an enzyme-linked immunosorbent assay (ELISA) that effectively measures human HGF in serum. The levels of serum human HGF increased in patients with a variety of liver diseases, especially ALF [9]. Measurement of these serum HGF concentrations is useful to predict the prognosis of patients with ALF [10], making this measurement an important laboratory marker for the early diagnosis and outcome prediction of ALF. The ELISA procedure, however, requires approximately 6 h to obtain patients' serum HGF levels. As the clinical measurement of serum HGF levels is usually performed by a commercial laboratory, several days are necessary for doctors to obtain patients' serum HGF values. As the status of patients with ALF often progresses rapidly, rapid assessment at the bedside of serum HGF levels in patients with various liver diseases would be of great clinical importance. Therefore, we developed a novel assay using immunochromatography for the semi-quantification of serum HGF levels. This study also evaluated the usefulness of this method in the assessment of patients with acute hepatic injury, such as fulminant hepatitis (FH) or acute hepatitis (AH).

2. Subjects and methods

2.1. Definition of disease type

The most widely accepted definition of ALF requires evidence of coagulation abnormalities and encephalopathy in a patient without pre-existing cirrhosis that develops within 26 weeks [1,2,11]. Patients with vertically acquired HBV may be included in this diagnosis despite the possibility of cirrhosis. Additional names for this condition include fulminant hepatic failure and FH [2,11]. According to Japanese diagnosis criteria, a diagnosis of FH was made in patients developing an encephalopathy of grade II or greater and a prothrombin time (PT) <40% of normal within 8 weeks of AH onset [12,13]. Acute liver failure on chronic liver disease (AOC) was diagnosed by the same criteria as FH, but developing following a diagnosis of chronic hepatitis. We excluded those AOC patients with liver cirrhosis from this study. As a number of reports have described ALF to include cases with pre-existing symptomless chronic liver conditions [14,15], we included patients with AOC who exhibited underling chronic liver disease without liver cirrhosis in the definition of ALF.

2.2. Subjects

We examined 13 patients with FH, 6 with AOC, 40 with AH, and 2 with acute exacerbation of chronic hepatitis without hepatic failure (Table 1). Six patients with FH and five with AOC exhibited serum markers of hepatitis B virus (HBV) infection. Ten FH patients and all six AOC patients died. One patient with alcoholic hepatitis died due to accidental bleeding without liver failure. Of the patients with acute hepatitis, 6 were positive for hepatitis A virus infection, 13 were HBV-positive, 3 were positive for hepatitis C virus, and 4 were diagnosed with alcoholic hepatitis. Two patients with acute exacerbation of chronic hepatitis were also infected with HBV.

Table 1
Demographics of the 61 patients with acute hepatic injury enrolled in this study

Diagnosis	N	Etiology[a]	Death
Acute liver failure			
Fulminant hepatitis	13	B; 6	3
		Unknown; 7	7
AOC[b]	6	B; 5	5
		Unknown; 1	1
Non-acute liver failure	36	A; 6	0
Acute hepatitis		B; 13	0
		C; 3	0
		Unknown; 14	0
Acute alcoholic hepatitis	4	Alcohol; 4	1
Acute exacerbation of chronic hepatitis[c]	2	B; 2	0
Total	61		17

[a] A, hepatitis A virus; B, hepatitis B virus; C, hepatitis C virus.
[b] Acute liver failure on chronic liver disease.
[c] Excluding acute liver failure.

Sixty-one serum samples were collected from 61 patients prior to receiving therapy, including blood transfusion. Of these samples, 40 were collected on the day of admission (day 1) or the next day (day 2), while 15 were obtained on days 2–4, 4 were taken on days 5–8, and 2 were acquired on either day 12 or day 28. In addition, 16 serum samples were sequentially collected before and after receiving therapy from 7 of the 61 patients during hospitalization. All 77 samples were stored at −80 °C until experimentation. Serum HGF concentrations were measured in each sample using both a semi-quantitative immunochromatographic (IC) assay and a human HGF ELISA (Otsuka Pharmaceutical Co., Tokushima, Japan) [9]. Ten healthy volunteers were also examined as normal controls.

2.3. Methods

We developed a semi-quantitative IC assay kit consisting of sample hole, a lane labeled H, and two windows, T and C (Fig. 1). In lane H, an excess amount of colloidal gold-labeled anti-human HGF mouse monoclonal antibody (Au-anti-HGF) was applied; this antibody (Au-anti-HGF) is movable. In window T, an excess amount of anti-human HGF mouse monoclonal antibody (anti-HGF) was fixed. An anti-mouse IgG rabbit monoclonal antibody (anti-IgG) is fixed in window C (Fig. 1A). These kits need to be stored at 4 °C, but can be used after approximately 10 min at room temperature according to the following procedure. One hundred microliters of serum is dropped into the sample hole. Serum proteins, including HGF, diffuse to lane H, reacting there with the Au-anti-HGF. This complex moves to window T. As the epitopes recognized by the Au-anti-HGF and anti-HGF antibodies differ from each other, these two

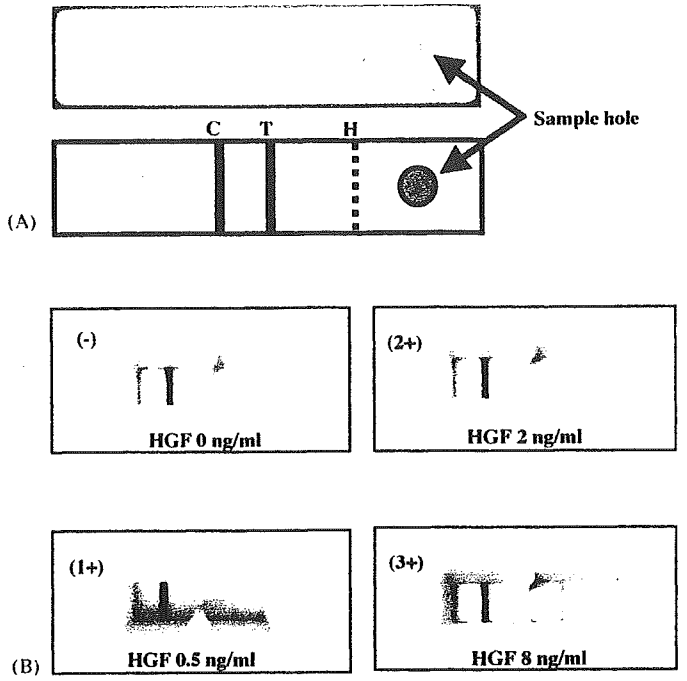

Fig. 1. The immunochromatographic assay device. (A) lane H: a movable colloidal gold-labeled anti-human HGF monoclonal antibody; T: anti-human HGF monoclonal antibody was fixed in a line at window T; C: anti-mouse IgG antibody is fixed at window C. (B) Evaluation of the immunochromatographic (IC) assay using purified human HGF standards. The intensity of the red test line was dependent on the concentration of purified HGF.

antibodies form a sandwich complex surrounding human HGF [(Au-anti-HGF)–HGF–(anti-HGF)], becoming fixed at window T. Therefore, the total amount of HGF in serum samples is detected as the intensity of the red-colored band. The remaining Au-anti-HGF antibody moves to window C and reacts with anti-IgG. This reaction serves as a control to guarantee the assay system; a strong band should always be detected in window C. The intensity of the red band in window T is compared to a standard HGF solution 20 min after loading. A standard HGF solution (32 ng/ml) is prepared at 8.0, 2.0, and 0.5 ng/ml (Fig. 1B). Recombinant human HGF, (rhHGF), kindly provided by Mitsubishi Pharma Co. (Tokyo, Japan), was used as the standard solution. The HGF levels determined from serum samples obtained from patients with a variety of liver diseases were classified into the following ranges according to the intensity of the bands: (−) <0.5 ng/ml, (1+) = 0.5 or > 0.5 ng/ml, (2+) = 2.0 or >2.0 ng/ml, and (3+) = 8.0 or > 8.0 ng/ml.

2.4. Statistical analysis

Statistical parameters were ascertained using StatView J-4.5 software (Abacus Concepts Inc., Berkeley, CA). The relationship between the semi-quantitative serum HGF levels measured by the IC assay and the quantitative serum HGF levels measured by ELISA was analyzed by one-factor ANOVA and Scheffe's test. The relationship between serum HGF levels by IC assay and either disease severity or prognosis was analyzed by the χ^2-test or Fisher's exact test, as appropriate. p-Values <0.05 were considered to be statistically significant.

3. Results

3.1. Evaluation of semi-quantitative IC assay using the standard concentrations of recombinant human HGF

We performed semi-quantification of the standard concentrations of rhHGF using the IC assay. While samples without any rhHGF did not produce a detectable red band in window T, a red band was observed in window C (Fig. 1A). Samples containing low concentrations of rhHGF (0.5 ng/ml) resulted in a trace red band in window T (Fig. 1B). When the rhHGF concentration was increased to 2.0 ng/ml, the red band was clearly observed; the intensity of the band was enhanced in parallel with increases in rhHGF concentrations (Fig. 1B). Therefore, the minimum limit of detection for rhHGF is <0.5 ng/ml in our semi-quantitative IC assay method. This assay method can semi-quantitatively measure HGF concentrations up to 32 ng/ml rhHGF.

In healthy subjects, serum HGF levels, which were determined to be less than 0.40 ng/ml (lower limit of detection) by human HGF ELISA, were negative (−; no red line) according to the semi-quantitative IC assay (data not shown).

Table 2
Relationship between the serum levels of HGF measured by ELISA and those obtained by immunochromatographic assay

Semi-quantitative evaluation of HGF*	Number of samples	HGF concentration**, † (mean ±SD, range)
–	2	0.77 ± 0.52 (0.40-1.13)
1+	41	0.73 ± 0.44 (0.30-2.10)
2+	26	2.01 ± 1.21 (0.33-5.23)
3+	8	5.04 ± 1.54 (3.10-7.33)

Seventy-seven serum samples, which included 16 sequential samples were evaluated by (*) immunochromatographic assay or (**) ELISA.
†Significant differences ($p < 0.001$) existed between the groups based on one-factor ANOVA or '¶' $p < 0.001$ Scheffe's test.

3.2. The correlation of semi-quantification of HGF determined by IC assay with concentration of HGF determined by ELISA in patients with acute hepatic injury

We next examined the serum HGF levels in patients with acute hepatic injury using the semi-quantitative IC assay. Seventy-seven serum samples taken from 61 patients, which included 16 sequential samples before and after treatment, were used. The serum HGF levels using IC assay were compared with those determined by human HGF ELISA. According to the observed increases in intensity in the IC assay, mean serum HGF levels increased in patients with acute hepatic injury (Table 2). The mean serum HGF values of the categories assigned to patient serum samples by the IC assay results were significantly different by ELISA (Table 2) ($p < 0.001$).

