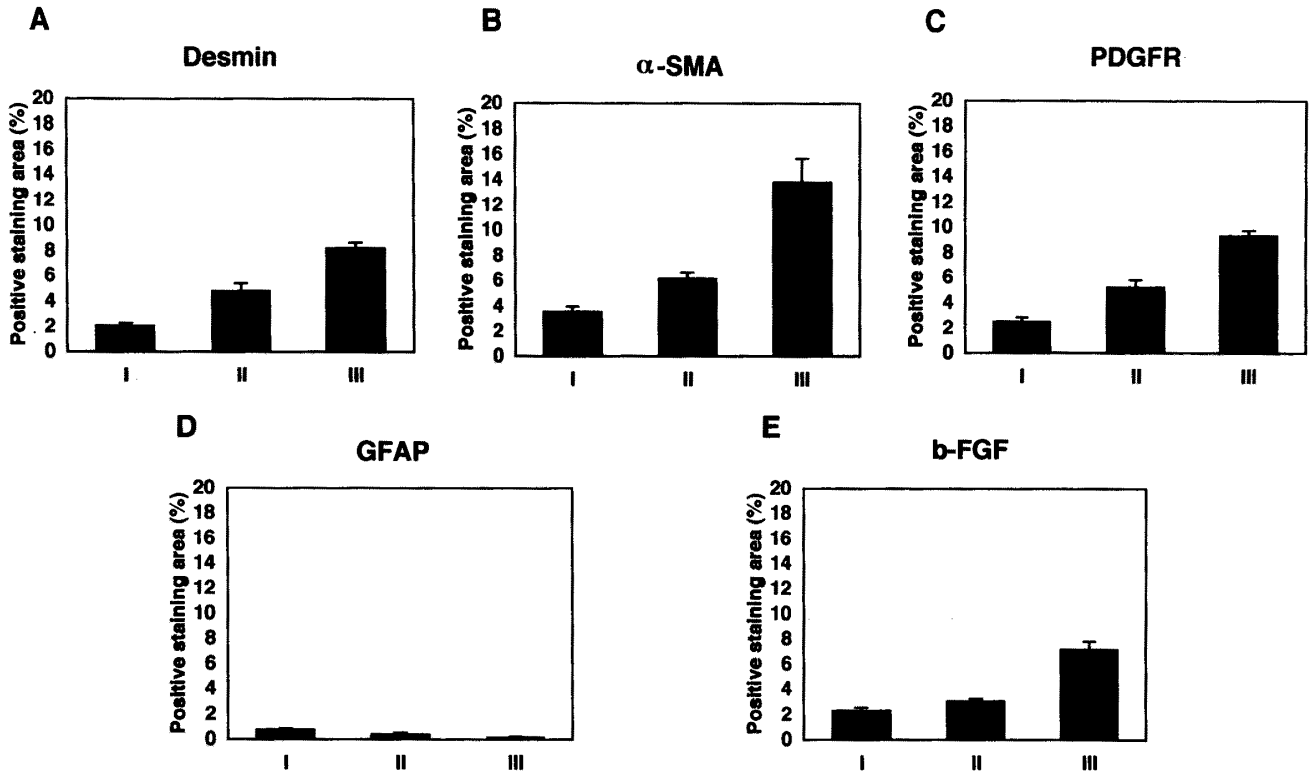

Fig. 4 The positively stained area in the fibrous septae is represented by % of the total section area occupied by positively stained cells in control rats and after CCl_4 administration.

cholangioles (Figs. 6D, E, F).

Lobular HSC markers in normal human liver and in human cirrhosis

In the normal human liver, α-SMA$^+$ cells were recognized not only in the portal tracts and the central veins but also in the perisinusoidal spaces. In contrast, there was no expression of desmin and much less expression of GFAP by human HSC.

In human cirrhosis, similar patterns of expression were seen in PBC and AIH (Fig. 5A). In the lobular areas, PDGFR expression was significantly increased compared to normal human liver but GFAP and bFGF expression was not increased. Lobular α-SMA$^+$ cells were not increased in cirrhosis compared to normal human liver.

