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Expression of Hepatocyte Growth Factor Activator and Hepatocyte Growth Factor Activator Inhibitor Type 1 in Human Hepatocellular Carcinoma

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Hepatocyte growth factor activator inhibitor type 1 (HAI-1), a Kunitz-type serine protease inhibitor for hepatocyte growth factor activator (HGFA), is responsible for proteolytic activation of hepatocyte growth factor. We examined the expression of HGFA and HAI-1 in liver tissues of chronic liver diseases including hepatocellular carcinoma (HCC). HGFA expression was detected not only in the liver tissues of chronic hepatitis and cirrhosis and in the nontumorous liver tissues surrounding HCC, but also in HCC tissues. On the other hand, none of the liver tissues of hepatitis and cirrhosis and none of the nontumorous tissues surrounding HCC were stained with anti-HAI-1. However, 35% of HCC tissues were stained with anti-HAI-1, and HAI-1 positivity increased as the histological grade decreased and as serum α-fetoprotein increased. Transduction of antisense HAI-1 inhibited the growth of human hepatoma cells. These results suggest the possibility that HAI-1 plays an important role in the progression of HCC.

Key Words: **HGF; HGFA; serine protease; HAI-1; serine protease inhibitor; hepatocellular carcinoma; chronic hepatitis; liver cirrhosis.**

Hepatocyte growth factor (HGF) is secreted from mesenchymal cells of the liver as an inactive single-chain form of HGF (pro-HGF) and normally remains in this form associated with the extracellular matrix (1). Once tissue injury occurs, pro-HGF is converted to active heterodimeric HGF (mature HGF) by two homologous serine proteases, HGF activator (HGFA) and blood coagulation factor XII (1–3), and other serine proteases such as plasminogen activators (4, 5). HGFA is produced by hepatocytes as an inactive form and is also activated mainly by thrombin and partly by blood coagulation factor Xa in response to tissue injury (6, 7). The levels of HGFA mRNA in the liver increase during liver injury induced by carbon tetrachloride (8). Recently, two HGFA inhibitor (HAI)-1 and -2, which were Kunitz-type serine protease inhibitors having two Kunitz domains, were isolated (9, 10). HAI-1 mRNA was strongly expressed in the kidney, pancreas, prostate, small intestine, lung and placenta. In human liver tissues, biliary epithelial cells in portal triads were immunohistochemically stained with anti-HAI-1 antibody, but hepatocytes were not stained (11).

HGF is known to play an important role in the migration and invasion of tumor cells (12–14). Recently, several investigators have reported that overexpression of HGF as well as transforming growth factor-α, another hepatotropic growth factor, promoted carcinogenesis in transgenic mouse models (15–17), and that the HGF levels in the liver and serum increased during diethylnitrosamine-induced hepatocarcinogenesis (18). However, growth inhibitory effects of HGF on various cancer cells *in vitro* have been also demonstrated (19–21), and Satori-Rugiu *et al.* have shown that HGF overexpression in c-myc-transgenic mice reduces hepatocarcinogenesis (22). Moreover, the infusion of recombinant HGF inhibited cell proliferation of rat HCC

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Abbreviations used: HGF, hepatocyte growth factor; HGFA, hepatocyte growth factor activator; HAI, hepatocyte growth factor activator inhibitor; HAI-1, hepatocyte growth factor activator inhibitor type 1; HCC, hepatocellular carcinoma; AFP, α-fetoprotein.

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induced by diethylnitrosamine (23). Since HAI is a potent inhibitors of HGFA and serine proteases/serine protease inhibitor systems are considered to be important for tumor progression (9, 24, 25), HGFA and HAIs may regulate the mature HGF levels in tumorous and surrounding nontumorous tissues, resulting in the contradictory effects of HGF on carcinogenesis or tumor progression.

In humans, the development of HCC is strongly associated with chronic liver disease, particularly cirrhosis, which commonly occurs as a result of hepatitis B virus or hepatitis C virus infection. The level of serum HGF in HCC patients increases approximately twofold compared to the level in normal subjects but is similar to the level in patients with liver cirrhosis (26). On the other hand, overexpression of c-Met, a specific receptor for HGF, in HCC tissues was immunohistochemically detected in approximately 40% of HCC patients (27–29) and was related to the poor prognosis of HCC patients after hepatic resection (30). These investigations suggest that HGF may play an important role in hepatocarcinogenesis and the progression of HCC. In the present study, to clarify the involvement of HGFA and HAI-1 in liver tissues during the development and progression of HCC, we investigated the expression of HGFA and HAI-1 in liver tissues from patients with hepatitis and cirrhosis, and in both HCC and the nontumorous liver tissues surrounding HCC. Moreover, we examined the effect of antisense HAI-1 transduction on the growth of human hepatoma cells.