3.3. The correlation between HGF levels measured by IC assay and prognosis in patients with acute hepatic injury

To evaluate the usefulness of the IC assay in the prediction of patient outcome, we examined the correlation between HGF levels measured by IC assay and the prognosis of patients in acute hepatic injury. We analyzed 61 blood samples collected from 61 patients before receiving therapies, such as blood transfusion. The rate of ALF increased with increases in HGF values determined by IC assay (Table 3A). In addition, increased serum HGF levels measured by IC assay correlated with an increased rate of nonsurvivors (Table 3B).

4. Discussion

ELISA is currently used in clinical practice to measure serum HGF levels [9,10]. These samples, however, are not typically measured at the bedside, because ELISA is not a simple method. Instead, HGF is evaluated in most patients by external commercial laboratories, which may require several days to receive the results. Therefore, current testing methods do not allow the prompt diagnosis that is necessary to improve the prognosis of patients with ALF. To address this problem, we developed a rapid, simple assay device that can semi-quantify serum HGF levels using immunochromatography. This assay can be performed in ordinary clinical/hospital settings. In this study, our evaluation of the semi-quantification of serum HGF levels using a new IC assay demonstrated that measured values correlated well with the concentration of serum HGF determined using ELISA. Our assay device can therefore provide rapid and easy access to the patient HGF levels at the bedside.

Serum HGF levels in patients with a variety of liver diseases, such as FH and AH, are significantly elevated [9,10,16–19]. These serum levels are significantly elevated in patients with FH, over those seen in AH [16,18,19]. Serum HGF levels are not always elevated in patients with acute hepatitis, even when the laboratory data such as a PT reflects an enhanced severity of the liver disease. In this study, semi-quantification of HGF levels using the IC assay demonstrated significantly elevated values in patients with ALF from the values seen in patients without ALF (Table 3A). The HGF values in patients with acute hepatitis, whose PT were <40%, remained low by IC assay (data not shown). These results suggest the clinical utility of this IC assay for the prompt discrimination of FH from acute hepatitis.

In addition, measurement of serum HGF is useful for predicting the outcome of patients with ALF [10]. In patients with a poor prognosis FH, especially in subacute type [20], HGF is an important factor in the decision between plasmapheresis and liver transplantation. Therefore, measurement of HGF is a useful and important test, contributing significantly

Table 3
Relationship between serum HGF levels measured by immunochromatographic assay and either diagnosis (A) or prognosis (B) of patients with acute hepatic injury

Part A			Part B		
Semi-quantitative evaluation of HGF[a]	ALF[b] ($n=19$)	Non-ALF[b] ($n=42$)	Semi-quantitative evaluation of HGF[a]	Death ($n=17$)	Alive ($n=44$)
–	0	2	–	0	2
1+	2	28	1+	1	29
2+	13	11	2+	12	12
3+	4	1	3+	4	1

[a] Sixty-one serum samples, collected from 61 patients before receiving therapies such as blood transfusion were evaluated by immunochromatographic assay.
[b] Acute liver failure (ALF) included fulminant hepatitis and acute liver failure on chronic liver disease.

to the survival rates of such patients. In this study, serum human HGF values determined by IC assay were significantly different between nonsurvivors and survivors in patients with acute hepatic injury. Semi-quantification of HGF by IC assay is thus a useful predictor of outcome in acute hepatic injury, as is the determination of HGF concentration by ELISA [10].

The serum HGF level of patients with FH is known to be >1 ng/ml. These levels in all of the nonsurvivors in ALF, with one exception, were reported to be >3 ng/ml [9,16]. Although the categories were not set at either 1 or 3 ng/ml in our study, we also observed that the rate of patients in liver failure was higher in patient groups whose HGF values measured by IC assay were 2+ or 3+ [17/29 (58.6%)] than in patients with HGF values of either (−) or (1+) [2/32 (6.3%)] ($p < 0.001$) (Table 3A). Furthermore, the mortality of patients with IC assay HGF values of (2+) or (3+) was higher [16/29 (55.2%)] than that observed for patients with values of (−) or (1+) [1/32 (3.1%)] ($p < 0.001$) (Table 3B). These results suggest that the measurement of HGF values by IC assay is similar to ELISA in diagnostic accuracy and more rapid and simple to perform. Further studies, including the re-calibration of the standard solution, the use sequential measurements, and the evaluation of this methodology in a large-scale trial, will be necessary to demonstrate the clinical utility of this IC assay.

We conclude that this newly developed IC assay is applicable to the detection of serum human HGF levels, in a manner similar to ELISA. In addition, this method will allow the wide application of bedside HGF testing in a clinical setting, which will be especially useful for the early diagnosis and prognosis prediction of patients with acute hepatic injury.

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HEPATOLOGY

Hepatocyte growth factor accelerates the proliferation of hepatic oval cells and possibly promotes the differentiation in a 2-acetylaminofluorene/partial hepatectomy model in rats

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Abstract

Background: Hepatocyte growth factor (HGF) is the primary agent promoting the proliferation of mature hepatocytes. The purpose of the present paper was to clarify the effects of HGF on the proliferation and differentiation of hepatic oval cells using a 2-acetylaminofluorene/partial hepatectomy (2-AAF/PH) model in rats.

Methods: Recombinant human HGF (0.2 mg/day) was administered to 2-AAF/PH rats for 7 days using osmotic pumps intraperitoneally implanted in conjunction with hepatectomy (day zero).

Results: Periportal basophilic areas consisting of oval cells were significantly enlarged by treatment with HGF on day 8. In control animals, expression of α-fetoprotein (AFP) in the liver was gradually upregulated, leading a marked increase on day 12. In HGF-treated rats, AFP expression was stimulated at an earlier date and decreased to an undetectable level on day 12. Conversely, expression of albumin transcripts, which was stimulated by HGF-treatment at a later date, continued to increase even after HGF administration ceased, leading to an extremely high level on day 12. Moreover, treatment with HGF also stimulated the expression of hepatocyte nuclear factor-1α and -4α at an early date.

Conclusions: These results indicate that, besides the proliferation of hepatic oval cells, HGF possibly promotes the differentiation to hepatocytes *in vivo*, suggesting that recombinant human HGF accelerates the regeneration of severely damaged livers, a situation in which the proliferation of mature hepatocytes is impaired.

Key words: 2-acetylaminofluorene, differentiation, hepatocyte growth factor, oval cell, proliferation.

INTRODUCTION

Adult mammalian livers can fully regenerate following surgical resection or injury although normal adult liver cells are essentially quiescent. In rodents, following liver tissue loss due to surgical or chemical treatments, the regeneration response begins with the activation of growth of mature hepatocytes and is followed by proliferation of biliary epithelial cells and sinusoidal cells restoring normal tissue mass and architecture.[1,2] However, when 2-acetylaminofluorene (2-AAF) is continuously administered to animals, the process is slowed due to the lack of epithelial cell division; a new source of cells, oval cells, appears to facilitate liver repair. Oval cells are characterized by their ovoid nuclei, small size (relative to hepatocytes) and high nuclear to cytoplasm ratio, and they are thought to play an important role in hepatic growth and development.[3–5] Furthermore, hepatic oval cells are capable of differentiating to several lineages, including hepatocytes and biliary epithelial cells.[6–8] Therefore, oval cells are thought to be a candidate population for hepatic stem cells in rats. In

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humans, oval-like cells are also present in injured liver tissues and are thought to play an important role in the regeneration of severely damaged livers.[9-12]

Hepatocyte growth factor (HGF) was first purified as a potent mitogen for hepatocytes from the plasma of patients with fulminant hepatic failure.[13,14] This protein is characterized as a broad-spectrum and multifunctional growth factor acting as a mitogen, motogen and morphogen for a variety of cells through binding to c-Met, a specific receptor for HGF, on the cell membrane.[15,16] Hepatocyte growth factor is one of the primary agents promoting the proliferation of mature hepatocytes[17-19] and acts in concert with transforming growth factor-α and heparin-binding epidermal growth factor-like growth factor during liver regeneration *in vivo*.[20,21] In addition, HGF has been shown to stimulate the proliferation of hepatic oval cells.[22-24] Recently, several investigators have shown that HGF plays an important role in liver development[25,26] and is an important factor in the *in vitro* differentiation of hematopoietic or embryonic stem cells to hepatocytes.[27,28] These investigations suggest the possibility that, in addition to proliferation of both mature hepatocytes and oval cells, HGF induces the differentiation of oval cells to hepatocytes during liver regeneration. However, the effect of HGF on the differentiation of oval cells is not well understood.

Recombinant human HGF will be available for patients with severe liver diseases, such as fulminant hepatic failure and liver cirrhosis, in the near future in Japan. Therefore, in the present study we examined whether treatment with recombinant human HGF stimulates oval cell proliferation, and we also investigated whether HGF accelerates the differentiation of oval cells to hepatocytes, using a 2-AAF/partial hepatectomy (2-AAF/PH) model in rats.

METHODS

Animals

Eight-week-old male Fisher rats were obtained from Kyudo (Kumamoto, Japan). Rats were maintained under constant room temperature (25°C) and provided with free access to a standard diet and tap water in accordance with institutional guidelines; rats were acclimatized to these conditions for 7 days prior to any involvement in experimental studies. The Ethics committee of University of Miyazaki (Miyazaki, Japan) approved all aspect of the present study. Rats were given 2-AAF (1.5 mg/day; Sigma Chemical, St Louis, MO, USA) i.p. five times a week for 2 weeks, and two-thirds partial hepatectomy was performed 7 days after the beginning of 2-AAF treatment (day zero).

Administration of recombinant human HGF and measurement of human HGF in the serum and liver tissues

Recombinant human HGF (0.2 mg/day) in phosphate-buffered saline (PBS) or PBS alone was given for 7 days by osmotic pumps (Alzet, Palo Alto, CA, USA) implanted in the peritoneal cavities in conjunction with partial hepatectomy on day zero. Human HGF levels in the serum (days 0, 2, 4, 7 and 12) and the liver tissue (day 7) were measured using a commercially available ELISA kit (Otsuka Pharmaceutical, Tokushima, Japan), in which only human HGF, but not rat HGF, was detected.[29]

Tyrosine phosphorylation of c-Met

Tyrosine phosphorylation of c-Met was evaluated by western blotting. Liver tissues were obtained from 2-AAF/PH rats treated with or without HGF on day 8, and were solubilized in lysis buffer containing 50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1% nonidet P-40 (NP40), 0.5% sodium deoxycholate, 1 mmol/L Na_3Vo_4, 1 mmol/L dithiothreitol, 1 mmol/L phenylmethylsulfonyl fluoride, and 10 μg/mL each of leupeptin, aprotinin and pepstatin A. Post-nuclear supernatants were precleared with protein A-agarose and immunoprecipitated with antic-Met antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and protein A-agarose. Immunoprecipitated materials were washed five times with 0.1% NP40 and 0.05% sodium deoxycholate and eluted by boiling in Laemmli Sample Buffer (BIO-RAD, Hercules, CA, USA). Samples were separated by 7.5% sodium dodecylsulfate (SDS)–polyacrylamide gel electrophoresis and blotted onto a nitrocellulose filter. After blocking membranes with 1% bovine serum albumin, filters were incubated with horseradish peroxidase-conjugated antiphosphotyrosine antibody and subjected to ECL Western Blotting Detection (Amersham Life Science, Buckinghamshire, England).

