In vivo transfer of hepatocyte growth factor gene accelerates proliferation of hepatic oval cells in a 2-acetylaminofluorene/partial hepatectomy model in rats

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Abstract To clarify the effect of hepatocyte growth factor (HGF) on proliferation of hepatic oval cells, we transferred HGF gene into liver of the Solt–Farber rat model. Male Fisher 344 rats were infected with a recombinant adenovirus carrying the cDNA for HGF (pAxCAHGF) from tail vein. HGF mRNA showed its peak at 4 days, and diminished thereafter. The total and proliferating cell nuclear antigen-positive hepatic oval cells were significantly elevated in HGF-transferred rats, in which stem cell factor and c-kit mRNA increased at each time point. Our results suggest that in vivo transfer of the HGF gene into liver accelerates proliferation of hepatic oval cells in the Solt–Farber model in rats.

Key words: Hepatocyte growth factor; Oval cell; Solt–Farber model; Liver regeneration

1. Introduction

Liver regeneration is usually accomplished by the proliferation of differentiated hepatocytes. However, when hepatocyte proliferation is impaired, hepatic oval cells proliferate, invade into the adjacent liver parenchyma, and differentiate into hepatocytes or biliary epithelial cells [1–4]. One of the most widely used models of oval cell activation is the modified Solt–Farber regime [5,6]. In the presence of 2-acetylaminofluorene (2-AAF), oval cells rather than hepatocytes proliferate after 2/3 partial hepatectomy (PH), and oval cells play an important role in liver regeneration when liver is severely damaged and hepatocyte proliferation is inhibited. In humans, oval cells which are similar in morphology and antigenic profile to those seen in rodents emerge in many liver diseases [2,7].

Oval cells express receptors for transforming growth factor α (TGFα), TGFβ, acidic fibroblast growth factor and hepatocyte growth factor (HGF) [2]. Although there seems to be a paracrine loop between Ito cells and oval cells, oval cells may be autonomous in their requirement for mitogens and morphogens since putative embryological oval cell equivalents are able to proliferate in serum-free conditions [8,9]. HGF, a potent mitogen for mature hepatocytes, proved to be a multifunctional growth factor [10–12]. HGF is a powerful mitogen for mature hepatocytes and biliary epithelial cells [13,14]. Since HGF is a morphogen during embryological tissue development [15], and therefore probably has a fundamental influence on oval cell behavior. Indeed, HGF plays an important role in the control of proliferation and differentiation of erythroid progenitor cells in fetal liver [16], and acts in synergy with stem cell factor (SCF) [17]. There is evidence that hepatic oval cells may be derived from bone marrow [18]. Since several growth factors enhance the mitogenic response of oval cells [19] and the SCF/c-kit system is an important regulator of oval cell activation [20,21], we examined the effect of HGF gene transfer on hepatic oval cell proliferation, and also clarified expression of SCF/c-kit in this situation.

2. Materials and methods

2.1. Chemicals and animals

2-AAF pellets (75 mg/pellet over a 21 day release) were purchased from Innovative Research (Sarasota, FL, USA). Fisher 344 rats, 8 weeks old and adult males weighing 150–180 g, were obtained from Charles River Japan (Yokohama, Japan). Rats were maintained in a temperature-controlled room with a 12 h light/dark illumination cycle. Rats were fed a CE-2 diet (CLEA Japan, Tokyo, Japan) and were allowed to drink water ad libitum.

2.2. Construction of pAxCAHGF and pAxCALacZ

The cassette cosmid for constructing recombinant adenovirus of the E1 substitution type is an 11 kb charomid vector bearing an Ad5 genome spanning (mu) 0–99.3 map units with deletions of E1 (mu1.3–9.3) and E3 (mu79.6–84.8) [22]. Two plasmids expressing rat HGF [23] and *Escherichia coli* LacZ were constructed by inserting the rat HGF gene and the LacZ gene downstream from the CAG promotor, respectively. The CAG expression unit was inserted at the deleted E1 region. After insertion of an expression unit into the cassette cosmid, the cassette cosmid bearing an expression unit was cotransfected into 293 cells, human embryonic kidney cells transformed

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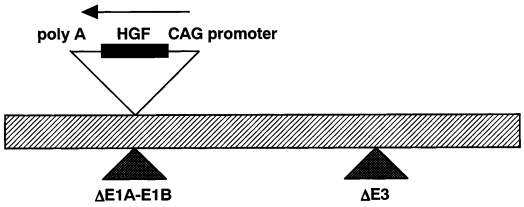


Fig. 1. Construct of pAxCAHGF. Rat HGF cDNA was inserted in CAG promotor driving adenovirus vector lacking E1A, E1B and E3. In case of pAxVALacZ, LacZ cDNA was inserted instead of HGF cDNA.