PATIENTS AND METHODS

Patients. Liver tissues were obtained from 32 patients with chronic liver disease, including 9 patients with chronic hepatitis, 6 patients with liver cirrhosis and 17 patients with HCC. The diagnosis was based on the findings of blood biochemistry, imaging studies and histological examination. Hepatitis B surface antigen was positive in 1 patient with chronic hepatitis, 2 patients with liver cirrhosis and 7 patients with HCC; anti-hepatitis C virus antibody was positive in 7 patients with chronic hepatitis, 2 with liver cirrhosis and 7 with HCC. Seven of the HCC patients had liver cirrhosis. According to the histological findings, the 17 HCC patients were divided as follows: 4 patients were well differentiated, 7 were moderately differentiated and 6 were poorly differentiated. The tumorous and the surrounding nontumorous liver tissues from patients with HCC were obtained by surgical resection, and liver tissues from patients with chronic hepatitis and liver cirrhosis were obtained by needle biopsy.

The liver tissues used were fixed in formalin for histological and immunohistochemical examinations. Four sets of tumorous and the surrounding nontumorous liver tissues were snap frozen in liquid nitrogen and stored at −80°C for Northern blot analysis and *in situ* hybridization.

Immunohistochemistry. Immunohistochemical staining for HGFA and HAI-1 was performed on formalin-fixed paraffin sections using the labeled polymer method, as described previously (11). Briefly, after antigen retrieval (5 min of autoclaving in 10 mM citrate buffer, pH 6.0), the sections were treated with 3% H_2O_2 in PBS for 10 min and washed in PBS twice, followed by blocking in 3% BSA in PBS for 1 h at room temperature. Then, the sections were incubated with monoclonal antibodies against HAI-1(10 μg/ml) (9, 11, 31) and HGFA (10 μg/ml) (32) for 16 h at 4°C. The sections were then washed in PBS and incubated with Envision-labeled polymer reagent (Dako, Carpinteria, CA) for 45 min at 37°C. The reaction was detected by ImmunoPure Metal Enhanced DAB Substrate Kit (Pierce, Rockford, IL) and counterstained with Mayer's hematoxylin.

Northern blot analysis. Total cellular RNA was isolated by QuickPrep Total RNA extraction kit (Amersham–Pharmacia Biotech Inc., Piscataway, NJ). HAI-1 transcripts were detected using a human HAI-1 cDNA probe (11).

In situ hybridization. The procedures used for *in situ* hybridization were described previously (11). For the probe, a 622-bp HAI-1 cDNA corresponding to bases 144–765 of the human HAI-1 cDNA sequence was generated by PCR. The PCR product was subcloned into pCR2.1 (Invitrogen; San Diego, CA), and *in vitro* transcription to generate digoxigenin-labeled probes was carried out according to the manufacture's instruction (Boehringer Mannheim GmbH, Mannheim, Germany). After the hybridization, the sections were treated with 30 μg/ml of ribonuclease A at 37°C for 30 min, followed by blocking non-specific binding sites with 3% BSA in Tris-buffered saline. Signals were detected by alkaline phosphate-conjugated anti-digoxigenin sheep IgG Fab fragment and BM Purple (Boehringer Mannheim GmbH).

Cell culture. Human hepatoma cells (HepG2, Hep3B, PLC/PRF/5, HLF, huH1/cl.2, Huh6/cl.5, and HuH7) and PT67 retrovirus packaging cells were cultured in RPMI 1640 (Sigma, St. Louis, MO)/5% fetal calf serum (FCS) and Dulbecco's modified Eagle's medium (Sigma)/5% FCS, respectively.

