J Korean Med Sci 2001; 16: 439-47
ISSN 1011-8934

Parenchymal and Nonparenchymal Cellular Responses in Human Hepatic Regeneration

To characterize cellular responses during hepatic regeneration, we examined 13 explant livers and 5 liver allografts by immunohistochemistry for cytokeratin 7, HepPar1, CD68, α-smooth muscle actin (α-SMA) and proliferating cell nuclear antigen as well as reticulin and Masson-trichrome staining. Within a week after liver damage, elongated CD68-positive cells were detected along the border of necrotic area. The number of α-SMA-positive cells was slightly increased along the sinusoids. Ductular proliferation or fibrosis was negligible. After one or two weeks, the size and number of CD68-positive cells were markedly increased. α-SMA-positive cells increased in number within lobules and portal tracts. Ductular proliferation occurred predominantly at the limiting plate or along the border of necrotic areas. After one month, necrotic parenchyma was replaced by many ductules, CD68-positive cells, α-SMA-positive cells. Nodules of regenerating hepatocytes and irregular fibrosis were diffusely present. Other nonparenchymal cells were not significantly changed. These observations indicate that chronological interaction between nonparenchymal and parenchymal cells occur during the course of human hepatic regeneration and suggest extensive porto-periportal fibrosis more than a few months after the onset of fulminant hepatitis is a major indicator of chronic functional impairment necessitating liver transplantation.

Key Words : *Liver Failure; Hepatitis; Liver Regeneration; Kupffer Cells; Hepatic Stellate Cell; Hepatic Stem Cells; Bile Ducts; Epithelium*

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Received : 6 February 2001
Accepted : 2 April 2001

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INTRODUCTION

Diffuse liver cell damage can develop due to many causes including overwhelming viral infection, superinfection with a second virus, microcirculatory failure, and various drugs and toxins (1). Sequential pathophysiological responses to diffuse liver cell necrosis have been well documented. Cellular kinetics of hepatic stellate cells (HSCs) in association with hepatic fibrogenesis and stem cells with hepatic regeneration have been carefully elucidated mostly in animal models (2, 3). There are fewer studies dealing with these responses in human liver because it is practically difficult to obtain and ethically improper to manipulate human tissues at different time courses of liver regeneration. Since liver transplantation has become a popular therapeutic modality for acute or chronic liver cell damage, failed liver explants or a native liver in auxiliary partial orthotopic liver transplantation are available at different stages of liver regeneration (4).

It has been suggested that liver regeneration can occur either by differentiated adult hepatocytes which retain the capability for several rounds of replication or by hepatic progenitor (stem) cells, depending on the type of the injurious agent, the nature of liver disease or the number of hepatocytes lost (5). Direct stimuli leading to proliferation of parenchymal cells and the mechanisms of liver regeneration, however, have not been clearly demonstrated. Previously overlooked to a great extent, nonparenchymal cells are emerging as major regulators in intermediary metabolism, growth, and response to injury of hepatocytes. Thus the interaction between parenchymal and nonparenchymal cells is essential in a wide variety of physiologic and pathologic responses in hepatic regeneration (6). Many cytokines produced by nonparenchymal cells or hepatocytes may promote or inhibit hepatocyte or nonparenchymal cell growth (7, 8), and HSCs play an important role in hepatic fibrogenesis (9, 10). In addition to various liver diseases, primary nonfunctioning allograft is an excellent human model of diffuse liver cell necrosis in which the onset of liver failure is clearly defined, i.e., at the time of transplantation (11).

In this study, we describe diverse cellular responses in explanted livers due to acute and chronic liver cell damage of variable duration. In early stage of regeneration, influx or proliferation of nonparenchymal cells including CD68-posi-

tive cells was predominant. During hepatic regeneration, small parenchymal cells in ductular arrangement increased prominently and some of them appreared to transform to hepatocytes in later stage. These cellular responses in human livers mirror sequential changes in animal models of massive hepatic necrosis and appear to be associated with time course after diffuse liver cell damage rather than the etiology of liver cell damage. Furthermore marked increase in number of intralobular HSCs and portal myofibroblasts may be closely related with irreversible hepatic fibrogenesis leading to liver transplantation compared with chronological histopathologic changes that were observed in completely regenerated native liver (4).

MATERIALS AND METHODS

Cases

Formalin-fixed, paraffin-embedded tissues from 13 livers that were explanted because of fulminant hepatic failure and 5 liver allografts because of primary nonfunctioning were selected from the files of the Department of Diagnostic Pathology at the Asan Medical Center, Seoul, Korea. Liver explants were obtained 1 day to over 1 yr after the onset of fulminant hepatitis. Preoperative clinical diagnosis and duration from the onset of jaundice or hepatic encephalopathy until the operation are summarized in Table 1.

Explanted livers were grossly examined and representative sections were taken from necrotic as well as viable areas. Serial sections were cut at a thickness of 4-6 μm and stained with hematoxylin and eosin for routine light microscopy.

