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Differing Distribution of Hepatocyte Growth Factor-positive Cells in the Liver of LEC Rats with Acute Hepatitis, Chronic Hepatitis and Hepatoma

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Using anti-rat hepatocyte growth factor (HGF) antibody, we investigated the distribution of HGF-positive cells in the liver tissues of LEC rats at various phases of liver diseases. During the phase of fulminant hepatitis, HGF-positive cells increased remarkably, and many of them were localized at the portal triads; these cells were identified from their shape as non-epithelial cells. A reduced number of HGF-positive cells was observed during the phase of chronic hepatitis, while no HGF-positive cells were seen in the tissue of cholangiofibrosis. During the phase of carcinoma, staining revealed that both the hepatocellular carcinoma cells and the non-epithelial cells in cancerous liver tissue were HGF-positive. These results suggest that, in LEC rats, HGF may play an important role in the regeneration of hepatocytes as well as in the development of hepatocellular carcinoma.

Key words: LEC rat — HGF — Immunohistochemistry — Hepatitis — Hepatoma

The LEC rat is characterized by the spontaneous development of acute and chronic hepatitis,[1,2] and the subsequent development of liver cancer in animals which survive for more than one year after recovering from acute hepatitis.[3] About 50% of all LEC rats die of fulminant hepatitis at the age of 4 to 5 months.[1,2] We have discovered an abnormal accumulation of copper in the liver of LEC rats, 40 times greater than that in control LEA rats, while the levels of ceruloplasmin in LEC rat serum are markedly low.[4,5] Such findings indicate that the liver disorder in LEC rats is very similar to Wilson's disease.[4–7]

Hepatocyte growth factor (HGF) is considered to be a major hepatotrophic factor that induces mitosis of hepatocytes during liver regeneration.[8,9] HGF also induces cell movement,[10] acting as a motogen, and further induces formation of tissue-like structures,[11,12] acting as a morphogen, for various cell types. HGF was first identified in the serum of partially hepatectomized rats[13] and has been purified from rat platelets[14] and human plasma.[15]

Levels of serum HGF are elevated in human patients with fulminant hepatitis, chronic hepatitis and liver cirrhosis,[16] and HGF mRNA is present in various tissues.[17] It has already been reported that HGF production increases in rats injured by hepatectomy or CCl_4, and that non-parenchymal cells produce HGF.[18–22] The levels of HGF mRNA and the activity of HGF rise prior to liver regeneration.[20] It was also reported that recombinant HGF remarkably promoted liver regeneration in rats with artificially induced liver damage.[9] In the present paper, we describe how immunological staining enabled us to detect distributional changes of HGF-positive cells in the liver of LEC rats at various stages of liver diseases.

We used male and female LEC rats aged from 10 weeks to 2 years. They were maintained under conventional conditions at the Center for Experimental Plants and Animals of Hokkaido University. Female LEC rats aged 10 weeks old were anesthetized with ether. The medial and left hepatic lobes were removed according to the method devised by Higgins and Anderson.[23] We developed a polyclonal antibody in rabbits by immunizing them with highly purified recombinant rat HGF. Mono-specific antibody to rat HGF was purified by affinity chromatographies using Protein A-Sepharose immobilized with highly purified rat HGF.

The livers were quickly removed from killed LEC rats. Serial 4–6 μm frozen sections of freshly frozen liver tissues were cut in a cryostat, immersed in ice-cold acetone for 15 min and stored at $-80°C$. The sections were incubated first with the polyclonal antibody against recombinant rat HGF, secondly with a biotinylated anti-rabbit IgG (Vector Laboratories Inc., Burlingame), and thirdly, with an alkaline phosphatase-conjugated streptoavidin. Finally, they were immersed in a solution containing 25 mg of Levamisole, 0.25 ml of 4% $NaNO_2$, 0.25 ml of 4% new Fuchsin, 25 mg of Naphthol AS-BI triphosphate, and 0.25 ml of N,N-dimethylformamide in 0.2 *M*