Portal and fibrous septal expression of HSC markers in normal and cirrhotic human liver

The portal tracts of normal human livers showed low levels of expression of PDGFR and GFAP (Fig. 5B). The portal tracts of normal human liver had moderate numbers of α-SMA$^+$ HSCs. In both PBC and AIH, the α-SMA$^+$ cells were significantly increased in the fibrous septae and in the perisinusoidal areas in surrounding nodules (Figs. 5B and 7A, B). GFAP staining was detectable in the fibrous septae, especially those located at the limiting plate (Figs. 7C, D). In the fibrous septae of human cirrhotic liver, a significant upregulation in PDGFR and bFGF expression was observed (Figs. 5B and 7E, F) in both PBC and AIH.

Discussion

The expression of multiple markers of HSCs in normal liver was compared with that in biliary and hepatitic cirrhosis and between animal models and human cirrhosis. The comparison of HSC markers in human and rat liver showed that the

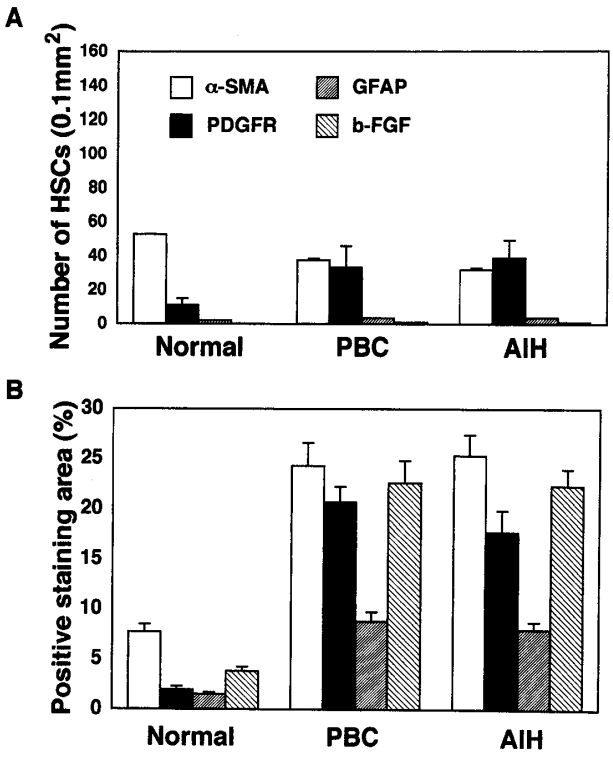

Fig. 5 A: Number of HSCs positively stained by five markers per 0.1 mm² in lobules of normal human liver and in tissue sections from patients with PBC and AIH.
B: The positively stained area in the fibrous septae is represented by % of the total section area occupied by positively stained cells in normal liver and diseased liver (PBC and AIH).
Vertical bars represent 1 SEM.

most efficient markers of HSCs were α-SMA and PDGFR in humans and desmin and PDGFR in rats.

In rat liver cirrhosis

In rat models of cirrhosis, immunostaining for desmin and GFAP revealed a minor population of desmin$^+$/GFAP$^-$ HSCs in a quiescent state which was expanded in cirrhosis.

Although the main increase in ECM deposition in cirrhosis occurs in the portal tract regions[25], the findings here clearly show an increase in HSC in the lobular regions within regenerating nodules. This is consistent with the observations of major changes in pericellular ECM formation and composition within regenerating lobules during the cirrhotic process[1)~3)]. Notably, the perisinusoidal basement membrane is thickened and has an altered composition in cirrhosis. In the experimental models studied here, the use of desmin as a marker indicated a major increase in HSC within the lobular region.

In contrast to PDGFR and desmin immunostaining, the proportion of lobular HSCs which expressed GFAP decreased in the experimental models. In both BDL and CCl_4-induced cirrhosis, the level of GFAP remained constant in spite of the increased numbers of HSCs identified by desmin and PDGFR expression. This finding has been reported previously[23)] and clearly indicates that GFAP is downregulated on lobular HSCs during the evolution to experimental cirrhosis.