Production of recombinant retrovirus and retroviral infection of human hepatoma cells. The doxycycline-regulated retroviral vector, Rev-Tet system (Clontech, Palo Alto, CA), was used in this study (33). To construct pRevTRE HAI-1(AS), the human HAI-1 coding region was amplified by PCR using the human HAI-1 cDNA as a template and the following primers: 5′-AGGCCCGCGCTCTGAAGGTGA-3′ and 5′-AGCTCGGGAGGACAGGGTTGG-3′. After *Hin*dIII–*Eco*RV digestion, the PCR product was inserted into the *Hin*dIII–*Hpa*I site of pRev TRE (Clontech) in an antisense orientation. pRevTRE HAI-1(AS) or pRevTet-On (Clontech) were transfected into PT67 packaging cells, followed by incubation with hygromycin (200 μg/ml) (Gibco-BRL, Rockville, MI) or G418 (400 μg/ml) (Wako, Osaka, Japan), respectively. The amphotropic recombinant retroviruses, RevTRE HAI-1(AS) and RevTet-On, were harvested from established virus producer cells.

Hep3B human hepatoma cells were infected with the RevTet-On retrovirus and the G418-resistant colonies were cloned. The cells were then infected with the RevTRE HAI-1(AS) retrovirus, followed by incubation with hygromycin (200 μg/ml). The pooled populations, Hep3B/TH-1(AS), resisting both G418 and hygromycin, were subjected to further studies.

Western blot analysis. Hep3B/TH-1(AS) or parental cells were cultured in the presence or absence of doxycycline (100 ng/ml) for 48 h, respectively. After solubilization, total proteins were subjected to SDS–PAGE and blotted to nitrocellulose filters. To detect HAI-1 protein, filters were incubated with anti-HAI-1 antibody (11) and then subjected to enhanced chemiluminescence Western analysis (Amersham–Pharmacia Biotech Inc.).

In vitro cell growth assay. Hep3B/TH-1(AS) and parental cells (4×10^4) were seeded into 6-well plates (day 0). On day 2, Hep3B/TH-1(AS) cells were cultured with doxycycline (100 ng/ml). The number of cells was counted on day 6.

Statistical analysis. Statistical parameters were ascertained with the Statview J-4.5 software (Abacus Concepts, Inc., Berkeley, CA). Statistical analysis was performed by the χ^2 test, Kruskal–Wallis test and Mann–Whitney U test. $P < 0.05$ was considered to be statistically significant.

TABLE 1

Immunohistochemical Detection of HGFA and HAI-1 in Chronic Hepatitis, Liver Cirrhosis, and Hepatocellular Carcinoma

Subjects	No. of samples	HGFA (%)	HAI-1 (%)
Chronic hepatitis	9	9 (100)	0 (0)
Liver cirrhosis	6	6 (100)	0 (0)
Hepatocellular carcinoma			
Nontumorous tissue	17	17 (100)	0 (0)
Tumorous tissue	17	17 (100)	6 (35)

Note. The paraffin-embedded liver tissues of patients with chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma were stained with anti-HGFA or anti-HAI-1. Immunohistochemical staining of the liver tissues with the exception of biliary epithelial cells was analyzed.

RESULTS

HGFA and HAI-1 Expression in the Liver Tissues

We examined the expression of HGFA and HAI-1 proteins in HCC and the surrounding nontumorous tissues from 17 HCC patients and in liver tissues from 6 patients with liver cirrhosis and 9 patients with chronic hepatitis by immunohistochemistry (Table 1). The liver tissues from all patients with chronic hepatitis and liver cirrhosis were stained with anti-HGFA antibody (Figs. 1A and 1C). Both HCC and the nontumorous tissues surrounding HCC from all HCC patients were also stained with anti-HGFA antibody (Figs. 1E and 1G). The cytoplasm of hepatocytes or HCC cells was homogeneously stained with anti-HGFA antibody.

Although the biliary epithelial cells were stained with anti-HAI-1, as previously reported in normal human livers (11), none of the liver tissues from patients with chronic hepatitis or cirrhosis were positive for HAI-1 (Figs. 1B and 1D). However, 6 of 17 HCC tissues (35%) were stained with anti-HAI-1 antibody, while the nontumorous liver tissues surrounding HCC from all HCC patients were negative for HAI-1 (Table 1 and Figs. 1F and 1H).