Table 1. Summary of 18 cases examined in this study

	Case No.	Etiology	Duration*
Group 1	1	Primary graft nonfunctioning	2 days
	2	Primary graft nonfunctioning	2 days
	3	Primary graft nonfunctioning	2 days
	4	Primary graft nonfunctioning	3 days
	5	Primary graft nonfunctioning	4 days
Group 2	6	Unknown	1 week
	7	Drug-induced toxic hepatitis	10 days
	8	Unknown	2 weeks
	9	Drug-induced toxic hepatitis	3 weeks
	10	Wilson's disease	3 weeks
	11	Drug-induced toxic hepatitis	3 weeks
Group 3	12	Unknown	1 month
	13	HBV infection	1 month
	14	Drug-induced toxic hepatitis	6 weeks
	15	Drug-induced toxic hepatitis	3 months
	16	Drug-induced toxic hepatitis	4 months
	17	Drug-induced toxic hepatitis	8 months
	18	Budd-Chiari syndrome	>1 yr

*time interval from the first episode of fulminant hepatic failure until the operation

Reticulin staining was performed to evaluate the status of hepatic reticulin framework, and Massontrichrome staining to grade intralobular as well as portal fibrosis.

Immunohistochemistry

Parellel sections were stained with a panel of 5 primary antibodies: anti-CD68 (DAKO, Carpinteria, CA, U.S.A.), anti-CK7 (DAKO), HepPar 1 (12), anti-α-SMA (DAKO), and anti-proliferating nuclear antigen (anti-PCNA, DAKO) antibodies. CD68 is a marker for circulating macrophages and Kupffer cells in the liver. CK7 is exclusively expressed in bile duct epithelium. α-SMA has been used as a marker of activated HSCs. These antibodies were employed using an avidin biotin complex (DAKO LSAB kit). Diaminobenzidine in PBS with 0.3% v/v hydrogen peroxide was used as a chromogen. All of the slides were counterstained with Harris hematoxylin.

All of the fields in the slides were examined and assessed in terms of spatial relationship between parenchymal and nonparenchymal cells.

RESULTS

Thirteen cases of liver explants that were removed during transplantation showed diverse histopathologic features in viable areas. We could not detect any specific histopathologic features indicating underlying liver diseases. But five cases of primary nonfunctioning allograft displayed either periportal or centrilobular hepatocellular ischemic necrosis only. We selected the sections with representative histopathologic features and categorized 18 explanted liver tentatively into 3 groups according to the duration since the first episode of fulminant hepatic failure as follows: group 1, within a few days (<7 days) as in 5 cases of liver allograft with primary nonfunctioning; group 2, from 1 to 4 weeks; group 3, more than 1 month. In all cases, there was no evidence of acute rejection.

Reticulin framework of the liver

The reticulin framework was intact in cases of group 1, although parenchymal necrosis was diffuse. In some cases of group 2, multifocal collapse of the reticulin framework within the lobule was detected in either necrotic areas or viable parenchyma. In most cases of group 3, condensation of reticulin fibers was observed multifocally where cellular components distributed sparsely.

Distribution of CD68-positive cells

In cases of group 1, CD68 positive cells were identified mostly along the border of necrotic area and partly in the

sinusoid of the necrotic area (Fig. 1A). Most CD68-positive cells were in elongated shape with slender cytoplasms. In cases of group 2, CD68-positive cells were present diffusely in necrotic areas (Fig. 1B) and became polygonal with plump cytoplasm (Fig. 1C). The number and distribution pattern of CD68-positive cells were similar in cases of group 3.

Distribution of CK7-positive cells and HepPar 1-positive cells

In cases of group 1, the number of bile ducts was not increased, while singly scattered cells or a few clusters of cells adjacent to limiting plates were strongly positive for CK7 (Fig. 2A). In cases of group 2, CK7-positive cells were arranged in irregular shaped ductules, and were mostly located at the border of necrotic areas (Fig. 2B). In cases of group 3, there were two types of epithelial cells: CK7+/CAM5.2+cells and CK7-/CAM5.2+cells (data not shown). CK7-positive cells were much more widely distributed and were arranged mostly in ductular structure (Fig. 2C). In cases of group 1, viable hepatocytes were weakly positive by HepPar 1 (Fig. 3A). In cases of group 2, no epithelial cells were positive by HepPar 1, while in cases of group 3, grouped small regenerating epithelial cells were positively stained by HepPar 1 (Fig. 3B). Some of CK7-positive cells abutted on HepPar1-positive cells that had plump cytoplasm and multiple nuclei with prominent nucleoli (Fig. 4).