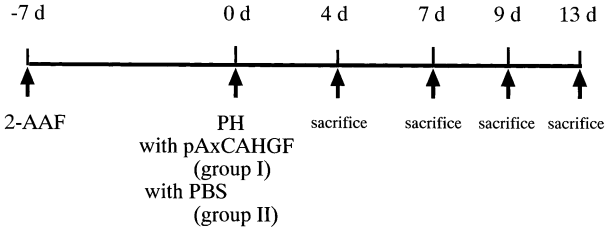


Fig. 2. Experimental schedule. 2-AAF: 2-acetylaminofluorene, PH: 70% partial hepatectomy.

by E1A and E1B genes, together with EcoT221-digested DNA-TPC of Ad5-dlX. The construction of resulting recombinant adenoviruses pAxCAHGF and pAxCALacZ was shown in Fig. 1. The reason why we used adenovirus vector is that adenovirus infectivity to liver is extremely high and adenovirus vectors are commonly used for in vivo gene therapy in experimental liver disorders [24].

2.3. Experimental procedures

The experimental protocol is shown in Fig. 2. For induction of oval cells, the rats were treated according to the method for the AAF/PH model [5,6]. AAF is used as a mito-inhibitor for adult rat hepatocyte in this model. Briefly, 2-AAF pellets were subcutaneously inserted 7 days before 70% PH. Just after PH, pAxCAHGF (1×10^9 plaque forming units (pfu)) or pAxCALacZ (1×10^9 pfu) in 0.1 ml of phosphate-buffered saline (PBS) was administered into tail vein of rats (group I). In rats of group II, 0.1 ml of PBS without pAxCAHGF was administered into vein. Five rats each were killed at 4, 7, 9 and 13 days after PH. PH alone, AAF administration without PH, and pAxCALacZ administration with AAF/PH regime were carried out as controls. All surgical procedures were performed under sodium pentobarbital anesthesia. Experimental procedures were approved by the Animal Care Committee of Tottori University School of Medicine and done in compliance with the guidelines of our university. Animals received human care according to the criteria outlined in the 'Guide for the Care and Use of Laboratory Animals' prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23 revised 1985). The number of oval cells was counted in 10 periportal areas selected randomly in each specimen stained with hematoxylin and eosin by using NIH Image 1.55 (National Institutes of Health, USA). The value was expressed as the number per periportal field (0.25×0.25 mm^2). The proliferating cell nuclear antigen (PCNA)-positive oval cells were counted in the same way.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total cellular RNA was isolated from snap-frozen liver tissue by using ISOGEN (Nippon Gene Co., Toyama, Japan) according to the manufacturer's instructions. The RNA samples were digested with DNase (Nippon Gene Co.) to remove DNA from the samples. 1 μg of RNA was converted to complementary DNA and was amplified by using RNA LA PCR™ kit (TaKaRa, Kyoto, Japan) and a thermocycler (Program Temp Control System PC-700, ASTEC, Japan). Primer sequences for rat HGF, c-met, SCF, c-kit and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), used in PCR on complementary DNA, size of PCR products and annealing temperature in each reaction were shown in Table 1. The cycle number of PCR was 30 for all genes. The amount of expression of HGF mRNA was determined by using the NIH image version 1.58 computer software (Macintosh, USA).