In normal rat liver

In the quiescent state, many more lobular GFAP$^+$ cells than desmin$^+$ cells were present, indicating the existence of a significant GFAP$^+$/desmin$^-$ HSC subset. Our results here showed that the majority of 'resting' lobular HSCs were GFAP$^+$/desmin$^-$ cells, although minority populations of GFAP$^+$/desmin$^+$.

It is possible that the loss of GFAP expression in cirrhosis represents a downregulation of GFAP on desmin expressing cells or an expansion of the original GFAP$^-$/desmin$^+$ subset, or both. The functional characteristics of these different subsets with respect to ECM production, proliferation, endothelial-released cytokine and nitric-oxide production needs to be clearly defined. Such analyses are important for understanding the role of HSCs in the pathogenesis of cirrhosis and portal hypertension. On the basis of the data presented here it appears that the GFAP$^-$/desmin$^+$ subset is associated with the development of experimental biliary and hepatitic cirrhosis.

In normal human liver

Identification of HSCs in human liver was more

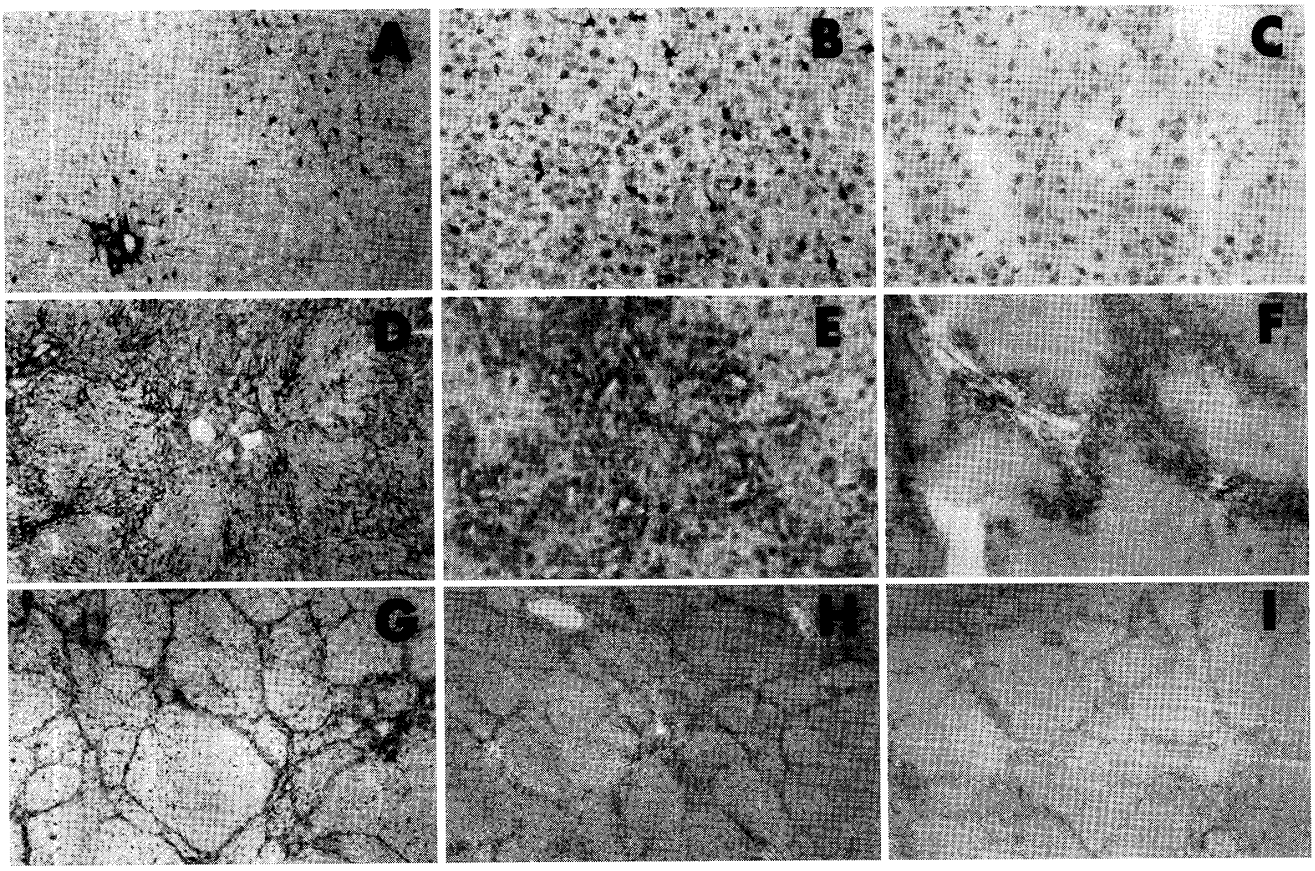

Fig. 6 Photomicrographs of immunostaining
For desmin in sham control (A), BDL rat model (D) and CCl_4 rat model (G); for GFAP in sham control (B), and CCl_4 rat model (C); for PDGFR in the BDL rat model (E) and CCl_4 rat model (H); for bFGF in the BDL rat (F) and CCl_4 rat (I).