Measurement of oval cell area in liver specimens

Liver tissues were obtained from 2-AAF/PH rats treated with or without HGF on days 4 and 8, and were stained with hematoxylin–eosin (HE). Oval cell areas were measured by video micrometer system (Olympus MV30, Olympus, Tokyo, Japan), and were auto-calculated by tracing the oval cell cluster borders with a video monitor at ×10 magnification. Measurement was performed in four different animal specimens from each group by two observers who were unaware of the experiment protocol. Statistical analysis was performed with a Mann–Whitney's *U*-test.

Immunohistochemistry

Liver tissues were fixed with 10% formalin and embedded in paraffin. For immunohistochemistry, sections were heated in a target retrieval solution (10 mmol/L citrate buffer, pH 6.0) for 5 min at 115°C or 121°C with an autoclave to facilitate antigen retrieval. The sections were treated with 3% H_2O_2 in PBS for 10 min and

washed twice with PBS, followed by blocking in 3% bovine serum albumin in PBS for 1 h at room temperature. The sections were then incubated with the following primary antibodies for 16 h at 4°C: mouse monoclonal anti-proliferating cell nuclear antigen (PCNA) antibody (Oncogene, Calbiochem, Cambridge, UK); and anti-α-fetoprotein (AFP) antibody (NeoMarkers, Fremont, CA, USA). After washing, the sections were incubated with Envision-labeled polymer reagent (Dako, Carpinteria, CA, USA) for 45 min at 37°C. The reaction was detected by ImmunoPure metal enhanced diaminobenzidine tetrahydrochloride substrate kit (Pierce, Rockford, IL, USA) and counterstained with Mayer's hematoxylin.

RNA isolation and quantitative or semiquantitative reverse transcription–polymerase chain reaction

To examine the expression of AFP and albumin mRNA, quantitative quantitative reverse transcription–polymerase chain reaction (RT-PCR) was performed. Total RNA was extracted from liver tissues, which were obtained from 2-AAF/PH rats treated with or without HGF on days 0, 4, 8 and 12, using Isogen reagent (Nippon Gene, Toyama, Japan). Total RNA (0.5 μg) was reverse transcribed using random hexamer priming and MMLV reverse transcriptase. The PCR primers and fluorogenic probes were designed according to published sequences using Primer Express software (Applied Biosystem, Foster City, CA, USA; Table 1). The fluorogenic probes contained a reporter dye (6-carboxyfluorescein, or FAM) covalently linked at the 5′-end and a quencher dye (6-carboxy-teramethyl-rhodamine, or TAMRA) covalently attached at the 3′-end. Extension from the 3′-end was blocked by attachment of a 3′-phosphate group. The PCR reactions contained at a final concentration 300 nmol/L forward and reverse primers, 200 nmol/L TaqMan probe, 200 μmol/L deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP) and deoxyguanosine triphosphate (dGTP), 400 μmol/L deoxyuridine triphosphate (dUTP), 0.025 U/mL AmpliTaq Gold, 0.01 U/mL uracil-*N*-glycosylase and 2.5 μL cDNA in a total volume of 25 μL. Each PCR amplification was performed in triplicate wells using the following temperature and cycling profile: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. The AFP and albumin transcripts were quantitated using an ABI PRISM 7700 Sequence Detection System (Perkin Elmer, Foster City, CA, USA) and were normalized to the levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Data are expressed as mean ± SD. The statistical analysis was performed with a Mann–Whitney *U*-test.

Expression of hepatocyte nuclear factor (HNF)-1α and -4α was evaluated by semiquantitative RT-PCR. Total RNA (0.5 μg) was reverse transcribed at 37°C for 60 min, and amplified by 35 cycles of PCR (94°C for 30 s, 53°C for 30 s, 72°C for 30 s) using a set of primers listed in Table 1. The reliability of these results was confirmed by performing assays in triplicate for each time-point studied.

RESULTS

Intraperitoneal delivery of human HGF increased the detection of human HGF in the serum and liver tissues and stimulated tyrosine phosphorylation of c-Met in the liver

Rats were given 2-AAF five times a week for 2 weeks, and a two-third partial hepatectomy was performed 7 days after the beginning of 2-AAF treatment (day 0). In order to evaluate the ability of intraperitoneally implanted osmotic pumps to deliver recombinant growth factor, serum levels of human HGF were measured by ELISA (Fig. 1a). The ELISA used could not

Table 1 Primers and probes

Name		Primers and probes for quantitative RT-PCR
AFP	Sense	5′-GAGAGTTGCCCAGCATACGAA-3′
	Antisense	5′-CCTTGTCATACTGAGCGGCTA-3′
	Probe	5′-CAGGGCGATGTCCATAAACACGTTCAT-3′
Albumin	Sense	5′-GAGACTGCCCTGTGTGGAAGA-3′
	Antisense	5′-CTTTCCACCAAGGACCCACTA-3′
	Probe	5′-TCTGTCTGCCATCCTGAACCGTCTGTG-3′
		Primers for semiquantitative RT-PCR
HNF-1α	Sense	5′-AGCTGCTCCTCCATCATCATCACA-3′
	Antisense	5′-TGTTCCAAGCATTAAGTTTTCTATTCTAA-3′
HNF-4α	Sense	5′-TGTCCATTCGCATCCTCGAT-3′
	Antisense	5′-CCCATGTGTTCTTGCATCAGG-3′
GAPDH	Sense	5′-ACTCTACCCACGGCAAGTTCA-3′
	Antisense	5′-GGCAGTGATGGCATGGACT-3′

AFP, α-fetoprotein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HGF, hepatocyte growth factor; HNF, hepatocyte nuclear factor; RT-PCR, reverse transcription–polymerase chain reaction.

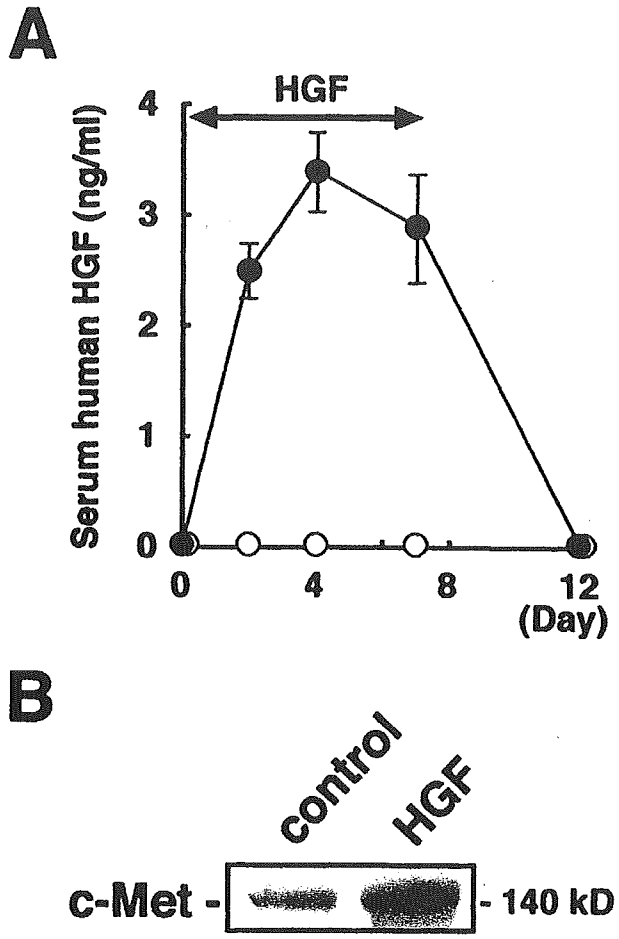

Figure 1 Intraperitoneal delivery of human hepatocyte growth factor (HGF) increased serum HGF levels, and stimulated c-Met tyrosine phosphorylation in liver tissues. Rats were given 2-acetylaminofluorene (2-AAF) five times a week for 2 weeks, and partial hepatectomy was performed after five doses of 2-AAF administration (day 0). Recombinant human HGF (0.2 mg/day) or phosphate-buffered saline (PBS) alone (control) was given for 7 days by osmotic pumps, which were implanted into the peritoneal cavities in conjunction with partial hepatectomy on day 0. (a) Serum HGF levels were examined by ELISA capable of detecting only exogenously administered human HGF as described in the Methods.[29] Rats treated with human HGF (●; $n = 4$) or PBS alone (○; $n = 4$) are shown. Recombinant human HGF was released from intraperitoneally implanted osmotic pumps, leading to detectable levels of human HGF. (b) Tyrosine phosphorylation of c-Met in liver tissues. Liver tissues were obtained from 2-AAF/partial hepatectomy (2-AAF/PH) rats treated with PBS alone (control) or recombinant human HGF on day 8 and were solubilized with lysate buffer containing protease inhibitors. Cell lysates were immunoprecipitated with an antic-Met antibody and blotted with an antiphosphotyrosine antibody. Intraperitoneal administration of human HGF stimulated c-Met tyrosine phosphorylation in the liver tissues.

detect endogenous rat HGF. Human HGF was not detected in the serum of rats treated with PBS alone at any time-point. In contrast, significant serum levels of human HGF were detected after implantation of osmotic pumps releasing recombinant human HGF, and levels continued to increase up to 7 days. The human HGF levels in liver tissues also increased to 2.0 ± 0.2 ng/g wet tissues on day 7.

c-Met, a specific receptor for HGF, is expressed on hepatocytes and is phosphorylated in response to ligation by HGF. Tyrosine phosphorylation of c-Met in liver tissues was examined by western blotting (Fig. 1b). As 2-AAF administration and partial hepatectomy increased endogenous rat HGF, some phosphorylated c-Met was detected within the livers of rats treated with PBS alone. Following the administration of human HGF to rats with 2-AAF administration and partial hepatectomy, c-Met tyrosine phosphorylation in liver tissues was enhanced.

Hepatocyte growth factor accelerated the proliferation of hepatic oval cells

We evaluated the effect of treatment with human HGF on the proliferation of hepatic oval cells. Periportal basophilic areas consisting of oval cells were observed in liver tissue after partial hepatectomy following 2-AAF administration (Fig. 2a). When human HGF was administered i.p., the basophilic areas were apparent on day 4 (Fig. 2a), and the oval cell clusters were significantly enlarged on day 8, in comparison with 2-AAF/PH rats treated with PBS alone ($P = 0.014$; Fig. 2a,b). Oval cells developed into basophilic hepatocytes and invaded the hepatic parenchyma on day 12, leading to a less substantial difference in the oval cell areas between PBS- and HGF-treated animals (data not shown). Immunohistochemically, hepatic oval cells strongly expressed c-Met on day 8, when compared with the surrounding mature hepatocytes regardless of HGF administration (data not shown). Conversely, most cells in oval cell clusters, the areas enlarged by HGF treatment, were stained with anti-PCNA antibody (Fig. 2c). The PCNA staining in mature hepatocytes surrounding oval cell clusters in the 2-AAF/PH rats was not observed, even when the rats were treated with HGF. These results indicate that i.p. administration of human HGF stimulated the proliferation of oval cells.