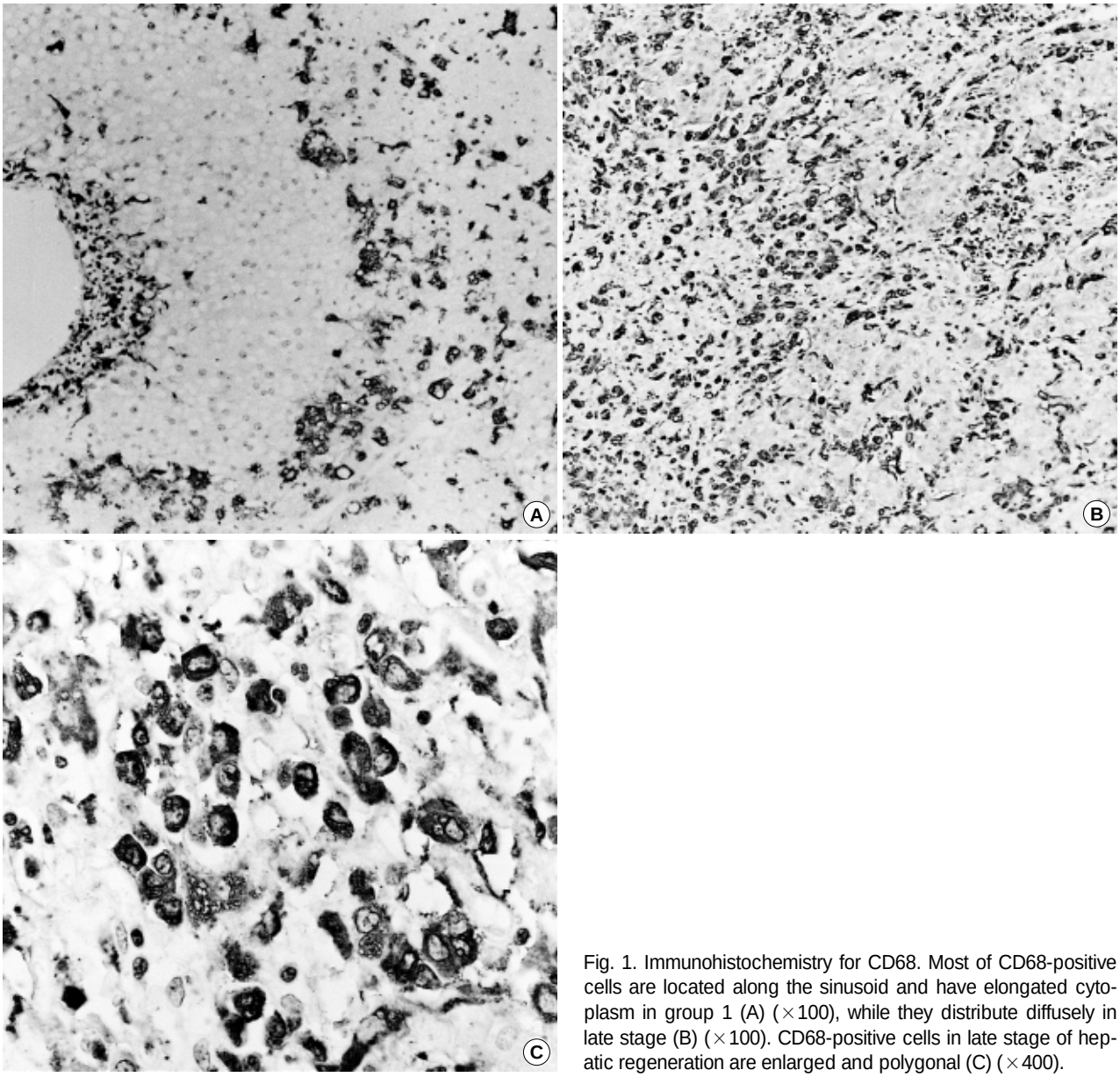

Fig. 1. Immunohistochemistry for CD68. Most of CD68-positive cells are located along the sinusoid and have elongated cytoplasm in group 1 (A) (×100), while they distribute diffusely in late stage (B) (×100). CD68-positive cells in late stage of hepatic regeneration are enlarged and polygonal (C) (×400).

Distribution of α-SMA-positive cells and fibrosis

In cases of group 1, α-SMA-positive cells were rarely detected in necrotic areas (Fig. 5A). They were flat with dendritic cytoplasmic processes and were located along the sinusoidal spaces. Within the portal tracts, α-SMA-positive cells were infrequently present. In cases of group 2, the number of α-SMA-positive cells was markedly increased, and most of α-SMA-positive cells were present within the lobules (Fig. 5B). In cases of group 3, the number of α-SMA-positive cells was markedly increased in areas of reticulin condensation within the lobule as well as portal areas where fibrosis was markedly advanced (Fig. 5C).

Distribution of PCNA-positive cells

In all primary nonfunctioning allografts, more than 90% of viable hepatocytes were strongly positive for PCNA, but cells in portal tracts were negative for PCNA (Fig. 6A). In cases of group 2, more than half of cells were strongly posivite for PCNA (Fig. 6B). Mitotic figures were occasionally present. A few PCNA-positive cells had elongated nuclei, which were suggestive of nonparenchymal cells (data not presented). In cases of group 3, the number of PCNA-positive cells was markedly decreased and the cells in portal tracts were mostly negative for PCNA (Fig. 6C).