2.5. Immunohistochemistry

Livers were fixed in 4% paraformaldehyde and embedded in paraffin. Sections (4 μm thick) were incubated in 0.3% H_2O_2 in methanol for 30 min. After washing in PBS, they were incubated with normal horse serum or normal goat serum (Vector Laboratories, CA, USA). The sections were then incubated with the following antibodies; a mouse monoclonal antibody against human CK-19 (Novocastra Laboratories, Newcastle, UK) diluted 1:40; a mouse monoclonal antibody against rat OV-6 [25] diluted 1:10; a mouse monoclonal antibody against human AFP (NEOMARKERS, Lab Vision Corporation, CA, USA) diluted 1:50; a mouse monoclonal antibody against human c-kit protein (NEOMARKERS, Lab Vision Corporation, Fremont, CA, USA) diluted 1:50; a mouse monoclonal antibody against rat PCNA (NCL-PCNA, Novacastra Laboratories, Newcastle, UK) diluted 1:100; a mouse monoclonal antibody against human desmin (YLEM, Rome, Italy) diluted 1:50; goat polyclonal antibody against SCF (Santa Cruz Biotechnology, CA, USA) diluted 1:100 and rabbit IgG fraction against rat albumin (Inter-Cell Technologies, NJ, USA) diluted 1:100, at 4°C overnight. To facilitate antigen retrieval, sections for the CK-19 and desmin immunostain were digested with 0.05% trypsin (Sigma Chemical, MO, USA) in 0.1 mol/l Tris–HCl and 0.1% $CaCl_2$, pH 7.8, for 5 min at 37°C before staining. All the sections stained in the present study were heated in 10 mmol/l sodium citrate buffer (pH 6.0) at 600 W for 15 min with a microwave oven. After washing in PBS, sections were incubated with biotin-conjugated horse anti-mouse or goat anti-rabbit immunoglobulin G (Vector Laboratories) for 60 min. Immunoreacted cells were visualized by a Vectastain ABC kit (Vector Laboratories). The expression of β-galactosidase was assessed by X-gal staining [26].

2.6. Statistical analysis

Statistical significances were assessed using Mann–Whitney's *U* test. $P<0.05$ was considered statistically significant.

3. Results

3.1. Expression of HGF mRNA and c-met mRNA

Serial analysis of HGF and c-met mRNA was performed (Fig. 3). In group I, expression of HGF mRNA showed its peak at 4 days after PH, and gradually decreased at 7 days and 9 days. In group II, expression of HGF mRNA was scarcely observed. Expression of HGF mRNA was much greater in group I than that in group II. The amount of HGF mRNA at 4 days was about 5-fold higher than that in control liver 2 days after PH without AAF. c-met expressed at a similar level between group I and II at 4 days, and slightly higher in group I than in group II at 7 and 9 days.

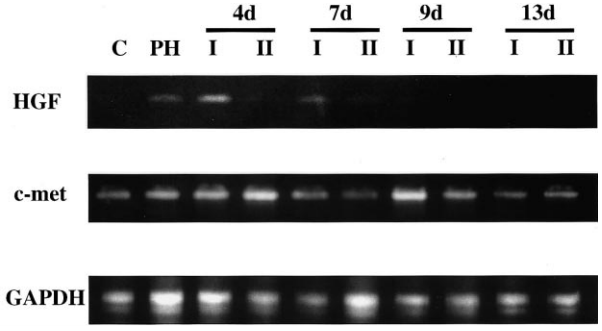


Fig. 3. RT-PCR analysis of HGF and c-met mRNA expression. GAPDH: glyceraldehyde-3-phosphate dehydrogenase, C: rat liver with no treatment, PH: rat liver 2 days after PH without AAF administration.