HGF stimulated the expression of AFP at an early stage, whereas increased albumin expression was observed at a later stage in rat livers treated with HGF

We examined the expression of AFP, a marker of oval cells, by immunohistochemistry (Fig. 3). On day 4, AFP expression was not observed in the liver of 2-AAF/PH rats treated with PBS alone (control), whereas, when the rats were treated with HGF, a small number of oval cells expressing AFP appeared in the periportal areas. The number of AFP-positive cells and the intensity of AFP expression increased on day 8 regardless of HGF administration. On day 12, although AFP expression was still sustained in control rats, the number of cells expressing AFP markedly declined in 2-AAF/PH

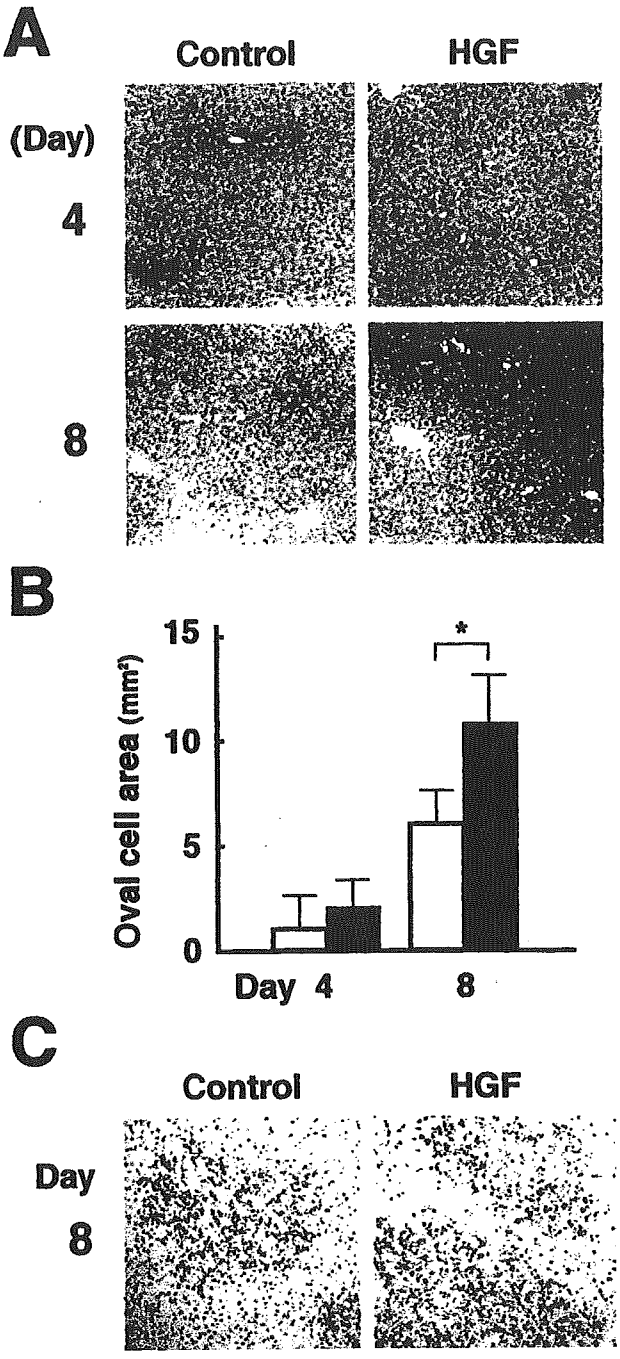

Figure 2 Administration of recombinant human hepatocyte growth factor (HGF) stimulated the proliferation of oval cells. (a) Liver tissues were obtained from 2-acetylaminofluorene/partial hepatectomy (2-AAF/PH) rats treated with phosphate-buffered saline (PBS) alone (control) or recombinant human HGF on days 4 and 8 and were stained with HE. When human HGF was administered i.p., the periportal basophilic areas consisting of oval cells were enlarged (original magnification ×400.) (b) The area occupied by oval cell clusters was calculated by tracing the area border under ×10 magnification as described in Methods. The measurement was performed in four different specimens of the liver tissues from PBS- (□; $n = 4$) or HGF-treated (■; $n = 4$) 2-AAF/PH rats) by two observers in a blind manner. The areas of oval cell clusters in the rat livers treated with human HGF were significantly larger than those without HGF on day 8 ($^*P = 0.014$). (c) Liver tissues from 2-AAF/PH rats treated with PBS or human HGF for 8 days were stained with anti-proliferating cell nuclear antigen (PCNA) antibody as described in Methods. Most cells in the oval cell clusters were positive for PCNA staining, and the area of oval cells were enlarged in HGF-treated animals (original magnification ×400).

rats treated with HGF. Conversely, expression of c-Kit, a marker of oval cells, or cytokeratin (CK)19 was observed in periportal areas on day 4, and the number of cells expressing c-Kit or CK19 increased on day 8, followed by a marked decrease in c-Kit or CK19 expression on day 12, in PBS-treated 2-AAF/PH rats (data not shown). However, expression of neither c-Kit nor CK19 was affected by administration of HGF.

To evaluate the sequential changes in AFP expression and albumin mRNA, we performed quantitative RT-PCR (Fig. 4). Expression of AFP mRNA in the 2-AAF/PH rat livers without HGF remained low on day 4, and increased during the experimental period, leading to a high level on day 12 (Fig. 4a). When 2-AAF/PH rats were treated with HGF, an increase in AFP expression was observed on day 4, and declined to an undetectable level on day 12. Conversely, expression of albumin mRNA remained low and was not affected by HGF treatment on day 4. Administration of recombinant human HGF, however, significantly stimulated albumin expression on days 8 and 12 (Fig. 4b). A marked increase in albumin expression was observed in 2-AAF/PH rats treated with HGF on day 12.

Expression of HNF-1α and -4α was stimulated at an early time-point in 2-AAF/PH rat livers treated with HGF

We examined the effect of recombinant HGF on the expression of liver-enriched transcription factors, HNF-1α and -4α, in the liver tissues of 2-AAF/PH rats by semiquantitative RT-PCR (Fig. 5). The HNF-1α transcripts were not detectable in normal rat liver tissues. In 2-AAF/PH rat livers without HGF treatment, expression of HNF-1α was observed after 4 days, and increased to a peak on day 8, followed by a mild decrease on day 12. When 2-AAF/PH rats were treated with HGF, HNF-1α expression was rapidly upregulated on day 4, and then declined to a low level on day 12. Trace amounts of HNF-4α transcripts were detected in normal rat liver tissues. Expression of HNF-4α was upregulated in the 2-AAF/PH rat livers on day 4, and increased on day 8. When 2AAF/PH rats were treated with HGF, a moderate stimulation of HNF-4α expression, compared to 2-AAF/PH rats without HGF, was observed on day 4, and continued on days 8 and 12. These results suggest that HGF induces the differentiation of oval cells predominantly to hepatocytes in the 2-AAF/PH rats.

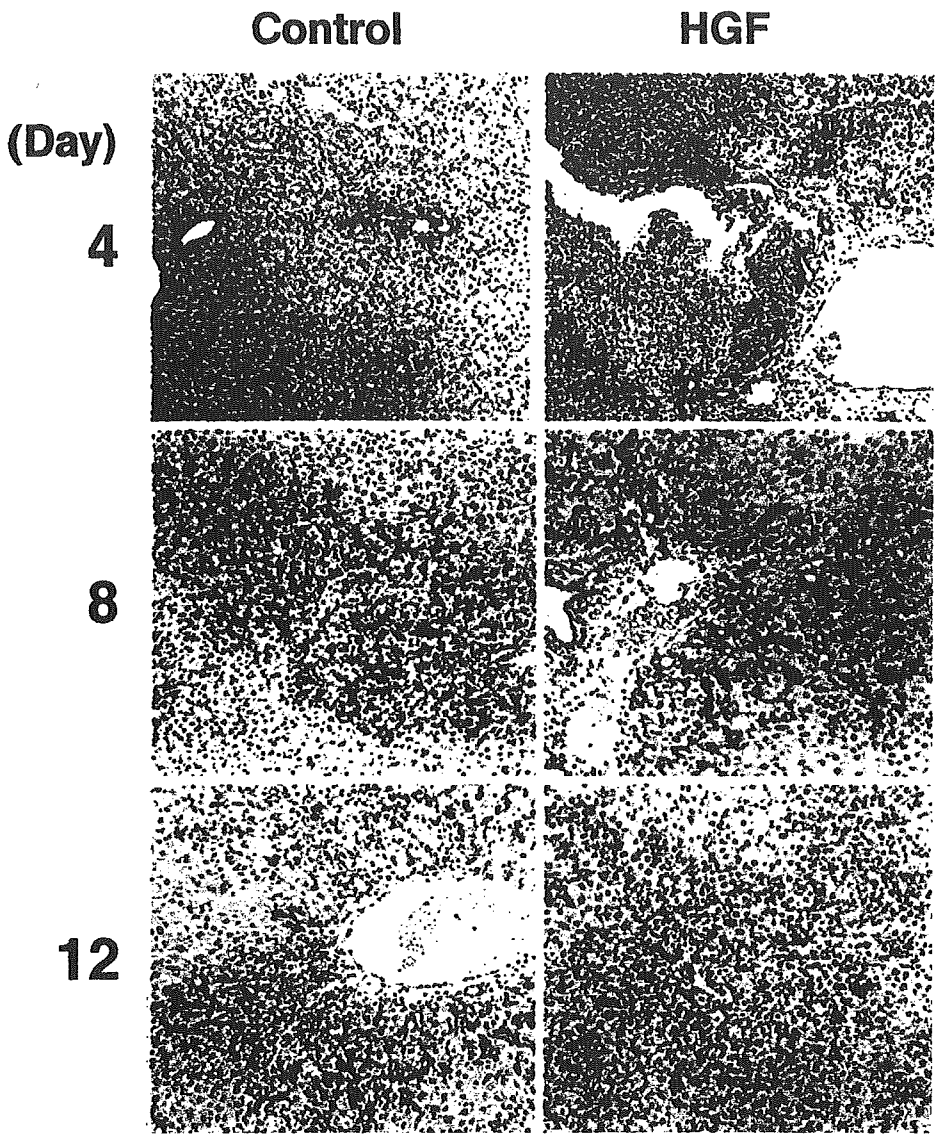

Figure 3 The α-fetoprotein (AFP)-expressing cells appeared in the rat livers treated with human hepatocyte growth factor (HGF) at an early stage. Liver tissues were obtained from 2-acetylaminofluorene/partial hepatectomy (2-AAF/PH) rats treated with phosphate-buffered saline (PBS) alone (control) or recombinant human HGF on days 4, 8 and 12, and were stained with anti-AFP antibody as described in Methods. Representative photographs are shown. In 2-AAF/PH rats treated with HGF, cells expressing AFP appeared on day 4. Although the number of AFP-positive cells and the intensity of AFP expression increased on day 8, expression of AFP decreased on day 12 in HGF-treated animals (original magnification ×100).