difficult than in the rat models, as desmin, which was expressed by both resting and activated rat HSCs, does not appear to be expressed by human HSCs[26]. The most efficient marker for human HSCs among those we examined was α-SMA, which was expressed on large numbers of lobular HSCs in normal and cirrhotic human liver as well as on vascular endothelium. Detection of lobular α-SMA$^+$ cells in normal liver is dependent on the technique used, as less sensitive techniques only detect their expression in the fibrous septae of cirrhotic livers while more sensitive techniques give similar results to those reported by Schmitt-Graff et al[19] and Yamaoka et al[26].

GFAP was not an efficient marker of human HSCs in either normal or cirrhotic liver compared to its labelling in the rat. GFAP expression in humans is detected in a limited number of HSCs in the perisinusoidal region at the edge of the limiting plate of cirrhotic nodules.

In human liver cirrhosis

Similar to the experimental models the PDGFR$^+$ cells in lobular areas increased in human cirrhosis. In cirrhotic human liver, bFGF stained the fibrotic septae strongly. However, this might not have been staining of HSCs, as bFGF binds to proteoglycan components of the extracellular matrix which are likely to be concentrated in areas of fibrosis[12]. In view of the marked increase in ECM production at portal tracts in cirrhosis, it is not surprising that these fibrous septae are strongly positive for the HSC markers, desmin, α-SMA, PDGFR and bFGF.