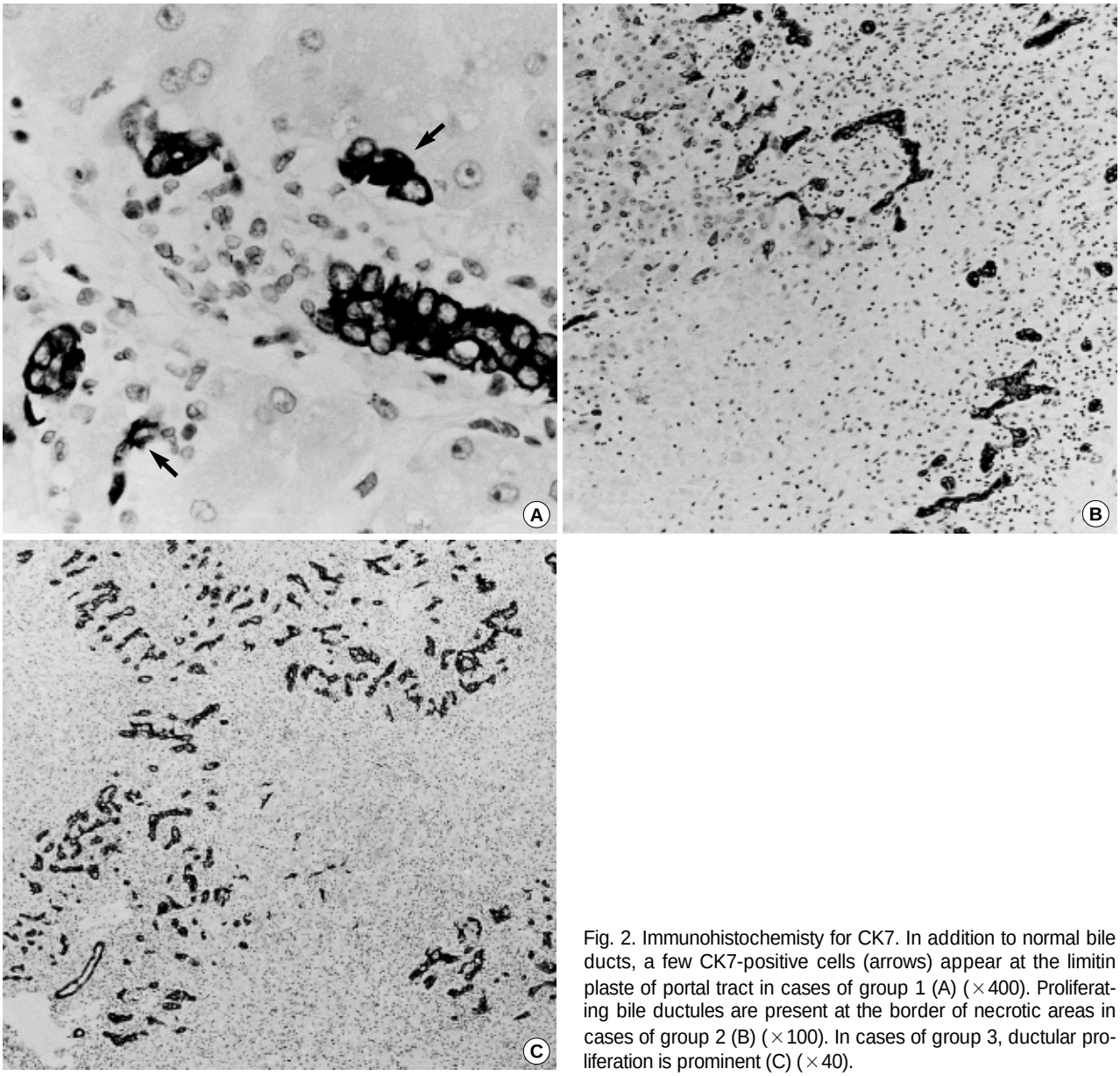

Fig. 2. Immunohistochemisty for CK7. In addition to normal bile ducts, a few CK7-positive cells (arrows) appear at the limitin plaste of portal tract in cases of group 1 (A) ($\times$400). Proliferating bile ductules are present at the border of necrotic areas in cases of group 2 (B) ($\times$100). In cases of group 3, ductular proliferation is prominent (C) ($\times$40).