DISCUSSION

Oval cells emerge in areas of injured livers where the proliferation of mature hepatocytes is impaired, and have been observed in several human liver diseases,[9–12] suggesting that these cells play an important role in liver regeneration. Therefore, agents that stimulate oval cell proliferation and differentiation, or transplantation of oval cells could be a potent therapeutic modality in the treatment of patients with fatal liver disease, such as fulminant hepatic failure. Recently, several studies have shown that *in vivo* transduction of HGF accelerated oval cell proliferation,[23] and HGF stimulated the growth of cultured oval cells through the phosphatidylinositol-3 kinase/Akt signaling pathway.[24] In the present study, we gave recombinant human HGF to 2-AAF/PH rats using osmotic pumps, which were intraperitoneally implanted, and demonstrated that the proliferation of oval cells was stimulated by this treatment. Recombinant human HGF was effectively released from the pumps, leading to a persistent increase in serum human HGF levels and accelerated proliferation of oval cells. Despite its short half-life (<3 min), recombinant HGF was predominantly distributed into the liver.[30] Also, in comparison with the surrounding mature hepatocytes, enhanced expression of c-Met was observed in oval cells (data not shown). Thus, an increase in serum levels of recombinant human HGF, which was released from intraperitoneally implanted osmotic pumps, stimulated c-Met tyrosine phosphorylation in liver tissue (Fig. 1), and, consequently, successfully affected oval cells.

Oval cells are known to have the ability to differentiate into either hepatocytes or biliary epithelial cells. In the present study, although sequential changes in the expression of AFP mRNA on days 4 and 8 were not in parallel with the number of AFP-positive cells (Figs 3,4a), HGF administration induced AFP expression and the appearance of AFP-positive cells at an early stage, followed by a decrease in both cell number and expression. In contrast, expression of albumin mRNA

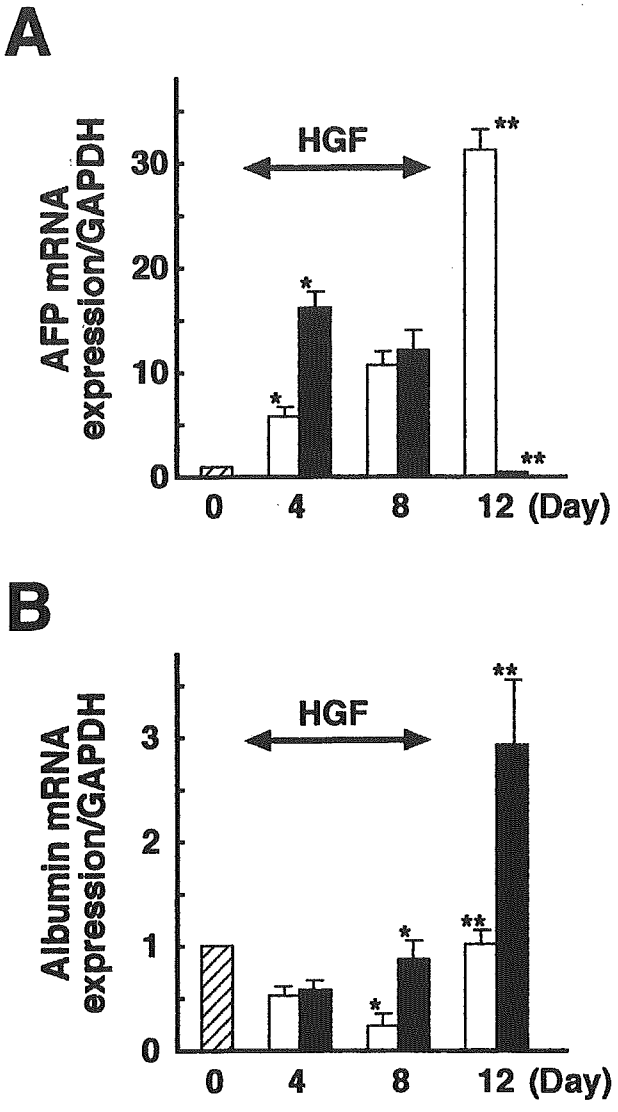

Figure 4 Hepatocyte growth factor (HGF) administration stimulated the expression of α-fetoprotein (AFP) at an early stage, whereas albumin expression gradually increased, achieving maximum expression at day 12. Total RNA was extracted from the 2-acetylaminofluorene/partial hepatectomy (2-AAF/PH) rat liver tissues treated with phosphate-buffered saline (PBS) alone (□; $n = 4$) or HGF (■; $n = 4$) on days 4, 8 and 12. Expression of AFP and albumin transcripts was evaluated by quantitative reverse transcription–polymerase chain reaction (RT-PCR) as described in Methods. The levels of AFP and albumin mRNA expression on day 0 were set to 1 (hatched column). The results were confirmed by performing assays in triplicate for each time-point studied. (a) The HGF administration stimulated AFP expression on day 4, followed by a decrease to an undetectable level on day 12, whereas expression of AFP gradually increased, reaching maximum expression on day 12 in PBS-treated rats (*$P = 0.021$; **$P = 0.021$). (b) Expression of albumin mRNA was stimulated by HGF treatment on day 8 and high levels of albumin expression were observed on day 12 (*$P = 0.041$; **$P = 0.041$).

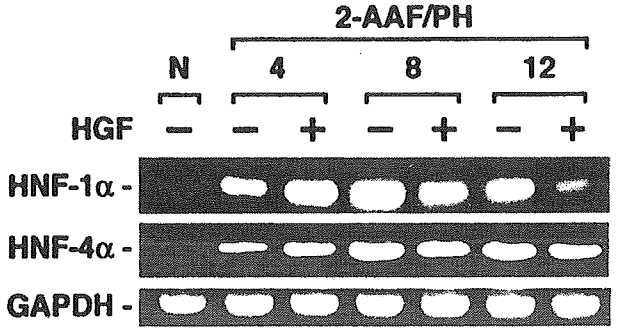

Figure 5 Hepatocyte growth factor (HGF) administration stimulated the expression of HNF-1α and -4α at an early stage. Total RNA was extracted from liver tissues of normal (N) and 2-acetylaminofluorene/partial hepatectomy (2-AAF/PH) rats treated with PBS alone or HGF on days 4, 8 and 12. Expression of hepatocyte nuclear factor (HNF)-1α and -4α was examined by semiquantitative reverse transcription–polymerase chain reaction (RT-PCR). Expression of HNF-1α was stimulated by HGF on day 4, and declined to a low level on day 12. Treatment with HGF also stimulated HNF-4α expression at an early stage (day 4).

was increased by HGF treatment at a later time. Additionally, expression of HNF-1α and HNF-4α, which are associated with the activation and differentiation of oval cells, respectively,[31] was also stimulated at an early stage (day 4; Fig. 5). These results suggest that, in addition to the stimulation of oval cell proliferation, recombinant human HGF induced the activation of oval cells at an early stage and accelerated the differentiation predominantly to hepatocytes. The effects of HGF on the hepatic differentiation of oval cells are supported by recent investigations. They have demonstrated that HGF plays a crucial role in liver development,[25,26] and HGF is also required for hepatic differentiation of hematopoietic and embryonic stem cells *in vitro*.[27,28] Additionally, Suzuki *et al.* demonstrated that HGF induced an early transition of albumin-negative hepatic stem cells, which were isolated from fetal mice, to albumin-positive hepatic precursors resembling hepatoblasts.[32] Further differentiation of these cells to mature hepatocytes required oncostatin M and extracellular matrix (ECM). Therefore, although treatment with recombinant HGF alone accelerated the differentiation of hepatic oval cells emerging in the 2-AAF/PH rats, other endogenous factors as well as ECM probably acted in concert with the administered HGF simultaneously or at different stages of differentiation. Conversely, in the present study, neither CK19 nor c-kit expression were affected by HGF treatment (data not shown). Because oval cell clusters consist of heterogenous cell populations, HGF may act on some subpopulations of the oval cells. However, it is still not clear whether HGF induced differentiation of oval cells to biliary epithelial cells *in vivo*, because the population of biliary epithelial cells in liver tissue is much smaller than that of hepatocytes. Our findings do not exclude the possibility that HGF affects oval cell differentiation to biliary epithelial cells, which is also an important step for liver regeneration.

Expression of albumin mRNA continued to increase even after HGF administration ceased, leading to an extremely high level on day 12 (Fig. 4). When the partially hepatectomized rats, in which liver regeneration is achieved by proliferation of mature hepatocytes, were treated with HGF, hepatocyte proliferation and albumin mRNA expression were stimulated.[19] In the present study, proliferation of mature hepatocytes was not observed in 2-AAF/PH rats treated with HGF (Fig. 2c), and the weight of livers on day 12 was barely affected by this treatment (data not shown), probably because of continuous 2-AAF administration. In contrast, oval cells emerged at an early stage, and the proliferation of oval cells was stimulated in the rat livers treated with HGF. Therefore, HGF accelerated both the proliferation and hepatic differentiation of oval cells, and the increased number of oval cells could continue differentiating to hepatocytes even after HGF treatment was stopped. These results also suggest that, although the rats were treated with HGF alone, other endogenous factors, including growth factors, cytokines, and ECM, worked cooperatively with HGF, and were all involved in the differentiation of oval cells to hepatocytes, acting either simultaneously or following the HGF stimulus.

In conclusion, administration of recombinant human HGF stimulated the proliferation of hepatic oval cells as well as mature hepatocytes and possibly accelerated the differentiation of oval cells to hepatocytes in an *in vivo* experimental model. These results indicate that HGF is able to promote the regeneration of severely damaged livers where the proliferation of mature hepatocytes is impaired. Because HGF is also known to facilitate the repair of liver injury[33–36] and fibrosis,[37–39] recombinant human HGF could be a potent therapeutic agent to treat patients with severe liver disease.

ACKNOWLEDGMENTS

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Molecular mechanisms of hereditary persistence of α-fetoprotein (AFP) in two Japanese families A hepatocyte nuclear factor-1 site mutation leads to induction of the AFP gene expression in adult livers

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Abstract

α-Fetoprotein (AFP) is produced abundantly in fetal liver but is hardly detectable in adults. In this study, we investigated two unrelated Japanese families with hereditary persistence of AFP. A g → a substitution at nucleotide −119 (−119g → a) in the hepatocyte nuclear factor (HNF)-1 binding site of the AFP promoter was identified in both families. The activity of the wild- or variant-type human AFP promoter was evaluated by in vitro and in vivo transfection experiments. This substitution in the AFP promoter significantly stimulated its transcriptional activity in human hepatoma cells, regardless of their prior AFP production. The variant-type AFP promoter was also active in adult mouse livers in vivo. Additionally, overexpression of HNF-1α stimulated the activity of both the wild- and variant-type AFP promoters in hepatoma cells. HNF-1α expression also activated both AFP promoters even in nonhepatoma cells, and this activation was suppressed by nuclear factor (NF)-I overexpression. These results indicate that an HNF-1 binding site mutation leads to induction of the AFP gene expression in adult liver, and suggest that competition between HNF-1 and NF-I in this region is involved in transcriptional regulation of the AFP gene during hepatic development.