To confirm the expression of HAI-1 mRNA, we performed Northern blot analysis using the liver tissues from 4 HCC patients (Table 2, patients 4, 11, 14, and 15). The HCC tissues from patients 14 and 15 were stained with anti-HAI-1 antibody, while those from patients 4 and 11 were not. The expression of HAI-1 mRNA was detected in the HCC tissues from patients 14 and 15 by Northern blot analysis (Fig. 2A). However, HAI-1 mRNA was not detected in the nontumorous liver tissues from patients 14 and 15, nor was it detected in either the HCC or the nontumorous tissues from patients 4 and 11. Moreover, we examined HAI-1 expression in the liver tissues from patient 15 by *in situ* hybridization. We found positive signals for HAI-1 mRNA in tumor cells of the HCC tissue (Fig. 2B, *a*) but not in the nontumorous tissue surrounding HCC (Fig. 2B, *c*). When using the sense probe, no signals were detected in the HCC (Fig. 2B, *b*) or the nontumorous tissues from this patient.

Relationship between HAI-1 Expression and Patient Profiles in HCC

To analyze the clinical significance of HAI-1 expression, we examined the relationship between HAI-1 positivity and each patient's profile. The profiles of 17 HCC patients and the findings of immunostaining for HAI-1 are shown in Table 2. Neither the age, underlying liver disease, status of hepatitis B and C virus markers, nor the tumor size was correlated with the immunohistochemical positivity for HAI-1. However, HAI-1 positivity was increased as the histological grade of the HCC decreased; 5 of 6 (83%) patients with poorly differentiated HCC were positive for HAI-1, while none of the 4 patients with well differentiated HCC were positive ($P < 0.01$). Also, HAI-1 positivity increased with an increase in the serum α-fetoprotein (AFP) levels; 4 of 6 (67%) HCC patients with greater than 400 ng/ml of serum AFP were positive for HAI-1, while none of 7 HCC patients with less than 10 ng/ml was positive ($P < 0.05$).

TABLE 2

The Profiles of Patients with Hepatocellular Carcinoma and Immunohistochemical Detection of HAI-1 in the Liver Tissues of These Patients

Case	Underlying liver disease (HBV or HCV)	Tumor size (cm)	AFP (ng/ml)	Histological grade	HAI-1 T	HAI-1 N
1	CH (HBV)	3.5	2	Well	−	−
2	CH (NBNC)	3.5	2	Well	−	−
3	CH (HCV)	2.0	6	Well	−	−
4	LC (NBNC)	2.2	336	Well	−	−
5	LC (HCV)	7.0	6	Moderate	−	−
6	CH (HBV)	14.0	6	Moderate	−	−
7	LC (HCV)	2.0	8	Moderate	−	−
8	CH (HBV)	12.0	12	Moderate	−	−
9	LC (HCV)	3.0	17	Moderate	+	−
10	LC (HCV)	2.4	18	Moderate	−	−
11	LC (HCV)	3.0	8,233	Moderate	−	−
12	CH (HBV)	2.0	3	Poor	−	−
13	CH (HBV)	2.5	573	Poor	+	−
14	CH (HBV)	2.6	879	Poor	+	−
15	CH (NBNC)	15.0	3,154	Poor	+	−
16	CH (HCV)	9.0	27,920	Poor	+	−
17	LC (HBV)	10	386,900	Poor	+	−

Note. Hepatocellular carcinoma was classified as well differentiated (well), moderately differentiated (moderate), or poorly differentiated (poor) by histological findings. CH, chronic hepatitis; LC, liver cirrhosis; NBNC, neither HBV or HCV; T, tumorous tissue; N, nontumorous tissue.