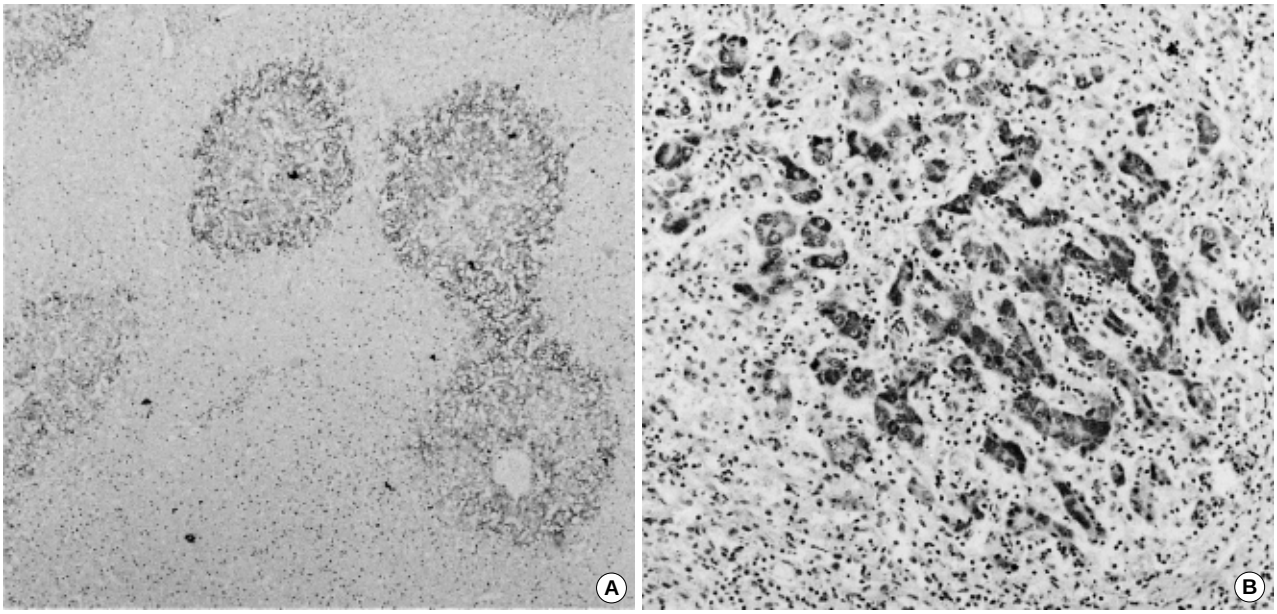

Fig. 3. Immunohistochemistry by HepPar1. In cases of group 1, viable hepatocytes are weakly positive (A) (×40). Irregularly arranged regenerating epithelial cells are positive in cases of group 3 (B) (×200).

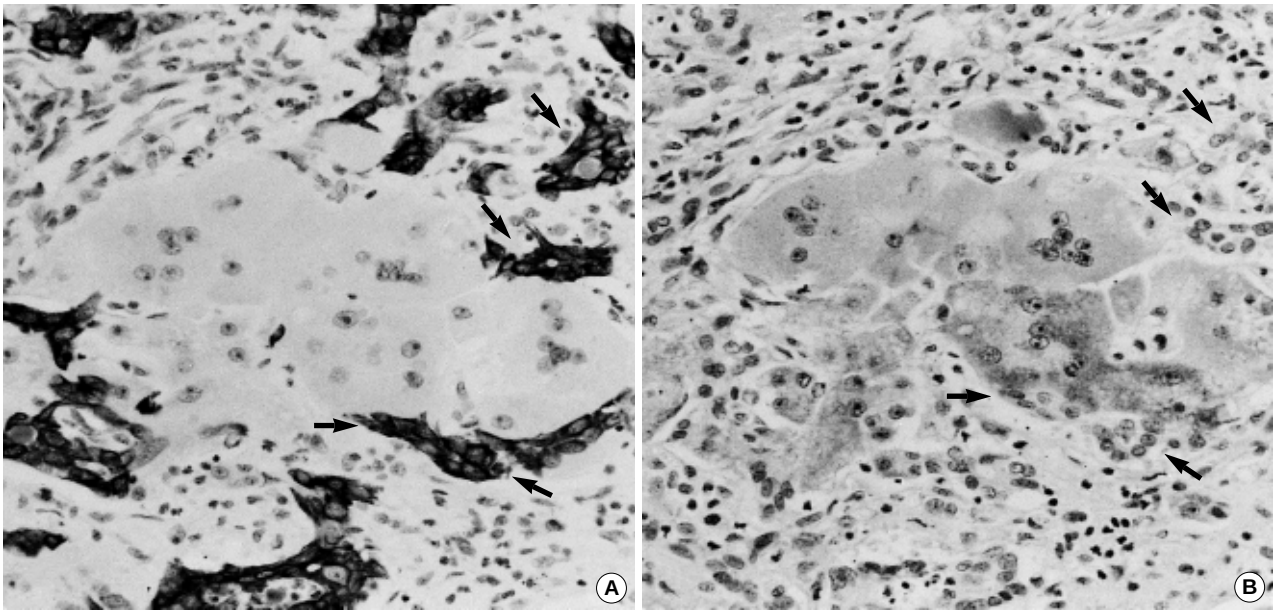

Fig. 4. Immunohistochemisty for CK7 (A) and HepPar1 (B) in cases of group 3 (×400). CK7-positive cells abut on HepPar 1-positive large multinucleated regeneratin hepatocytes.

DISCUSSION

In order to analyze cellular responses during hepatic regeneration, it is ideal to examine serial histopathologic features in the same cases. However, it was impossible in human cases ethically as well as practically due to the limitation of obtaining the samples. Thus broad spectrum of histopathologic changes during hepatic regeneration have been rarely documented in human livers (4, 11, 13, 14).