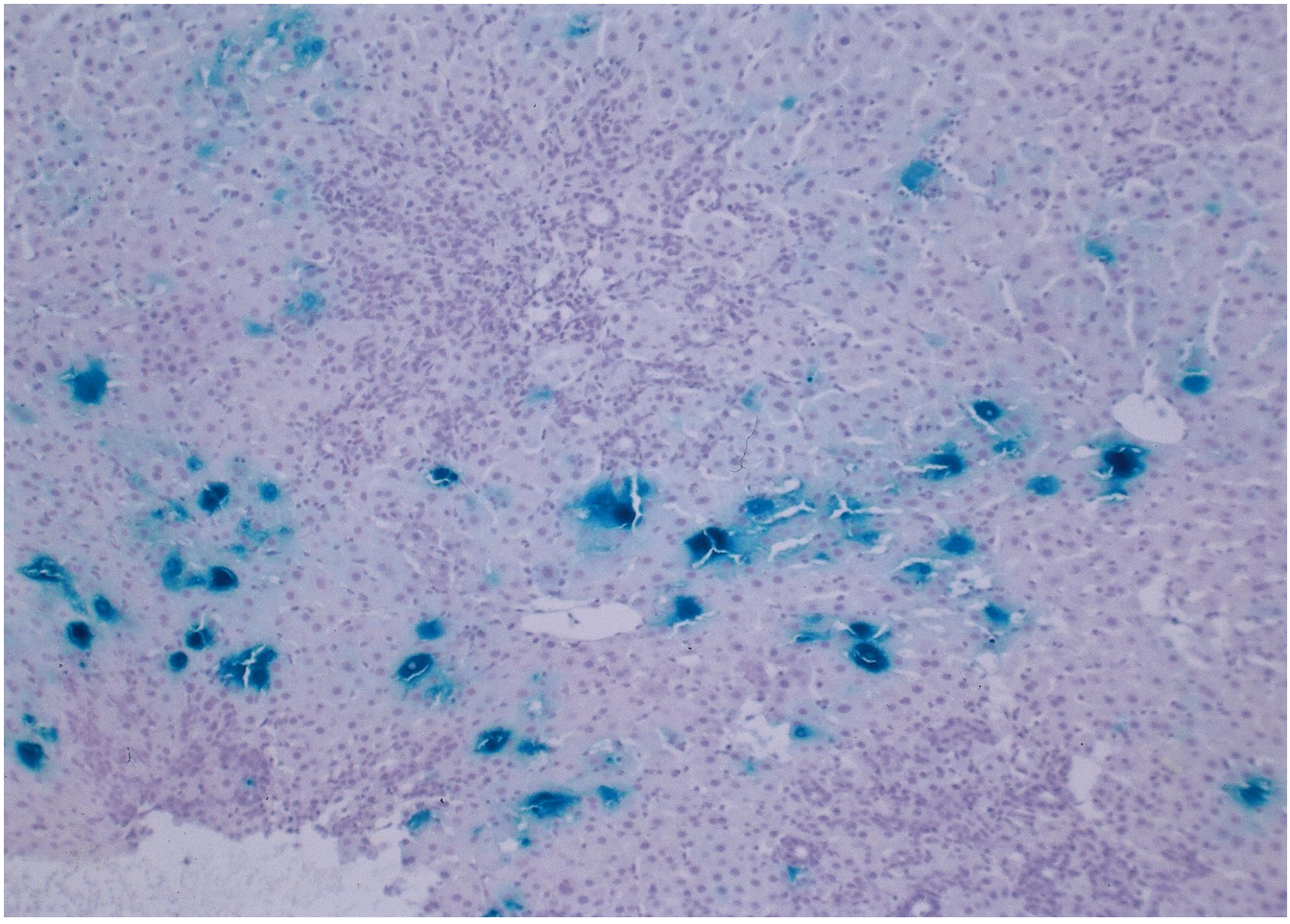


Fig. 4. Immunohistochemical staining of expression of β-galactosidase. The liver of rats at 7 days after pAxCAlacZ injection was used (×200).

3.2. Oval cell development

At 4 days after PH, small basophilic cells with a high nuclear:cytoplasmic ratio were evident around the portal tracts. These cells increased in number with time. They fanned out from each portal tract and penetrated deep into the liver acinus surrounding preexisting acidophilic hepatocytes at 9 days after PH. In AAF/PH rats in which pAxCALacZ was administered, expression of β-galactosidase was observed mostly in hepatocytes, but not in oval cells (Fig. 4). These cells were stained with anti-CK-19 antibody and anti-OV-6 antibody (Fig. 5C,D), and also stained with anti-AFP antibody (data not shown). At 9 days after PH, many and large clusters of oval cells were seen in group I (Fig. 5A), on the other hand, smaller and fewer clusters were seen in group II (Fig. 5B). Treatment with PH alone or administration of AAF without PH did not induce oval cells in rats (data not shown). In addition, in AAF/PH rats in which pAxCALacZ was administered instead of pAxCAHGF, the total and PCNA-expressing oval cells were almost equal to those in group II (data not shown). To evaluate the effect of HGF expression on oval cells, oval cells in the portal area were counted. The numbers of oval cells at 4, 7, 9 and 13 days after PH were 18.0 ± 6.3, 147.0 ± 17.0, 200 ± 25.4 and 150.0 ± 38.0 in group II, respectively. In group I, they were 19.7 ± 2.9, 192.0 ± 23.0, 410.0 ± 44.7 and 374.0 ± 93.1, respectively. Although oval cells reached their peak in number at 9 days, they were significantly increased in group I at 7, 9 and 13 days, compared to in group II ($P < 0.05$, $P < 0.01$ and $P < 0.01$, respectively, Fig. 6A). Furthermore, to assess the proliferating activity of oval cells in these livers, liver sections were stained with anti-PCNA antibody. PCNA-stained cells were restricted to the oval cells in the liver acinus (Fig. 7D). Interestingly, positive rates for