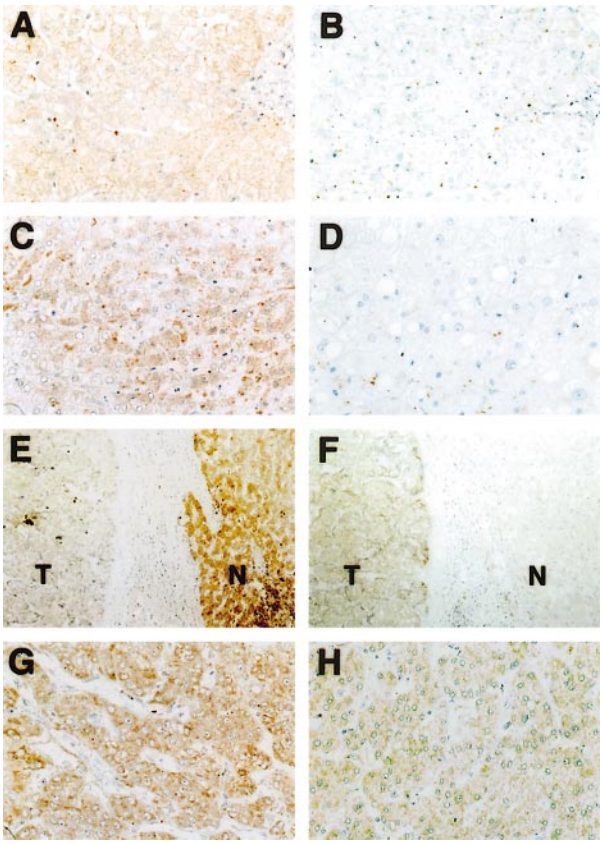

FIG. 1. Immunohistochemical staining for HGFA and HAI-1 in the liver tissues from patients with chronic hepatitis, liver cirrhosis and HCC. The liver tissues were obtained from the patients with chronic hepatitis (A and B), liver cirrhosis (C and D) and HCC (patient 16 in Table 2) (E–H) and were subjected to immunohistochemical staining for HGFA (A, C, E, and G) or for HAI-1 (B, D, F, and H). The areas of HCC tissues of E and F were magnified in G and H, respectively. HGFA expression was strongly detected in the liver tissue from chronic hepatitis and liver cirrhosis (A and C), whereas HAI-1 expression was not (B and D). Both HCC and the surrounding nontumorous tissues were also stained with anti-HGFA (E and G). However, HAI-1 expression was detected only in the HCC tissue but not in the nontumorous tissue (F and H). N, nontumorous liver tissue surrounding HCC; T, HCC tissue. [Original magnification (A–D, G, and H) 400 ×; (E and F) 150 ×.]

HAI-1 Expression in Human Hepatoma Cell Lines and the Effect of Inhibition of HAI-1 Expression on Cell Growth

We performed Northern blot analysis to examine HAI-1 expression in several human hepatoma cells. HAI-1 mRNA was strongly expressed in Hep3B and Huh6/cl.5 cells (Fig. 3, lanes 2 and 6), moderately in HuH7 (lane 7), and slightly in HepG2 and huH1/cl.2 (lanes 1 and 5), whereas expression of HAI-1 was not detected in PLC/PRF/5 and HLF (lanes 3 and 4).

To analyze the effect of inhibition of HAI-1 expression on growth of hepatoma cells, we constructed a recombinant retrovirus expressing antisense HAI-1 under the control of a tetracycline-regulated system and established antisense HAI-1-transduced Hep3B cells [Hep3B/TH-1(AS)]. When Hep3B/TH-1(AS) cells were treated with doxycycline to induce antisense HAI-1 expression, the amount of HAI-1 protein in Hep3B/TH-1(AS) was decreased to 60% of that observed in parental cells (Fig. 4A, $P < 0.05$), and the growth of Hep3B/TH-1(AS) was inhibited (Fig. 4B, $P < 0.05$). The HAI-1 expression and growth of Hep3B/TH-1(AS) without doxycycline were similar to HAI-1 expression and growth in parental cells (data not shown).

DISCUSSION

HGF is a pleiotropic factor that functions as a mitogen, motogen, and/or morphogen for a variety of cells, particularly epithelial cells bearing c-Met receptor tyrosine kinase. Because HGF is secreted in an inactive