We selected cases of fulminant hepatic failure without any specific histopathologic features in association with the etiologies, but with common histopathologic changes only in terms of the time interval between the onset of acute hepatic failure and liver transplantation. Thus, diverse cellular responses in our cases may be related to the duration since the onset of diffuse liver damage.

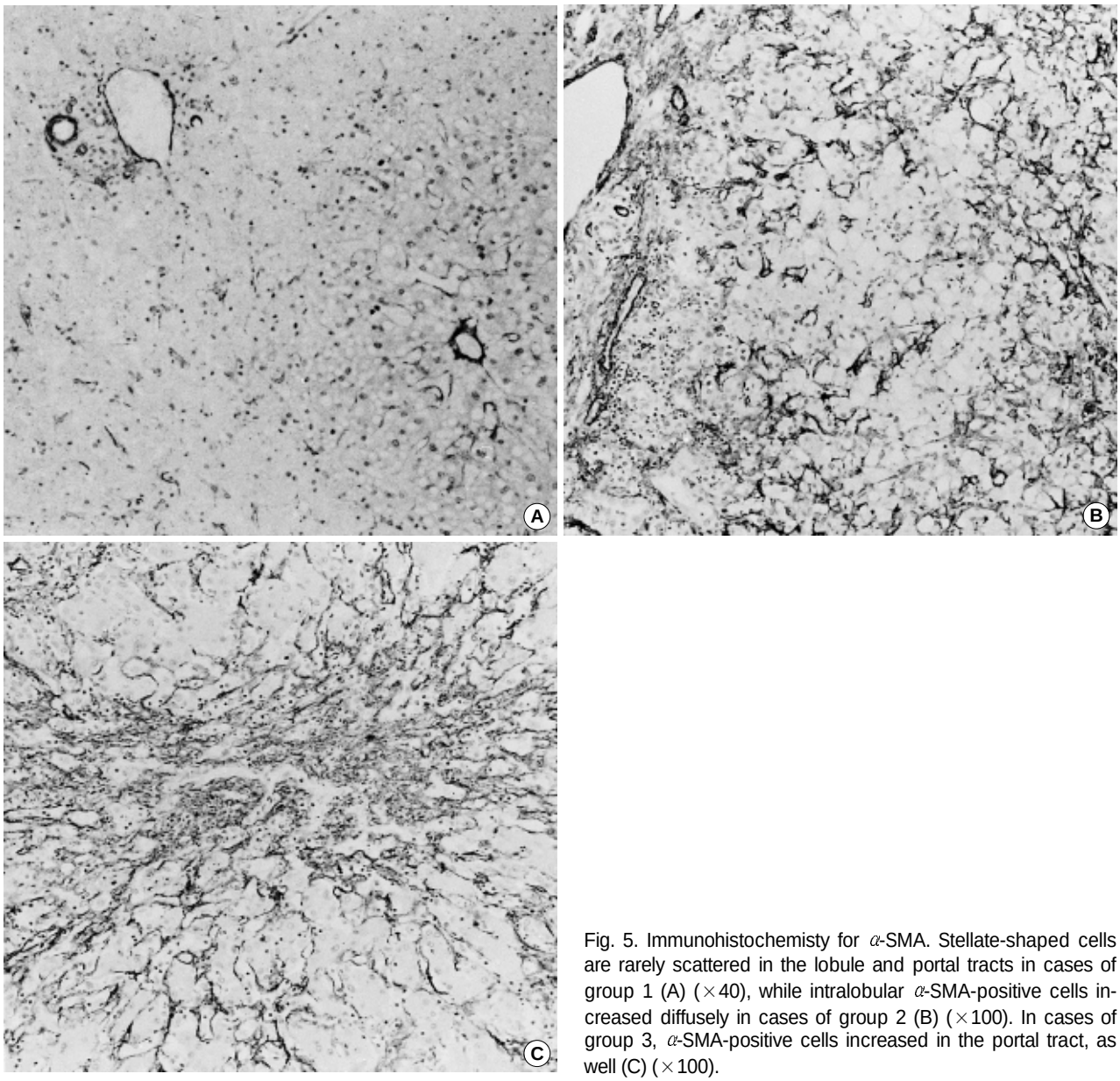

Fig. 5. Immunohistochemisty for α-SMA. Stellate-shaped cells are rarely scattered in the lobule and portal tracts in cases of group 1 (A) (×40), while intralobular α-SMA-positive cells increased diffusely in cases of group 2 (B) (×100). In cases of group 3, α-SMA-positive cells increased in the portal tract, as well (C) (×100).