Table 1
Primers and annealing temperature of PCR

Gene	Primer sequences		Size of PCR product (bp)	Annealing temperature (°C)
HGF	sense: 5′-ATGCTCATGGACCCTGGT-3′	antisense: 5′-GCCTGGCAAGCTTCATTA-3′	423	55
c-met	sense: 5′-CAGTGATGATCTCAATGGGCAAT-3′	antisense: 5′-AATGCCCTCTTCCTATGACTTC-3′	725	58
SCF	sense: 5′-CCGGGATGGATGTTTTGC-3′	antisense: 5′-TGCAACAGGGGGTAACAT-3′	382	55
c-kit	sense: 5′-TGCTCTGCGTCCTGTTGGTC-3′	antisense: 5′-CCTGGCGTTCGTAATTGAAGTC-3′	794	55
GAPDH	sense: 5′-CCTTCATTGACCTCAACTAC-3′	antisense: 5′-GGAAGGCCATGCCAGTGAGC-3′	496	57

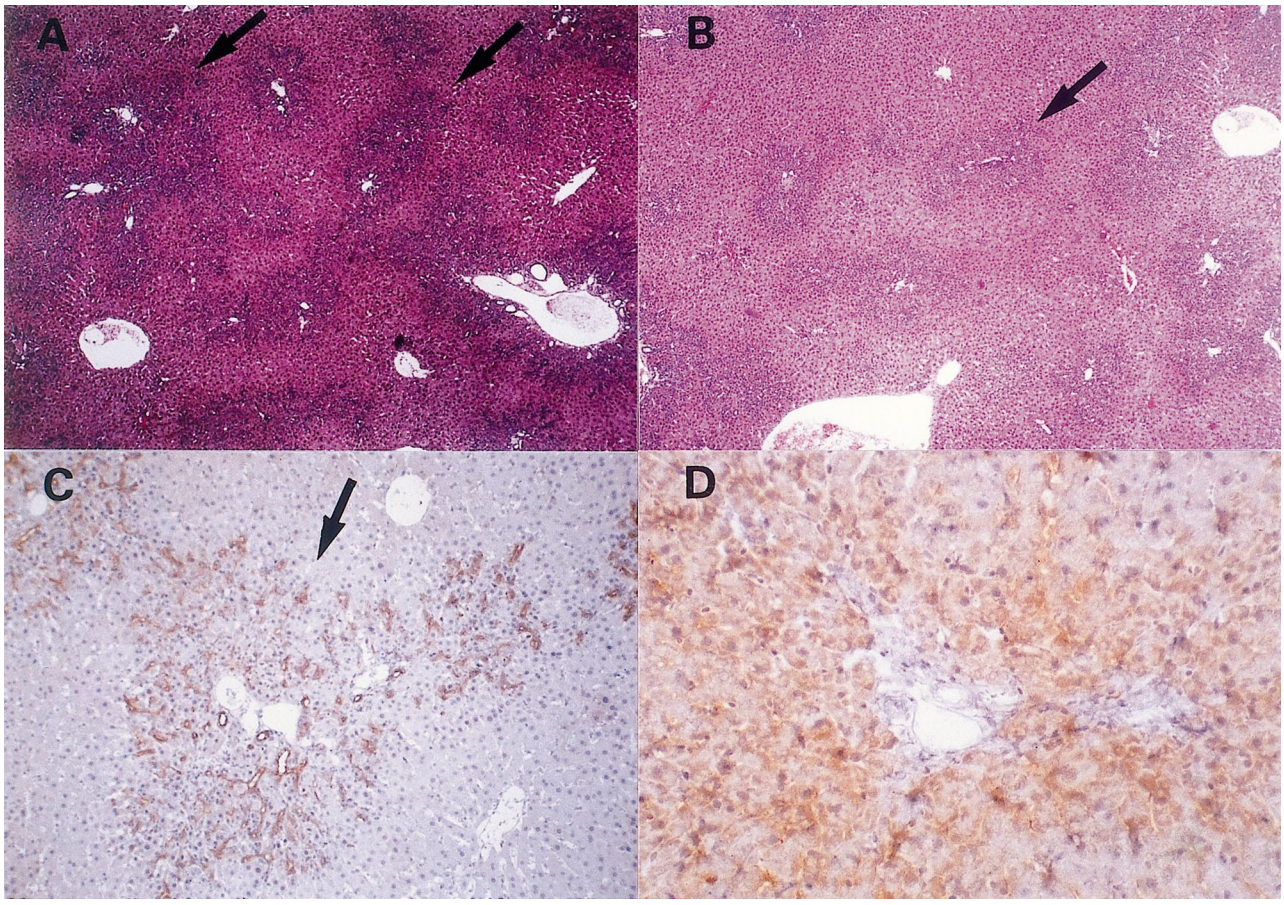
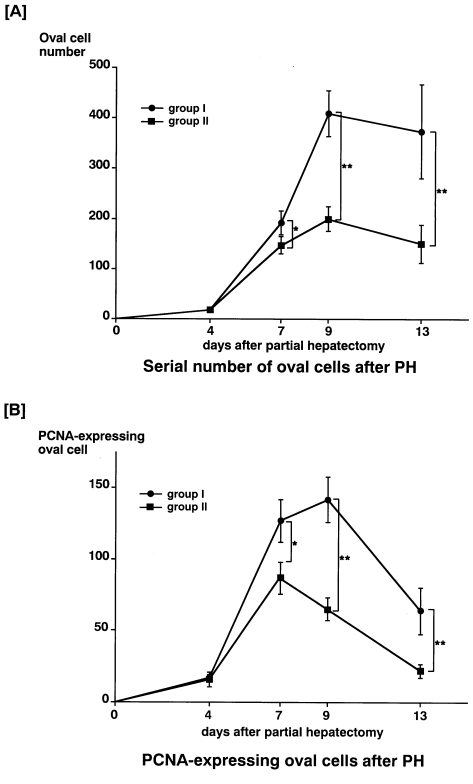