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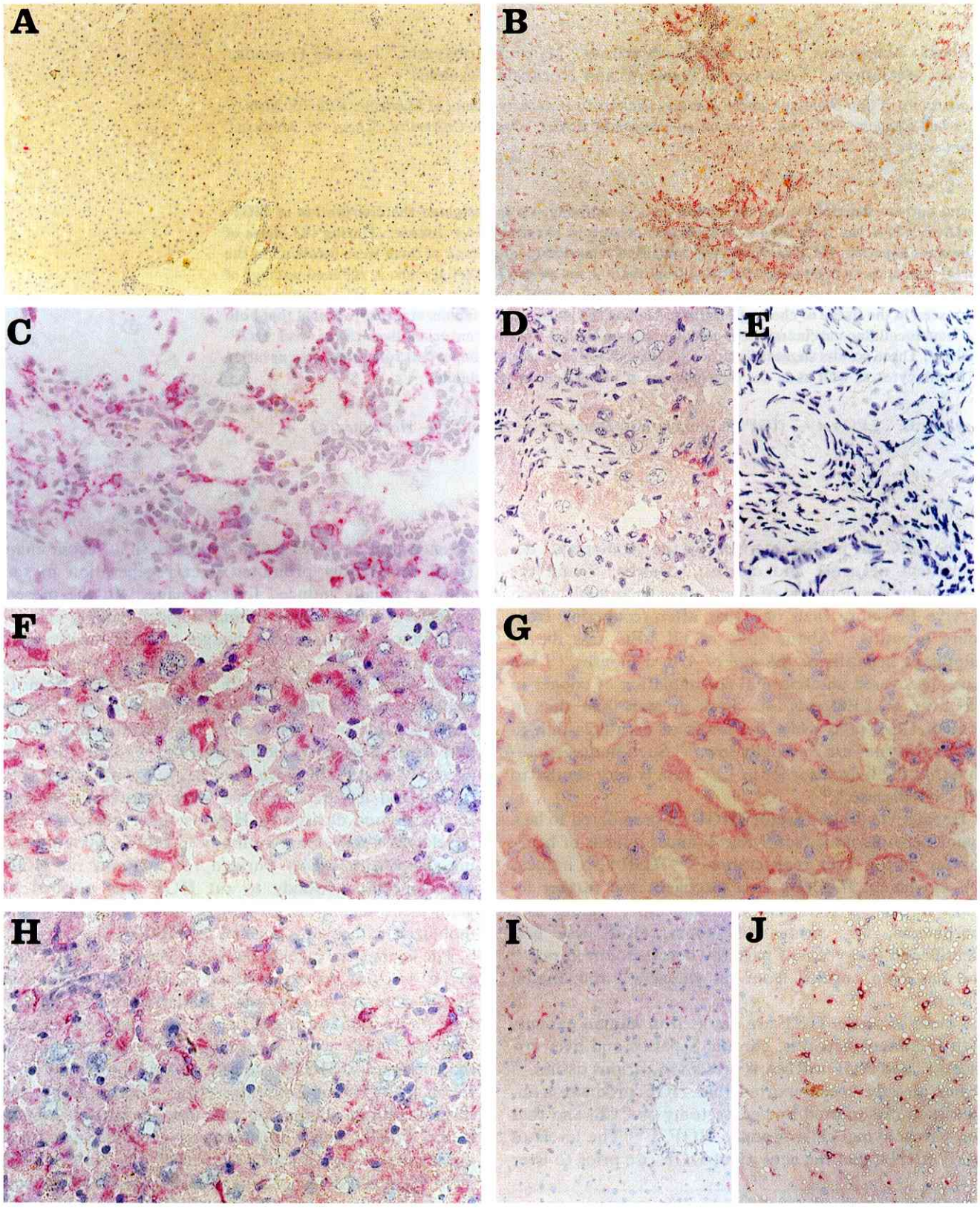
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Tris-Cl (pH 8.2–8.5) per 100 ml. Nuclear counter-staining was carried out with Mayer's hematoxylin solution.

During the pre-hepatitic phase, we observed a small number of diffusely scattered HGF-positive cells (Fig. 1A), and identified these cells according to their morphological characteristics as non-parenchymal cells such as Kupffer cells, macrophages and Ito cells. Two types of non-parenchymal cells stained HGF-positive; one of them had large nuclei, while the other had small, flattened nuclei. During the fulminant hepatitis phase, the number of HGF-positive cells increased markedly, especially around the portal triads in the liver (Fig. 1B). Fig. 1C shows a high magnification of the portal triads. We identified these HGF-positive cells as being histologically nonparenchymal. During the chronic hepatitis phase, we noted that the number of HGF-positive cells observed in the liver tissues decreased (Fig. 1D); most of these HGF-positive cells had small, flattened nuclei. Neither HGF-positive cells nor HGF-positive sites were found in any of three tissue samples of cholangiofibrosis from three different LEC rats (Fig. 1E).

We examined several lesions of three hepatocellular carcinomas (a, b, c) removed from two male LEC rats. There were HGF-positive foci where tumor cells were partially stained in the well-differentiated HCC (a) of a 108-week-old male LEC rat (Fig. 1F). This type of staining was also detected in a lesion of the moderately differentiated HCC (b) of a 91-week-old male LEC rat. In the HCC tissue (a) of the 108-week-old male LEC rat, the areas surrounding each cancer cell group were stained HGF-positive (Fig. 1G). This type of HGF-positive staining was also observed in a lesion of the same HCC (b) developed in the 91-week-old LEC rat. HGF-positive non-parenchymal cells were located in the liver cancer stroma (Fig. 1H). Such HGF-positive non-parenchymal cells were detected in two tumors (a, b), but not in the small tumor (c), a moderately differentiated HCC, of the 91-week-old LEC rat. HGF-positive cells increased significantly and diffusely in the liver of 10-week-old LEC rats 24 h after they had received partial hepatectomy (Fig. 1I, J).