In conclusion, both HSCs in experimental

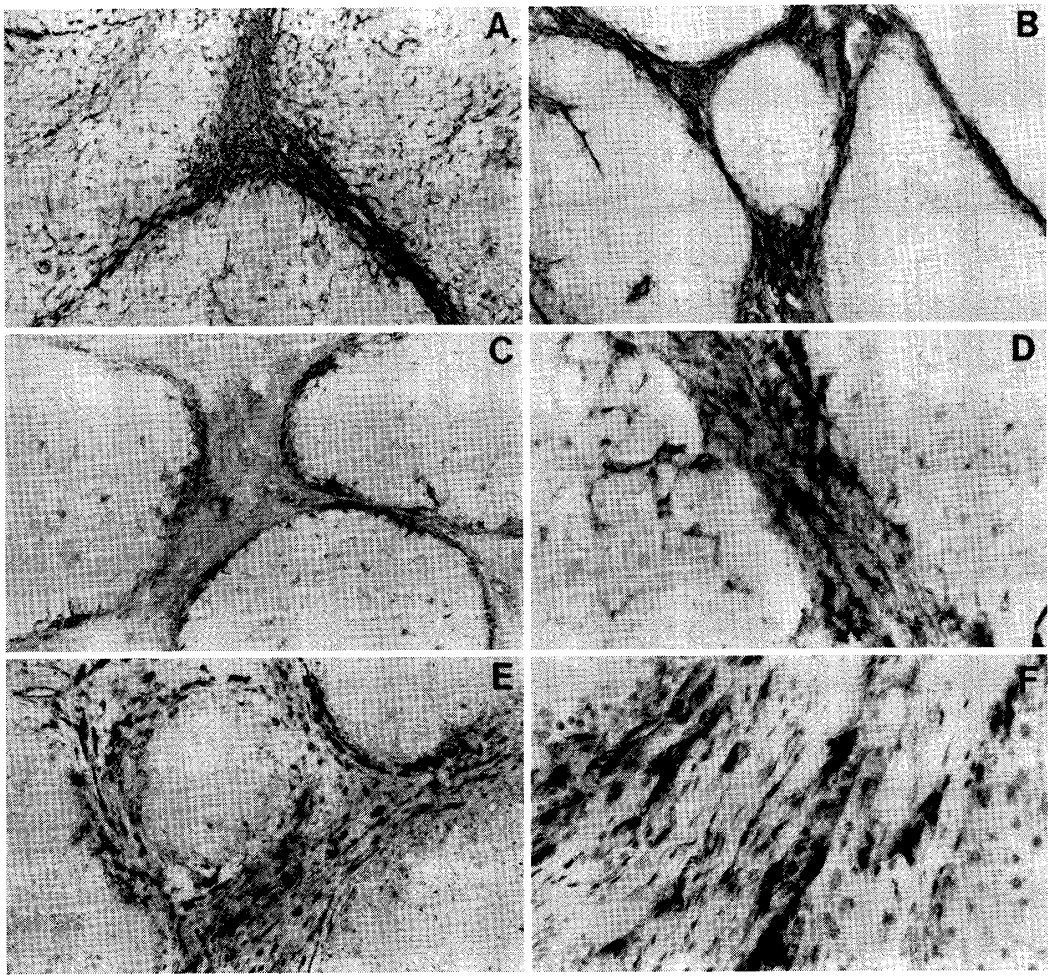

Fig. 7 Photomicrographs of immunoperoxidase staining
α-SMA in liver specimens from a patient with AIH (A) and a patient with PBC (B). GFAP staining in a patient with PBC (C). $GFAP^+$ cells are present in the fibrous septae and some are in the rim of HSCs (D). bFGF staining in a patient with AIH (E). $bFGF^+$ cells are limited almost to the fibrous septae with less strong expression compared to other markers (F).

model and human cirrhosis shared a similar surface marker such as PDGFR and bFGF suggesting the existence of many similar *in vivo* molecular fibrogenic pathways in fibrosis and tissue remodeling. Multiple markers of HSC are now available and their use *in vivo* to examine experimental and human cirrhosis is necessary to identify the role of HSC in liver remodeling. It is clear that more markers are required, particularly to detect HSCs in humans and those in all activation states. It will also be necessary to use multiple markers to clarify HSC subsets with particular physiological and pathological functions.

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多種のマーカーを用いたHSCの生体内での動物モデルおよびヒトの肝硬変における特性と定量化

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肝線維化発症のメカニズムにおいて，肝の非実質細胞の一つである hepatic stellate cell (HSC) が中心的役割を演ずることが知られている．そこで本研究では HSC が肝の線維化に伴ってどのように変化するかを検討した．HSC は，類洞の傍らの肝細胞と内皮細胞の間に，静止状態もしくは活性状態（活性型）の二つの状態で存在する．活性化は慢性的に肝に炎症が起こることで生じることが知られているが，HSC が活性化されると，HSC 自体が形態変化して樹枝状突起がのびた細胞へと変化する．この活性化された細胞の表面には PDGF や TGF-β に対するレセプターが提示され，細胞分裂は活発になりコラーゲン産生が亢進する．そこで本研究においてはラットを用いた二つの肝硬変モデル（胆汁鬱滞型モデルと肝炎型モデル）を作製し，線維化の各段階において活性型 HSC マーカー（desmin, bFGF, α-SMA, PDGFR）と静止期 HSC マーカー（GFAP）を用いて免疫染色を行い，定量化を行った．さらにヒトの肝硬変組織においても HSC マーカーを用いた免疫染色を行った．実験モデルにおいては，肝硬変線維化の進展に伴い，小葉内では desmin と PDGFR で染色される活性型 HSC が著明に増加し，GFAP で染色される静止期 HSC は減少した．fibrous septae では，desmin，α-SMA，PDGFR，bFGF 陽性の HSC が増加していた．一方ヒトの肝硬変組織では，PBC および AIH 症例ともに小葉内では PDGFR 陽性 HSC が増加しておりラット肝硬変モデルの結果と一致した．