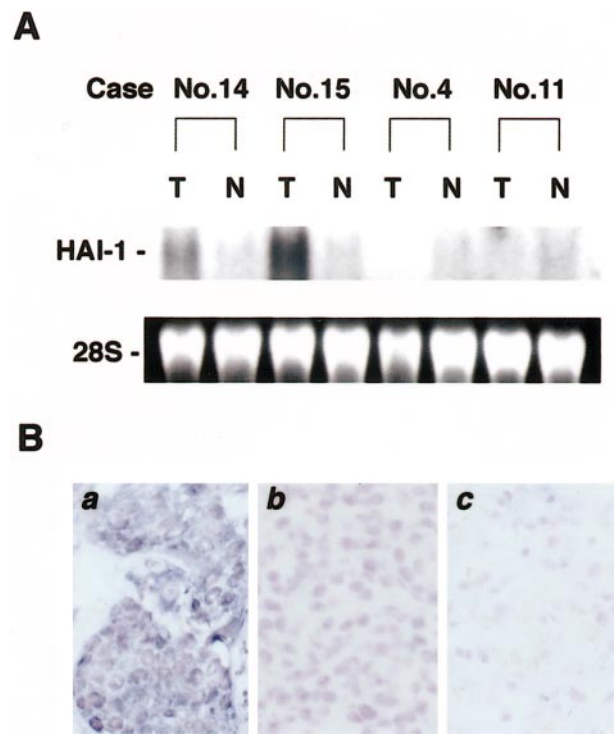

FIG. 2. Expression of HAI-1 mRNA in tumorous and nontumorous liver tissues surrounding HCC. (A) Northern blot analysis of HAI-1 mRNA in HCC and nontumorous liver tissues. Total RNAs (10 μg/ml) extracted from tumorous and nontumorous liver tissues from 4 HCC patients (patients 4, 11, 14, and 15 in Table 2) were analyzed for HAI-1 mRNA as described under Patients and Methods. The bottom panel shows the ethidium bromide staining for 28S ribosomal bands. Lanes 1 and 2, patient 14; lanes 3 and 4, patient 15; lanes 5 and 6, patient 4; and lanes 7 and 8, patient 11. N, nontumorous tissue surrounding HCC; T, HCC tissue. (B) *In situ* hybridization for HAI-1 mRNA in HCC and nontumorous liver tissues. The HCC (*a* and *b*) and nontumorous liver tissues (*c*) obtained from the HCC patient 15 in Table 2 was subjected to *in situ* hybridization for HAI-1 using an antisense mRNA probe (*a* and *c*) or using a sense probe (*b*). *In situ* hybridization was performed as described under Patients and Methods. (Original magnification 200 ×.)

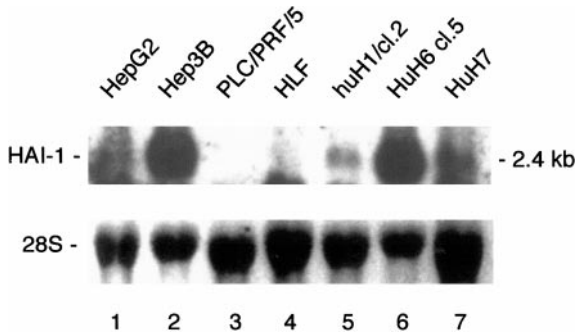

FIG. 3. Expression of HAI-1 mRNA in human hepatoma cell lines. Northern blot analysis of HAI-1 transcripts in human hepatoma cells. Total RNA (10 μg) was analyzed for HAI-1 mRNA, as described under Patients and Methods.

precursor form, proteolytic activation in the extracellular milieu is a critical limiting step in the HGF-induced signaling pathway. Therefore, HGFA and its potent inhibitor, HAI-1, must act in concert to regulate the HGF activity in the pericellular microenvironment existing not only in injured tissues but also in malignant tumors (1–3, 6, 7). Recently, we demonstrated that HAI-1 expression was detected in normal colorectal mucosa and was decreased in colorectal cancer (31) and that HGFA is expressed in colorectal mucosa and tumors and could be involved in the activation of HGF in colorectal cancers (34). In the present study, we showed that the hepatocytes in chronic hepatitis and cirrhosis were strongly stained with anti-HGFA antibody, in accordance with a previous report in which HGFA is expressed in the parenchymal cells of rat liver (8). Also both HCC and the surrounding nontumorous parenchymal tissues were positive for HGFA. In contrast, HAI-1 expression was detected only in HCC cells, but not in non-neoplastic hepatocytes, using immunohistochemical staining, Northern blot analysis and *in situ* hybridization. Furthermore, HAI-1 was immunohistochemically expressed in HCC tissues of 35% of HCC patients, where the HAI-1 positivity was increased in poorly differentiated HCC (83%) compared to well differentiated HCC and was also higher in HCC patients with high serum AFP levels than in those with low serum AFP. These results indicate that the HAI-1 expression in HCC tissues may be related to tumor progression rather than hepatocarcinogenesis. The concomitant upregulation of protease inhibitor and its target protease has been reported in several malignant tumors and also in the liver tissues during hepatic fibrosis. Tissue inhibitors of metalloproteases and matrix metalloproteases were increased in cases of gastric carcinoma (35) and HCC (36) as well as in fibrotic liver disease (37, 38). Plasminogen activator inhibitors and urokinase-type plasminogen activator were also upregulated simultaneously in malignant tumors (25), and high levels of plasminogen activator inhibitor type 1 have been reported to indicate a poor patient prognosis (39, 40). Recently, several investigators have demonstrated that plasminogen activator inhibitor type 1 was vital for the invasion and neovascularization of cancer though the underlying mechanism is undefined (41, 42). Moreover, protease nexin-II, a serine protease inhibitor having a Kunitz domain that is homologous to those in HAI-1 (9), is abundantly expressed in most of the cancer cell lines (43, 44).