From these results, we suggest that there is a marked difference in the distribution of HGF-positive cells at each of the stages of development of spontaneous hepatitis, as well as during hepatectomy. We have noted that, during the development of fulminant hepatitis, the number of HGF-positive cells increased markedly and these cells were localized around the portal triads. Since the hepatocyte is formed at the periportal tract rim, and the assembled unit then continues to stream along the acinus radius, travelling through the three acinus zones until reaching the terminal vein,[24] we suggest that, in LEC rats with fulminant hepatitis, the regeneration of liver cells starts at portal triads. On the other hand, although HGF-positive cells increased diffusely in the tissue of the liver after 70% hepatectomy, the cells were not localized around portal triads. This may indicate that the growth of hepatocytes occurs in the whole liver after hepatectomy. Although we are not able to explain the reason for the difference in the distribution of HGF-positive cells, we speculate that many of the inflammatory cells transported to the portal triads may produce HGF during the phase of fulminant hepatitis, since no inflammatory cells were observed at the portal triads during instances of hepatectomy. The mechanism responsible for the production of HGF in LEC rats with fulminant hepatitis seems to differ from that in LEC rats which have undergone hepatectomy.

Transforming growth factor (TGF)-β1 is the most potent growth inhibitor for hepatocytes,[25,26] but is an enhancer for extracellular matrix deposition,[27] and more importantly, it is a potent suppressor of the gene expression of HGF.[28] Therefore, during chronic hepatitis, persistently elevated TGF-β1 would induce the onset of hepatic fibrosis. This hypothesis is supported by the finding that during chronic hepatitic and subsequent

Fig. 1. HGF-positive cells in the liver of LEC rats visualized by alkaline phosphatase-conjugated streptavidin-new fuchsin (red). A. Pre-hepatitis liver of a 10-week-old female LEC rat; normal liver tissue, with a small number of HGF-positive cells distributed diffusely. (×100) B. Liver of a 21-week-old female LEC rat with fulminant hepatitis; many HGF-positive cells are observed and most of them are localized around the portal triads. (×100) C. A high magnification of the portal triads of the liver of a 21-week-old female LEC rat with fulminant hepatitis; most of the HGF-positive cells are non-parenchymal. (×400) D. Liver of a 74-week-old female LEC rat with features of chronic hepatitis, such as infiltration of inflammatory cells, necrosis of hepatocytes, and proliferation of oval cells. A reduced number of HGF-positive cells is apparent. (×400) E. Liver of a 74-week-old female LEC rat with typical pathological features of cholangiofibrosis; no HGF-positive cells are observed. (×400) F. A high-magnification view of an HGF-positive focus in well-differentiated hepatocellular carcinoma of a 108-week-old male LEC rat; tumor cells are partially stained positive. (×400) G. Well-differentiated hepatocellular carcinoma of a 108-week-old male LEC rat; the areas surrounding the cancer cell groups and non-parenchymal cells in stroma are stained positive. (×400) H. Well-differentiated hepatocellular carcinoma of a 108-week-old male LEC rat; HGF-positive non-parenchymal cells are observed in the cancer stroma. (×400) I. Liver of a 10-week-old female LEC rat before hepatectomy. (×200) J. Liver of a 10-week-old female LEC rat 24 h after partial hepatectomy; HGF-positive cells have increased diffusely. (×200)

cholangiofibrosis, the expression of HGF detected by immunohistochemical analysis was markedly decreased. We suppose that some hepatoma cells stained HGF-positive may have produced HGF. So far, there has been no report that hepatoma cells produce HGF, and false-positive staining can not be ruled out. We intend to examine our finding further by other methods, such as *in situ* hybridization. Although it is not clear yet whether HGF promotes or suppresses mitosis of these hepatoma cells in an autocrine manner, Shiota *et al.*[29] have shown that HGF inhibits the growth of hepatocellular carcinoma cell lines. We want to examine whether the HGF which we detected in the liver cancer stroma and on the surface of hepatocellular carcinoma cells represents low-affinity binding of HGF to the cells, since the major constituents of the extracellular matrix are heparan sulfate and glycosaminoglycan, which is related to heparin. Yoshinaga *et al.*[30] suggested that one of the relatively low-affinity binding sites may be heparan sulfate.

In human beings, the serum HGF concentration increases during both fulminant hepatitis and liver cirrhosis.[6] It will be of interest to see whether increased serum HGF has a positive effect on liver regeneration in patients with fulminant hepatitis, or whether HGF has any role in carcinogenesis in cirrhotic liver.

Kanda *et al.*[31] have demonstrated the transforming activity of HGF, utilizing immortalized but not fully transformed mouse hepatocytes. We believe that LEC rats provide a good animal model for investigating the roles of HGF in the whole spectrum of liver diseases.

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