Fig. 5. Immunohistochemical analysis of oval cells. A: Hematoxylin and eosin staining of liver tissue from 9 days after PH in group I (×40). The arrows show the cluster of oval cells. B: Hematoxylin and eosin staining of liver tissue from 9 days after PH in group II (×40). The arrow shows the cluster of oval cells. C: CK-19 staining at 9 days after PH in group I (×100). The arrow shows the CK-19-expressing oval cells. Oval cells were stained with anti-CK-19 antibody. D: OV-6 staining at 9 days after PH in group I (×200). Oval cells were stained with anti-OV-6 antibody.



PCNA in oval cells of both groups were high, reaching about 90% at 4 days, and reducing with time. The numbers of PCNA-positive oval cells at 4, 7, 9 and 13 days in group II were 16.4 ± 5.7, 78.0 ± 10.2, 65.0 ± 8.3 and 21.5 ± 5.4, respectively. On the other hand, in group I, they were increased to 17.2 ± 2.5, 126.7 ± 15.2, 142.0 ± 15.6 and 64.0 ± 15.9, respectively (Fig. 6B). Taken together, activation and expansion of oval cell population is enhanced by increased expression of HGF.

3.3. SCF and c-kit expression

Since SCF/c-kit has been reported to play an important role in development of oval cells in the AAF/PH model, expression of SCF and c-kit was examined in rat livers from group I and II (Fig. 8). As shown in Fig. 8, SCF mRNA was strongly expressed at 4 and 7 days after PH in group I, however, a small amount of SCF mRNA was also observed in group II.