Keywords: α-Fetoprotein; Hereditary persistence of AFP; Hepatocyte nuclear factor-1; Nuclear factor-I; Transcription; Hydrodynamic-based gene transfer

1. Introduction

α-Fetoprotein (AFP) is a major fetal serum glycoprotein produced by the embryonic yolk sac and fetal liver, where it is expressed at high levels [1]. After birth, transcription of the AFP gene decreases rapidly [2,3], decreasing serum AFP to its lowest point between 6 and 8 months of age [4]; in adults, serum levels of AFP are barely detectable (less than 10 ng/ml) [5]. An increase in adult serum AFP has been observed under some conditions, however, including liver regeneration, hepatocellular carcinoma (HCC), testicular cancer, and other malignancies [6–9].

Much progress has been made towards the characterization of *cis*- and *trans*-acting elements that regulate human AFP gene expression [10–14]. It has been shown that hepatocyte-specific enhancers exist in a region far upstream (−3.8 and −3.5 kb) of the AFP gene, and that the position-

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dependent silencer is located between the enhancer region and the hepatocyte promoter region. The silencer functions block the enhancer-induced activation of the AFP promoter, resulting in AFP gene repression in adult liver [14]. These transcriptional regulatory elements have been identified using AFP- and non-AFP-producing human hepatoma cell lines as an in vitro model of fetal and adult livers, respectively. Conversely, the AFP promoter is considered to be a 200-bp region immediately upstream of the AFP gene, containing two binding sites for hepatocyte nuclear factor-1 (HNF-1), both of which are indispensable for and play a key role in tissue-specific transcription [13,15–18]. Recently, naturally occurring mutations in the human AFP promoter have been reported, which increase transcriptional activity [19–21]. Members of a family showing persistent elevation of AFP in adult life, designated "hereditary persistence of AFP" (HPAFP), contain a g → a substitution at nucleotide −119 (−119g → a) or a c → a substitution at −55 (−55c → a); these mutations exist in the distal or proximal HNF-1 binding site of the AFP promoter, respectively. Both the −119g → a and −55c → a substitutions lead to an increase in sequence similarity to the consensus HNF-1 recognition site and to an increase in promoter affinity for HNF-1. Additionally, the distal HNF-1 binding site of the AFP promoter has been reported to partially overlap a recognition site for nuclear factor (NF)-I or CCAAT/enhancer binding protein (C/EBP), which appear to function negatively or positively, respectively, for the rat and mouse AFP promoters [16,18,22]. Thus, a −119g → a substitution may lead to a decrease in binding of NF-I or C/EBP as well as to an increase in affinity for HNF-1, leading to an overall increase in AFP promoter activity.

In the present study, we have identified a single nucleotide substitution in the AFP promoter, −119g → a, in two unrelated Japanese families with high serum AFP concentrations. We have examined the effect of a −119g → a substitution on human AFP promoter activity using in vitro and in vivo transfection experiments. Additionally, we have evaluated the effect of HNF-1 and/or NF-I expression on the activities of wild- and variant-type AFP promoters.

2. Materials and methods

2.1. Study subjects

2.1.1. Family 1

The proband was a 43-year-old male with a high serum AFP level (516 ng/ml), which was discovered in a routine check-up (Fig. 1). Despite elevated serum AFP that persisted for 2 years, clinical examinations, ultrasonography and computed tomography scans of the liver, and selective celiac angiography (which were each repeated periodically) were normal. Alanine aminotransferase and aspartate aminotransferase activities were normal, and hepatitis B surface antigen (HBsAg), antibody to hepatitis B surface antigen (anti-HBs), and anti-hepatitis C virus antibody (anti-HCV) tests were negative. Clinical and ultrasonographic examinations of the testes were normal. Because serum AFP levels remained elevated, a family study was performed with written informed consent. A brother and daughter exhibited high serum AFP levels (465 and 292 ng/ml, respectively) (Fig. 1). The parents, another brother, and the son did not agree to be tested for serum AFP levels.

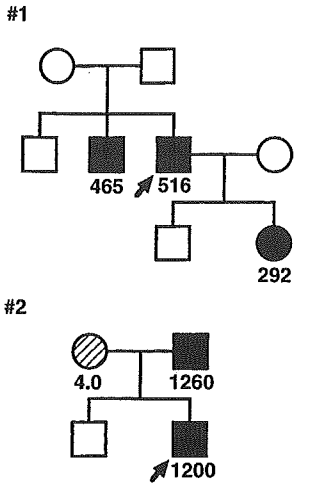

Fig. 1. Pedigrees of the HPAFP families. Numbers below each individual indicate serum AFP level (ng/ml). Arrows indicates the proband. #1 and #2, pedigrees of families 1 and 2, respectively.

2.1.2. Family 2

The proband was a 44-year-old male with a marked increase in serum AFP (1200 ng/ml) found during a routine check-up (Fig. 1). Physical examinations, including liver and testes, were normal. Ultrasonography and computed tomography scans of the liver and ultrasonography of the testes were normal. HBsAg, anti-HBs and anti-HCV were negative. Alanine aminotransferase and aspartate aminotransferase activities were normal. Because an increase in serum AFP levels was repeatedly confirmed and considered to occur in the absence of any other abnormality, a family study was performed with written informed consent. His father also exhibited elevated serum AFP (1260 ng/ml), but serum AFP of his mother was normal (4.0 ng/ml) (Fig. 1). His brother did not agree to be tested for serum AFP.

2.2. Amplification and sequencing of genomic DNA

Genomic DNA was isolated from peripheral blood mononuclear cells as previously described [23]. The 5′-flanking region of the AFP gene was amplified by 30 cycles of PCR (30 s at 94 °C, 30 s at 53 °C and 30 s at 72 °C) using the primers 5′-GCCCCAAAGAGCTCTGTGTC-3′ and 5′-CTATGCCAGTGTTCTGGATTC-3′ [19]. The resulting 294-bp fragment was characterized by direct sequencing using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems Japan, Tokyo, Japan).

2.3. Cell culture

The human hepatoma (HepG2 and huH1) and nonhepatoma (HeLa) cell lines were cultured in DMEM (Sigma, St. Louis, MO, USA) (HepG2 and HeLa) or RPMI 1640 (Sigma) (huH1) supplemented with 10% fetal calf serum under 5% CO_2 at 37 °C. Quantitative analysis of AFP secretion was performed in 24-well multiplates. The human hepatoma and nonhepatoma cells were plated at a density of 3×10^5 cells/well and then incubated for 24 h. After the incubation period, medium AFP was assayed by a commercially available chemiluminescent immunoassay (ABBOTT JAPAN, Tokyo, Japan).

2.4. Reverse transcription-PCR (RT-PCR)

Total cellular RNA was extracted from human hepatoma and nonhepatoma cells using ISOGENE (Nippon Gene, Tokyo, Japan). After reverse transcription using a random primer at 42 °C for 30 min, PCR (30 s at 94 °C, 32 s at 55 °C and 30 s at 72 °C) was performed using the primers, 5′-TAAGGTCCACGGTGTGCGCTAT-3′ and 5′-TGTTGATGACCGGCACACTCTG-3′ or 5′-GTGAGCGAGATGCAGAGCAAA-3′ and 5′-AGATGCCTCCTTCCATGTCCTC-3′, to amplify human HNF-1 (29 cycles) or NF-I (27 cycles) cDNA, respectively. Human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA was amplified by 23 cycles of PCR at 30 s at 94 °C, 30 s at 55 °C and 30 s at 72 °C using the primers 5′-CGGAGTCAACGGATTTGGTCGTAT-3′ and 5′-AGCCTTCTCCATGGTGGTGAAGAC-3′.

2.5. Plasmid construction

pAF0.3-luc contains the 0.3-kb human AFP gene promoter between −230 and +29 bp relative to the AFP 5′-flanking sequence, as previously described [24]. The variant-type 0.3-kb human AFP promoter, which contains a g → a substitution at nucleotide −119, was released from pLNAFM0.3TK [25] by digestion with BamHI–HindIII and inserted into the BglII–HindIII site of pGL3-basic vector (Promega, Madison, WI), resulting in pAF0.3M-luc (Fig. 3A).

The plasmid expressing human HNF-1α, GeneStorm Expression-Ready Clone, RG000612, was purchased from Invitrogen (Carlsbad, CA, USA). To make pcDNA-NF-I, which expresses human NF-I, human NF-I cDNA was released from the entry vector, Ultimate ORFCard series, IOH12484 (Invitrogen), and inserted into pcDNA3.2/V5-DEST (Invitrogen) by LR reaction.

2.6. Cell transfection and dual-luciferase reporter assays

Transfections were performed using 800 ng of the luciferase fusion plasmids and 80 ng pRL-TK (Promega) per well in 24-well multiplates (1×10^5 cells/well) using LipofectAMINE 2000 reagent (Invitrogen). After a 24-h incubation, the cells were harvested and a dual-luciferase reporter assay (Promega) was performed according to the manufacturer's instructions. In cotransfection experiments using the plasmid expressing HNF-1α or NF-I, the luciferase plasmids were reduced to 400 ng, and 400 ng of HNF-1α and/or NF-I expression vectors or mock were introduced simultaneously. HNF-1α siRNA and its control siRNA (5′-CCAGCUAGUGACCCACAUGCCAUUU-3′ and 5′-CCAGAUAGUCCCACACGUACGCUUU-3′, respectively) were obtained from Invitrogen. Twenty picomoles of siRNA was transfected using LipofectAMINE 2000 reagent 24 h before the transduction with luciferase plasmids. Results were confirmed by three independent experiments.

2.7. Animals

Male BALB/C mice, 6 weeks of age, were obtained from Japan SLC, Inc. (Shizuoka, Japan). The animals were maintained under constant room temperature (25 °C) and given free access to water and a standard diet throughout the study. The protocol for animal studies was approved by the ethical committee of the University of Miyazaki, Faculty of Medicine (Miyazaki, Japan). All animal experiments were performed after a 1-week acclimation period.

2.8. In vivo transfection experiments

To introduce exogenous genes in mouse livers, hydrodynamics-based transfections were performed as previously described [26]. In brief, 2.0 ml of Ringer's solution containing plasmid DNAs (50 μg of pSV-β-galactosidase (Promega) or mock vector, 40 μg of pAF0.3- or pAF0.3M-luc, and 4 μg of pRL-TK) was injected into mice via the tail vein. DNA injection was completed in less than 5 s, and the hepatic expression of β-galactosidase or luciferase was assessed 8 h after plasmid DNA injection. For histochemical analyses of β-galactosidase gene expression, 10 μm cryosections were made from frozen mouse liver tissues. The sections were stained in X-gal solution (1 mg/ml) for 3 h at 37 °C. To assay luciferase expression, 100 mg of frozen liver tissues was homogenized in 1 ml of Passive Lysis Buffer (Promega) and centrifuged at 4 °C at 12,000 × *g* for 10 min. The supernatants were subjected to the dual-luciferase reporter assay (Promega), as described above.