We examined primary nonfunctioning liver allografts in which liver injury presumably occurred at the time of liver transplantation because they are excellent models for early phase of hepatic regeneration. In the early phase of hepatic regeneration, the most prominent feature was that number of CD68-positive cells increased moderately along the border of the necrotic area and slightly in the sinusoid of the necrotic areas. Most of the CD68-positive cells within the necrotic areas are considered Kupffer cells on the basis of their location and morphologic characteristics such as elongated cytoplasm (15-17). However, a possibility that migrated macrophages might participate in the tissue reaction during hepatic regeneration can not be excluded, because necrotic hepatic parenchyma was filled with blood cells. In rats in which cross-sex or cross-strain bone marrow transplantation and hepatic injury using 2-acetylaminofluorene and CCl_4 protocol were performed, a proportion of regenerated hepatic cells were shown to be derived from the donor bone marrow stem cells (18). Thus, presence of bone marrow stem cells needs to be examined to evaluate the possibility of hepatic stem cells of bone marrow origin in human cases of massive hepatic necrosis.

The marked increase in PCNA-positive hepatocytes in early hepatic regeneration indicates that they are activated to repair intracellular damage as well as prepare to replicate because PCNA is involved in DNA repair and replication (13, 19-21). Sinusoidal PCNA-positive cells are considered to be activated Kupffer cells or macrophages on the basis of

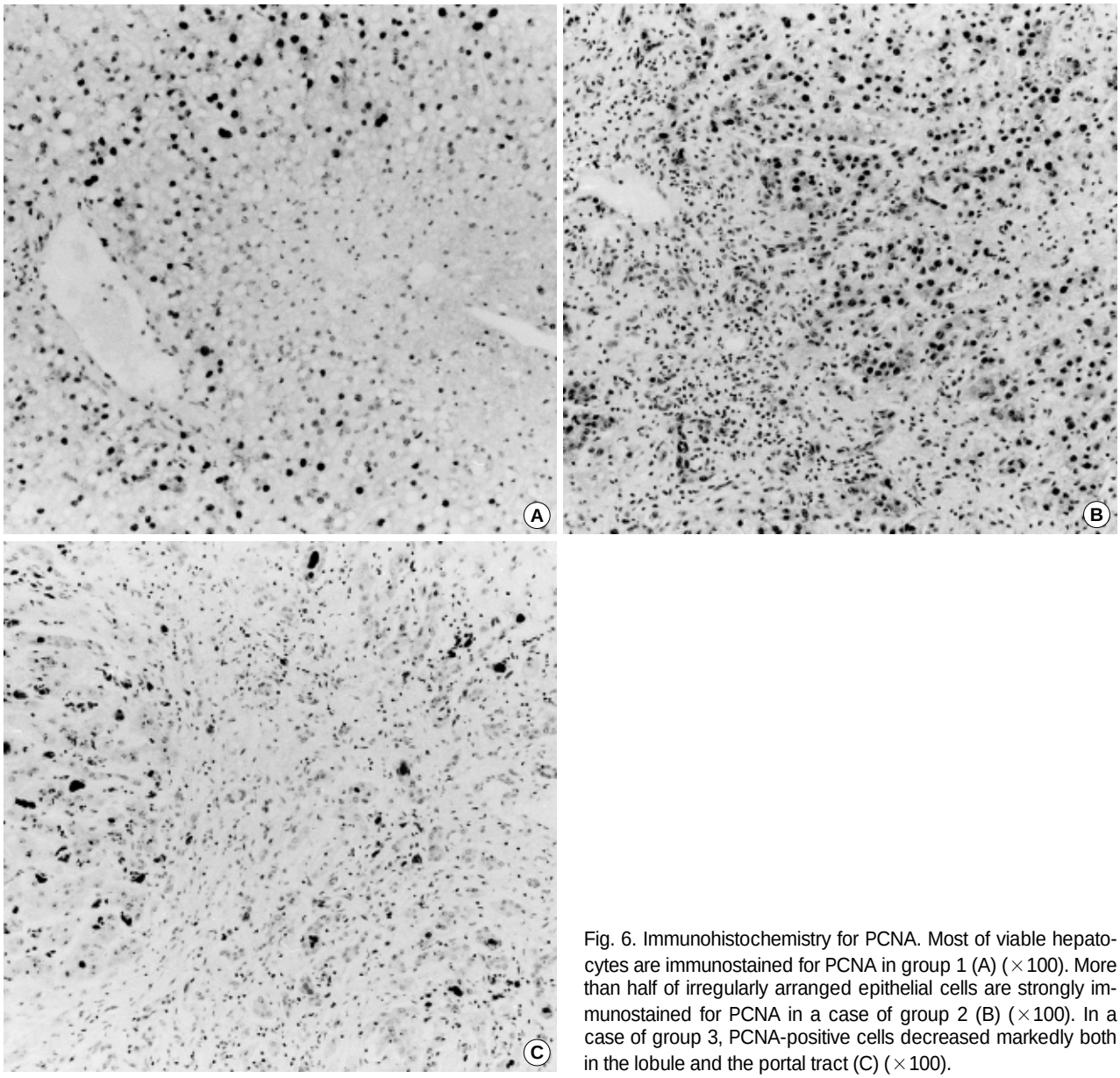

Fig. 6. Immunohistochemistry for PCNA. Most of viable hepatocytes are immunostained for PCNA in group 1 (A) (×100). More than half of irregularly arranged epithelial cells are strongly immunostained for PCNA in a case of group 2 (B) (×100). In a case of group 3, PCNA-positive cells decreased markedly both in the lobule and the portal tract (C) (×100).

their location and nuclear shape as indicated previously (19). Frequent mitotic figures in ductules that were present mostly at the limiting plates indicated that they might actively proliferate from hepatic stem cells. In late stage of hepatic regeneration, PCNA immunostaining decreased markedly and was confined within the hepatocytes. These sequential changes of the type of proliferating cells indicate that there should be a cross talk signaling cell proliferation between nonparenchymal and parenchymal cells.