←

Fig. 6. Serial changes of oval cells and PCNA-expressing oval cells. (A) Oval cell number in group I and II. The number of oval cells was counted in 10 periportal areas selected randomly in each specimen stained with hematoxylin and eosin by using NIH Image 1.55 (National Institutes of Health, USA). The value was expressed as the number per periportal field (0.25×0.25 mm^2). (B) PCNA-positive oval cell number in group I and II. The PCNA-positive oval cells were counted in the same way.

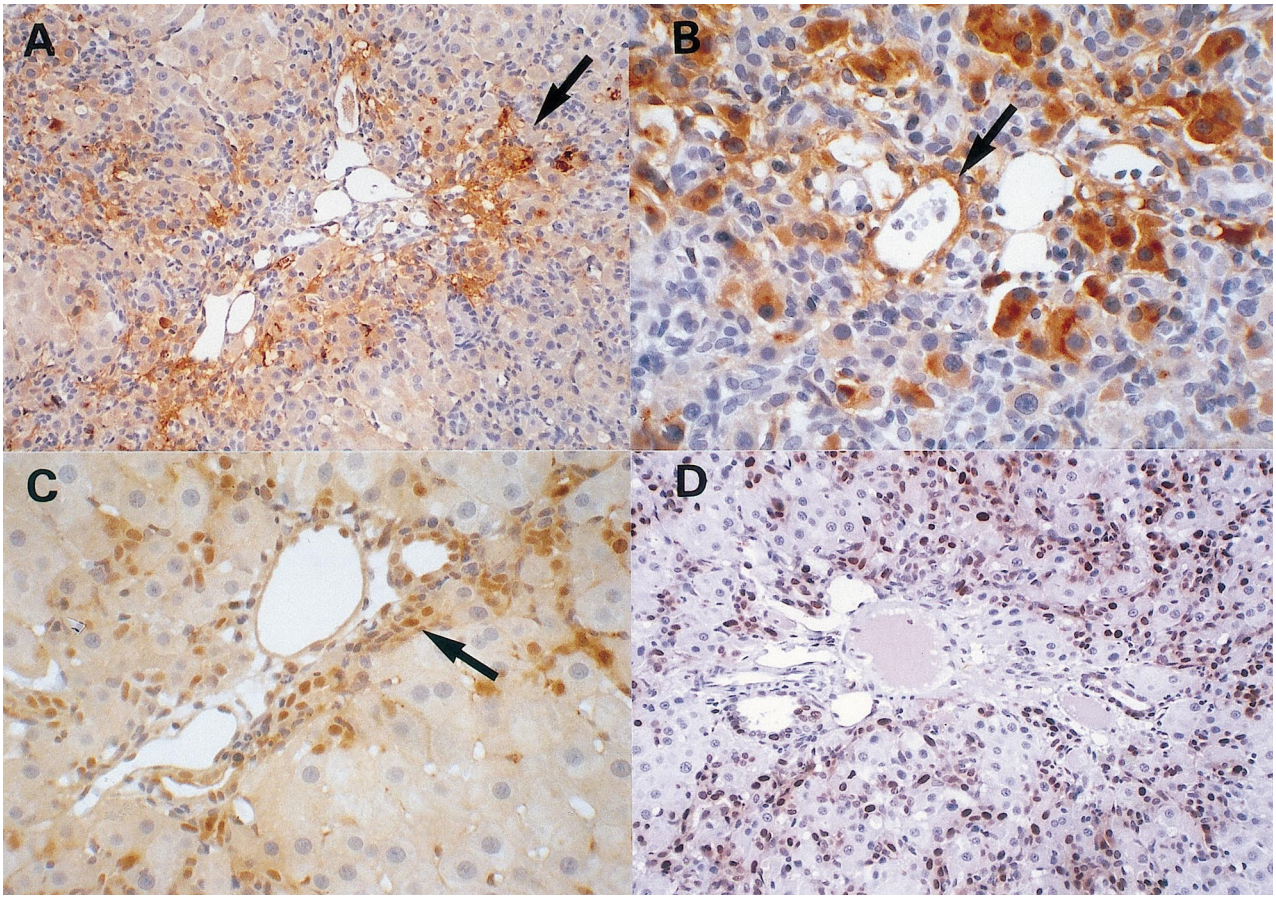


Fig. 7. Immunohistochemical study of oval cells. A: c-kit was expressed in oval cells and biliary epithelial cells (×40). The arrow shows the c-kit-expressing oval cells. B: c-kit was expressed in oval cells and bile duct epithelial cells (×400). The biliary epithelial cells are also positive for c-kit (arrow). C: SCF was expressed in oval cells and biliary epithelial cells (×200). The arrow shows the SCF-positive oval cells. D: PCNA was expressed in oval cells in portal area (×100).