3. Results

3.1. A g → a substitution at position −119 of the AFP promoter is identified in two Japanese HPAFP families

We examined nucleotide sequences of the 5′-flanking region of the AFP gene in two Japanese families (Fig. 2A).

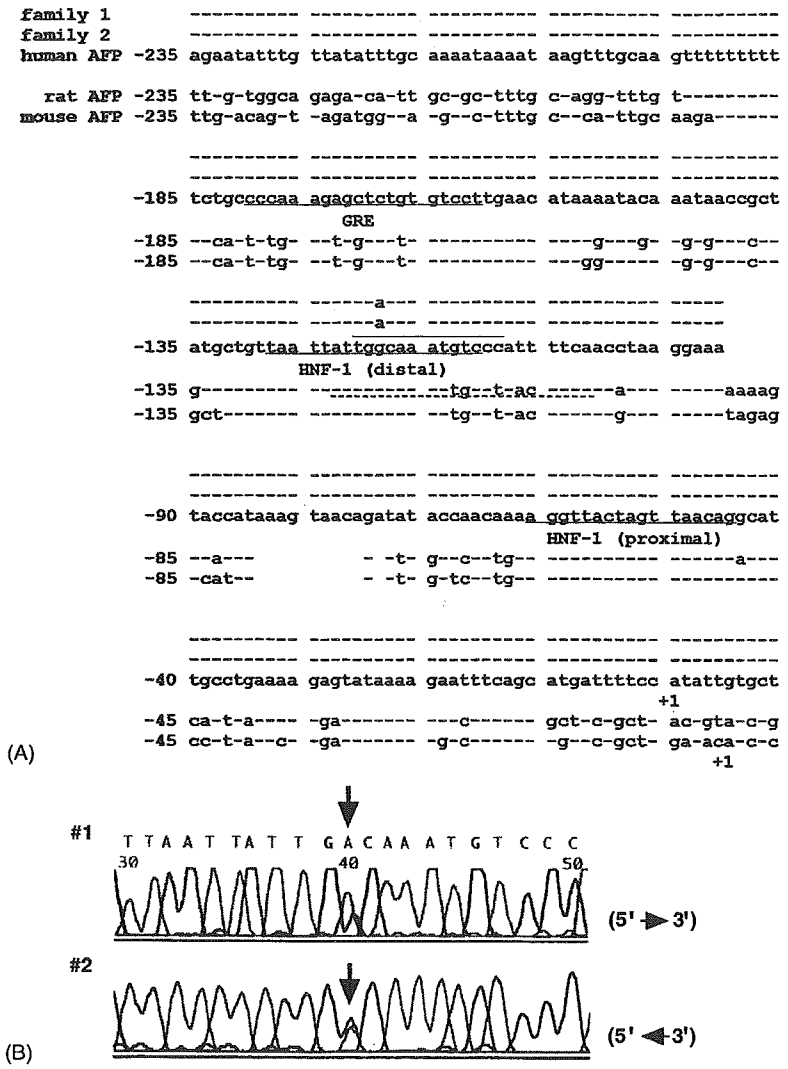

Fig. 2. (A) Sequence alignment of the AFP promoter from HPAFP families 1 and 2 with the wild-type sequence [10]. The cap site is indicated +1. A glucocorticoid responsive element (GRE) and two HNF-1 binding sites (distal and proximal) are underlined [13,15–18]. An NF-I binding site is upperlined. The dotted-underlined region of the rat AFP promoter has been reported to be occupied by recombinant C/EBPα and C/EBPβ [18]. The human AFP promoter also shows sequence similarity to the rat and mouse AFP promoters [16,27]. (B) Electropherograms of the probands of HPAFP families 1 and 2 (#1 and #2, respectively). The arrow indicates a g → a heterozygous substitution at nucleotide −119.

The human AFP promoter is highly homologous to the rat and mouse AFP promoters [10,16,27] and contains a glucocorticoid responsive element (GRE) and distal and proximal binding sites for HNF-1. In the probands of each family, the g at nucleotide −119 in the distal HNF-1 binding site was altered to an a (Fig. 2A and B). The same substitution was also observed in the brother and daughter in family 1 and in the father, but not mother, in family 2. We did not identify either a c → t mutation at nucleotide −65 or a c → a at −55 [21], and there was no nucleotide substitution in the two silencer regions at −0.31 and −1.75 kb [14] (data not shown).

3.2. A −119g → a substitution in the human AFP promoter stimulates transcriptional activity in hepatoma cells, but not nonhepatoma cells

To investigate the effect of a −119g → a substitution on the transcriptional activity of the human AFP promoter, we transfected the reporter plasmid, pAF0.3- or pAF0.3M-luc, into human hepatoma (HepG2 and huH1) and nonhepatoma (HeLa) cells (Fig. 3). HepG2 cells produced large amount of AFP, whereas huH1 cells secreted a smaller amount of AFP and HeLa did not produce AFP at all (Table 1). In the same manner as this ability to induce AFP production, the wild-type AFP promoter (AF0.3) was active in HepG2 and weakly

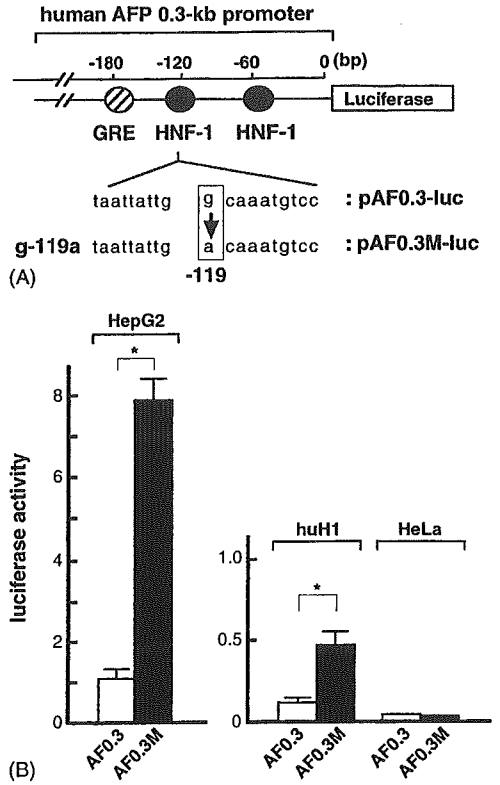

Fig. 3. (A) The structure of the pAF0.3- and pAF0.3M-luc reporter plasmids. The 0.3-kb human AFP gene promoter contains a GRE and two binding sites for HNF-1. The AFP promoter with or without a −119g → a substitution was inserted upstream of the luciferase gene, resulting in pAF0.3M- or pAF0.3-luc. (B) Transcriptional activity of the AF0.3 and AF0.3M promoters in human hepatoma and nonhepatoma cells. A −119g → a substitution in the AFP promoter significantly increased luciferase expression, in hepatoma cells specifically ($n=4$). $^{*}p<0.0001$.

in huH1, but not in HeLa (Fig. 3B). A −119g → a substitution in the AFP promoter (AF0.3M) significantly stimulated luciferase expression in HepG2 and huH1 cells ($p<0.0001$ and $p<0.0001$, respectively), while AFP promoter activity was not induced by this substitution in HeLa cells.

3.3. A variant-type human AFP promoter provides luciferase expression in adult mouse liver

We investigated whether a −119g → a substitution induced AFP expression in adult livers in vivo, in which expression of AFP gene was normally repressed. To examine the transduction efficiency of hydrodynamics-based DNA transfections [26], pSV-β-galactosidase or mock vector was injected intravenously into adult mice (Fig. 4A). Under these conditions, approximately 10% of hepatocytes were positive for β-galactosidase. Next, we introduced pAF0.3- or pAF0.3-luc into adult mouse livers using the hydrodynamics-based DNA transfections, and examined luciferase activity 8 h after transfection (Fig. 4B). When pAF0.3-luc was transfected, luciferase expression was barely detectable in liver tissues; the AF0.3M promoter, on the other hand, induced significantly high levels of luciferase expression ($p<0.0001$).

Table 1
AFP secretion in human hepatoma and nonhepatoma cell lines

Cell	AFP secretion[a] (ng/24 h/3 × 10^5 cells)
HepG2	2431.3 ± 89.9
huH1	18.0 ± 0.0
HeLa	<0.4

[a] Results are represented as the mean ± S.D. ($n=4$).

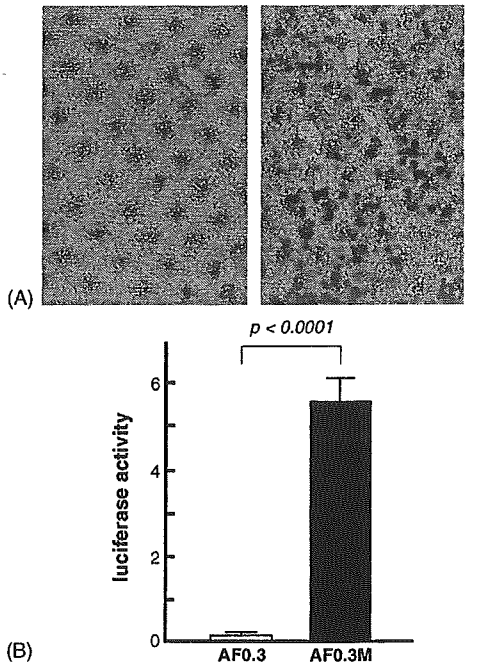

Fig. 4. (A) The efficiency of in vivo hydrodynamic gene transfection. pSV-βGal (b) or mock vector (a) was introduced into adult mouse liver, as described in Section 2. X-gal staining was observed in approximately 10% of hepatocytes (magnification: ×400). (B) Luciferase gene expression driven by the AF0.3 or AF0.3M promoters in adult mouse liver. Although luciferase expression from pAF0.3-luc was hardly detectable, the AF0.3M promoter induced luciferase expression at a high level ($n=4$).