We used CK7 and HepPar1 as a marker for parenchymal cells to evaluate their topological characteristics. CK7-positive cells along the interface of portal tracts were considered to be either progenitor cells or intermediate cells rather than bile duct cells on the basis of their cellular characteristics and immature organization. Both CK7- and HepPar1-positive cells in cases of group 3 may correspond to ductular hepatocytes (DHs) which are also known under the term 'biliary hepatocytes', 'transitional cells', 'hepatocyte-like cells', 'neocholangioles', or 'oval cells' (11, 14, 22-26). Fujita et al. (4) observed single regenerating hepatocyte first 2 months after the onset of fulminant hepatitis in native liver after auxillary partial orthotopic liver transplantation. On the basis of our observations, however, regeneration of liver cells appears to begin earlier at least 1 month after diffuse hepatic necrosis.

With progression of hepatic regeneration, reticulin framework was progressively collapsed, which was strongly corre-

lated with the proliferation of α-SMA-positive cells and deposition of collagen fibers. α-SMA-positive cells were distributed either within hepatic lobules or portal tracts. In acute liver injury following administration of CCl_4 and other toxins, the HSC may migrate into or actively proliferate predominantly in necrotic areas. In contrast, in models of chronic injury, progressive expansion of HSC population was mainly seen within fibrous septa as we described in cases of the late regenerative phase (27, 28). In the case of complete regeneration after massive hepatic necrosis without residual fibrosis, no increase of α-SMA-positive cells has been described (4), but fibrosis around the reorganized hepatic trabeculae at 6 months might be associated with intralobular α-SMA-positive cells. Thus, increase in α-SMA-positive cells in portal tracts and irregular fibrosis more than a month after the first episode of hepatic failure may be an indicator of irreversible hepatic fibrogenesis.

On the basis of the observation in the present study, a schematic sequence of events is suggested that CD68-positive cells are firstly involved very early following diffuse liver cell necrosis and then both HSCs and CK7-positive cells increase in number within a few weeks. Some CK7-positive cells are phenotypically transformed to hepatocytes 1 month after the onset of fulminant hepatitis. Increase of myofibroblasts in portal tracts in later phase of hepatic regeneration may cause irreversible hepatic fibrogenesis, while intralobular α-SMA may participate in forming intact perisinusoidal framework in cases of complete regeneration (Fig. 7). These parenchymal and nonparenchymal cellular responses against diffuse hepatic damage mirror the changes in experimental animal models of massive hepatic necrosis and appear to occur irrespectively to the etiology.

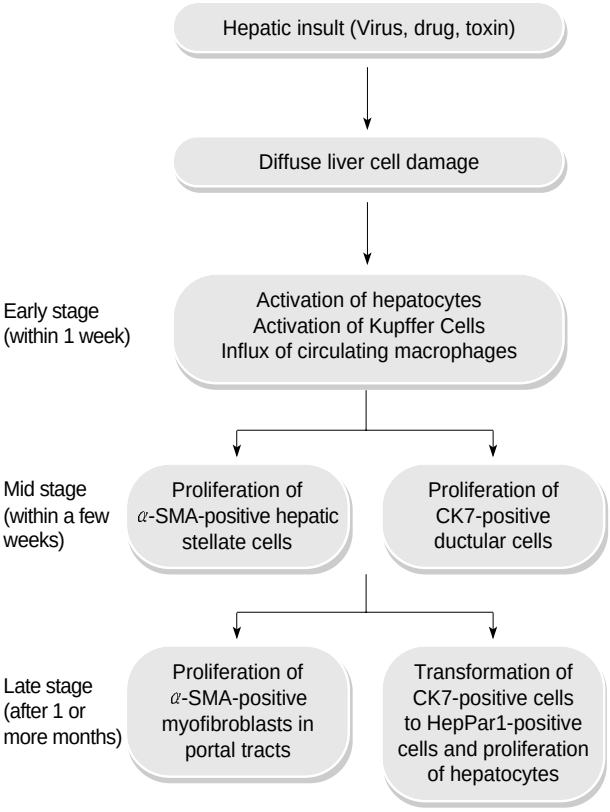

Fig. 7. Schematic sequence of events during human hepatic regeneration.

ACKNOWLEDGMENT

We thank Ms. Ji Young Jang for her technical assistance.

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