Expression of c-kit mRNA in group I was higher than that in group II at each time point. Western blot analysis also showed that c-kit protein was strongly expressed in livers from group I, especially at 9 days after PH (data not shown). Ito cells, which stain positively for desmin, have been shown to be closely associated with the development of oval cells in the AAF/PH model [21]. We examined their association with oval cells by staining Ito cells with desmin. The desmin-positive Ito cells were observed in the proximity of oval cells (data not shown). c-kit protein-expressing cells were oval cells and biliary epithelial cells in the portal area (Fig. 7A,B). In addition, SCF protein was also expressed in oval cells and biliary epithelial cells (Fig. 7C). These data suggest that SCF and c-kit on oval cells act in an autocrine fashion.

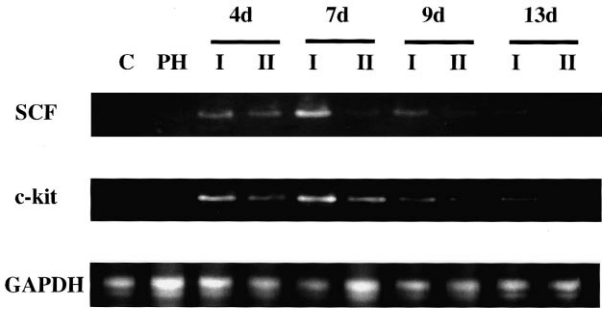


Fig. 8. Expression of SCF mRNA and c-kit mRNA. Analysis of SCF mRNA and c-kit mRNA was done by RT-PCR. C: rat liver with no treatment, PH: rat liver 2 days after PH without AAF administration.

4. Discussion

Oval cells are undoubtedly important for liver regeneration in severely injured liver. Oval cells emerge in situations where mature hepatocytes cannot proliferate, and they differentiate into hepatocytes [1–4]. In addition, in submassive necrosis, a typical feature of fulminant hepatic failure, and in human diseased liver, oval cells emerge in the portal area, suggesting that oval cells play a critical role in human liver regeneration [27]. Oval cells isolated from LEC rats differentiate into albumin-producing hepatocytes when returned to the livers of LEC/NAGASE analbuminemic double mutant rats [28]. This observation is very intriguing, since the authors presented the possibility that oval cells can be used as powerful tool to cure patients with hypoalbuminemia.

In the present study, we demonstrated that HGF gene transfer into liver causes increased proliferation of oval cells. Although HGF was initially found to be a mitogen for hepatocytes in vitro, it also proved to be a potent mitogen in vivo. We developed transgenic mice expressing HGF in liver

[29]. Using these mice, we demonstrated that HGF accelerates liver regeneration, protects against D-galactosamine-induced liver injury, and inhibits growth of c-myc-induced hepatocellular carcinoma, suggesting that HGF is active in vivo as well as in vitro [29–31]. In addition, gene therapy by HGF was reported to be potentially useful for treatment of experimental liver cirrhosis in rats [32]. In the present study, albumin-producing cells eventually appeared in oval cell population around portal field at 13 days in group I (data not shown). It is possible that HGF stimulates differentiation of the oval cells into hepatocytes. Taken together, ex vivo or in vivo gene therapy by HGF may be promising for treatment of liver diseases refractory to conventional therapy.

Why SCF and c-kit were up-regulated in the rat livers in group I remains to be resolved. There are two possibilities. Firstly, HGF directly stimulates SCF/c-kit via HGF/c-met signaling, resulting in activation of oval cells. Secondly, HGF activates oval cell proliferation, resulting in a concomitant and autocrine increase of SCF/c-kit. Recently, Matsusaka et al. have reported that the SCF/c-kit system plays an important role in development of oval cells, but reflecting a small role in stimulating proliferation of oval cells [21]. In the present study, in vivo transfer of HGF gene results in overexpression of SCF and c-kit in oval cells, suggesting oval cell activation and proliferation by HGF may be closely linked to the SCF/c-kit system.

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