3.4. HNF-1α expression stimulates transcriptional activity of the human AFP promoter

The distal binding site for HNF-1, encompassing the −119 nucleotide, partially overlaps an NF-I binding site (Fig. 5A) [18,19]. A −119g → a substitution increases the similarity to the consensus sequence for HNF-1 binding, rather than that for NF-I. We therefore examined expression of HNF-1 and NF-I in human hepatoma and nonhepatoma cells by semi-quantitative RT-PCR (Fig. 5B). Expression of HNF-1 was observed equally in HepG2 and huH1 cells, but not in HeLa cells. NF-I transcripts were expressed in both hepatoma and nonhepatoma cells; equally in HepG2 and huH1, and more abundantly in HeLa cells. To investigate a role for HNF-1 in AFP promoter activity, the plasmid expressing HNF-1α or siRNA inhibiting HNF-1α expression was cotransfected

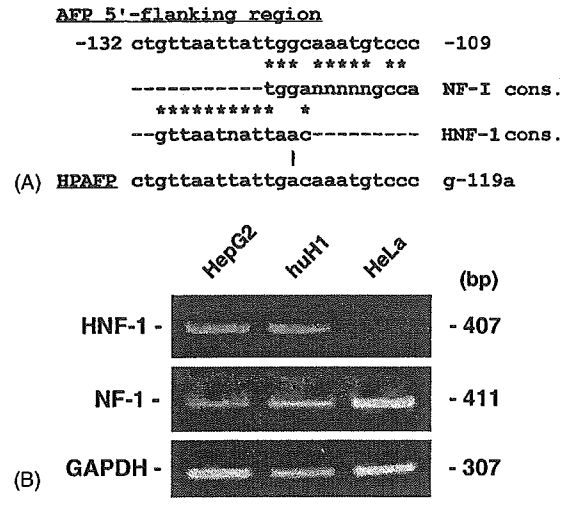

Fig. 5. (A) The binding site for HNF-1 overlaps that of NF-I. The −119g → a substitution increases the sequence similarity of this region to the consensus HNF-1 recognition site. (B) Expression of HNF-1 and NF-I was examined by RT-PCR in human hepatoma and nonhepatoma cells. HNF-1 expression was detected in HepG2 and huH1, but not HeLa, cells. NF-I was expressed in both hepatoma and nonhepatoma cells.

with pAF0.3- or pAF0.3M-luc (Fig. 6). Overexpression of HNF-1α significantly stimulated luciferase expression from pAF0.3- and pAF0.3M-luc in AFP-producing hepatoma cells (HepG2) ($p < 0.0001$ and $p < 0.0001$, respectively) (Fig. 6A). Despite the lower activities of the AF0.3 and AF0.3M promoters in non-AFP-producing hepatoma cells (huH1), HNF-1α overexpression also significantly upregulated both AF0.3- and AF0.3M-induced luciferase expression ($p = 0.0005$ and $p = 0.0004$, respectively). This stimulatory effect was more pronounced in huH1 cells than in HepG2 cells. Interestingly, in nonhepatoma (HeLa) cells, although the activities of the AF0.3 and AF0.3M promoters were hardly detectable, exogenous HNF-1α expression induced promoter activities of both AF0.3 and AF0.3M ($p = 0.0002$ and $p = 0.0001$, respectively) to the same levels as those in huH1 cells. Next, we introduced siRNA inhibiting HNF-1α expression into hepatoma cells (Fig. 6B). Inhibition of HNF-1α expression significantly reduced luciferase expression driven from the AF0.3 or AF0.3M promoters.

3.5. NF-I expression downregulates AFP promoter activity, possibly by influencing HNF-1 binding

We introduced plasmids expressing NF-I in combination with pAF0.3- or pAF0.3M-luc into hepatoma cells (Fig. 7A). Overexpression of NF-I significantly suppressed luciferase expression from both pAF0.3- and pAF0.3M-luc. To investigate the relationship between HNF-1 and NF-I and the transcriptional activity of the human AFP promoter in more detail, the HNF-1α or both HNF-1α and NF-I expression plasmids were cotransfected with pAF0.3- or pAF0.3M-luc into nonhepatoma (HeLa) cells (Fig. 7B). Although the activity from either the AF0.3 or AF0.3M promoter was hardly detectable in HeLa cells, transduction of the HNF-1α expression plasmid alone induced luciferase expression regardless of the substitution at nucleotide −119. When the plasmid expressing NF-I, in addition to that expressing HNF-1α, was introduced, luciferase expression driven from either the AF0.3 or AF0.3M promoter was significantly reduced.

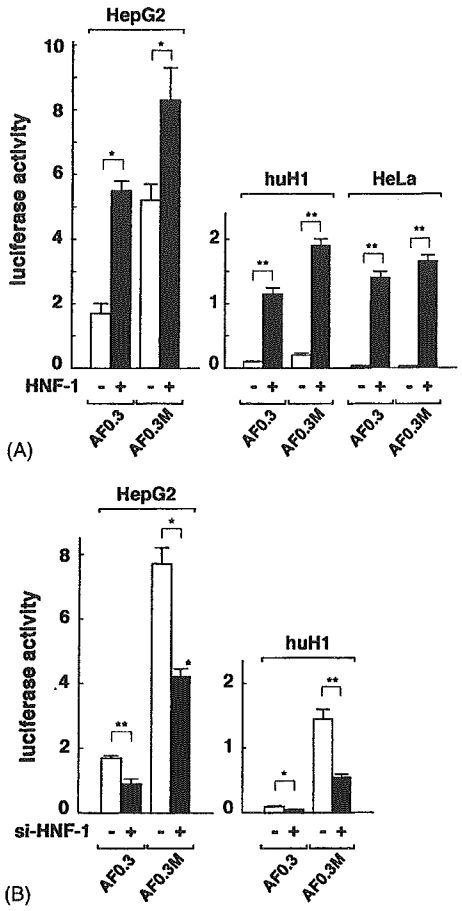

Fig. 6. HNF-1α contributes to an increase in human AFP promoter activity. (A) Overexpression of HNF-1α significantly stimulated both AF0.3 and AF0.3M promoter activities in hepatoma cells (HepG2 and huH1) ($n = 4$). Even in nonhepatoma cells (HeLa), transduction of the HNF-1α expression plasmid induced luciferase expression driven by either the AF0.3 or AF0.3M promoters ($n = 4$). $^{*}p < 0.0001$; $^{**}p < 0.001$. (B) Repression of HNF-1α expression reduced luciferase expression from both pAF0.3- and pAF0.3M-luc in human hepatoma cells ($n = 4$). $^{*}p = 0.0001$; $^{**}p < 0.005$.

4. Discussion

To date, three HPAFP families (Scottish, Spanish and Bengali) with a −119g → a substitution in the distal HNF-1 binding site of the AFP promoter have been reported [19–21]. HNF-1 binds DNA as a dimer and the HNF-1 binding site has a palindromic structure [28,29]. The first half of the distal

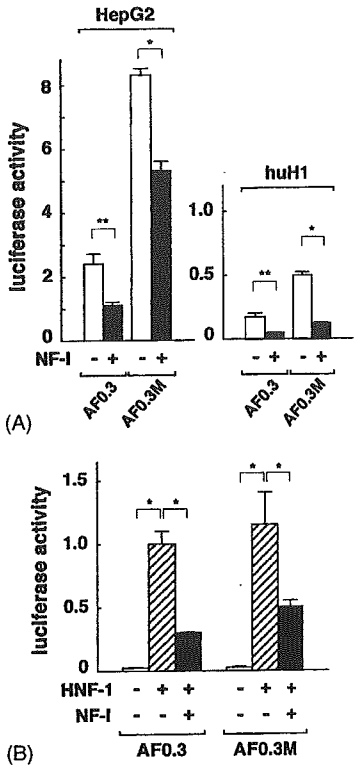

Fig. 7. NF-I negatively influences the human AFP promoter. (A) Overexpression of NF-I suppressed luciferase expression from either the AF0.3 or AF0.3M promoter in human hepatoma cells ($n=4$). $^{*}p<0.0005$; $^{**}p<0.002$. (B) In nonhepatoma cells, only introduction of HNF-1α activated both wild-type and variant-type AFP promoters ($n=4$). When the plasmid expressing NF-I was transfected simultaneously with HNF-1α expression vector, luciferase expression driven from AF0.3 or AF0.3M was significantly reduced ($n=4$). $^{*}p<0.0001$.

HNF-1 binding site of the AFP promoter completely aligns with the consensus HNF-1 recognition site, but the second half varies at nucleotides −118 and −119 (Fig. 3A). Therefore, the −119g → a substitution increases the sequence similarity to the HNF-1 consensus sequence. Consequently, this mutation increases the affinity of HNF-1 for the AFP promoter, leading to an increase in the AFP transcriptional activity in hepatoma cells (HepG2) [19]. We report here two unrelated Japanese families with HPAFP, both of which have a heterozygous −119g → a substitution in the AFP promoter. We showed that in hepatoma cells, this mutation stimulated AFP promoter activity regardless of prior AFP production. Additionally, in in vivo transfection experiments, the variant-type AFP promoter induced an approximately 40-fold increase in transcriptional activity over the wild-type promoter in adult mouse liver. Recently, another substitution in the AFP promoter responsible for HPAFP, −55c → a, was found in an Italian family [21]. The −55c → a substitution is located in the proximal HNF-1 binding site of the AFP promoter and also increases the sequence similarity to the consensus HNF-1 recognition sequence and likewise the affinity for HNF-1. Thus, these human studies of HPAFP allowed us to recognize an important role for HNF-1 in the induction of AFP expression in adult livers. Nakabayashi et al. have reported that repression of the AFP gene is caused by the silencer regions, which separate the enhancer regions from the promoter [14]. In the present study, the silencer sequences were not affected by the mutation (data not shown). Therefore, the HNF-1 site mutations in human AFP promoter may leads to derepression of the AFP gene expression in adult livers.

Although the far upstream enhancer and the position-dependent silencer regions have been known to be involved in the transcriptional regulation of the human AFP gene [10–12,14,30–33], transcriptional regulatory elements existing in ~200 bp of the human AFP promoter have not been studied in detail [10,13,14,19,21]. However, the rat and mouse AFP promoters have been more extensively investigated [16–18,22,34], and shows the high sequence similarity to the human AFP promoter (Fig. 2A). As in the rat and mouse AFP promoters, the distal HNF-1 binding site (encompassing nucleotide −119) of the human AFP promoter partially overlaps an NF-I recognition site (Figs. 2A and 5A). Therefore, we evaluated the effect of HNF-1α and/or NF-I on human AFP promoter activity in human hepatoma and nonhepatoma cells. Overexpression of HNF-1α stimulated the activities of wild- and variant-type AFP promoter in hepatoma cells, and this stimulatory effect was more pronounced in non-AFP-producing hepatoma cells (huH1) (Fig. 6A). In contrast, inhibition of HNF-1α expression or overexpression of NF-I suppressed the promoter activity (Figs. 6B and 7A). Exogenous HNF-1α expression alone activated the AFP promoter in nonhepatoma cells, which did not previously express either AFP or HNF-1, and this activation was significantly but not completely inhibited by NF-I overexpression (Figs. 6A and 7B). These results support the importance of HNF-1 for the transcriptional regulation of human AFP, as previously reported for the rat and mouse AFP genes [15,18]; they also indicate the possibility that NF-I competes with HNF-1 for binding of the distal HNF-1 recognition site, resulting in repression of AFP expression.

The −119g → a substitution increases the sequence similarity of the distal binding site for HNF-1 to the consensus HNF-1 recognition site. However, enhanced or reduced expression of HNF-1, or overexpression of NF-1, equally affected the activities of both wild- and variant-type human AFP promoters in both hepatoma and nonhepatoma cells. Several investigations have shown that the distal HNF-1 binding site of the rat and mouse AFP promoters is protected by NF-I protein in DNase I footprinting experiments using nuclear extracts from fetal or adult rat livers [17,35,36]. This footprint was not affected by excess amounts of the high-affinity HNF-1 binding site of the rat albumin promoter [18]. Also, the distal HNF-1 binding site of the rat AFP promoter partially overlaps the region occupied by the recombinant proteins C/EBPα and C/EBPβ, which induce the rat AFP promoter (Fig. 2A) [18]. The distal HNF-1 binding site exhibits much higher affinity to HNF-1 than does the proximal