Currently, it remains unclear whether the increased expression of HAI-1 causally contribute to, or rather are the consequence of, the malignancy in HCC. Nonetheless, two possibilities can be offered for a possible role of HAI-1 in the progression of HCC. First, HAI-1 might contribute to the growth of hepatoma cells via a function possibly independent of its HGFA-inhibitory activity. In the present study, the transduction of antisense HAI-1 also induced growth inhibition of human hepatoma cells, which strongly expressed HAI-1, in the absence of HGF. We have also observed that the overexpression of HAI-1 stimulated the growth of human fibrosarcoma cells, and that protease nexin-II could be involved in the growth of colon cancer cells *in vitro* and *in vivo* (unpublished observation), suggesting that possible link between Kunitz-type inhibitors and cellular growth. Second possibility is that HAI-1 might para-

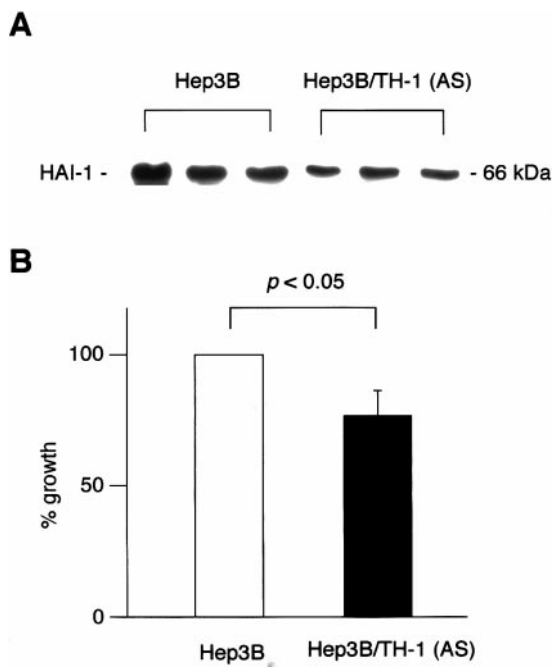

FIG. 4. The effect of antisense HAI-1 transduction in human hepatoma cells. (A) The expression of HAI-1 proteins in parental and antisense HAI-1-transduced Hep3B cells, Hep3B/TH-1(AS). Total proteins (15 μg) were extracted from Hep3B/TH-1(AS) and parental cells incubated with doxycycline for 3 days, and analyzed by Western blot analysis, as described under Patients and Methods. (B) The effect of antisense HAI-1 on the growth of Hep3B cells. Hep3B/TH-1(AS) and parental Hep3B cells were incubated with doxycycline (100 ng/ml) for 4 days, and then viable cells were counted. The number of parental cells was set as 100%.

doxically contribute to the pericellular activation of HGF that could have an important role in progression of HCC. Our recent investigations have shown that membrane-form HAI-1 acts not only as a cellular surface inhibitor of HGFA but also as a reservoir for this enzyme. This reservoir function is terminated by regulated shedding of HGFA/HAI-1 complex induced by protein kinase C activation, accompanying significant recovery of HGFA activity in the pericellular space (45). These properties of HAI-1 may resulted in the efficient localization and concentration of HGFA on the surface of hepatoma cells, which in turn may ensure the pericellular activation of HGF.

Recent studies have shown that the expression of HAI-1 was detected in the epithelial cells of pseudo-bile ducts and in the scattered hepatocytes with fulminant hepatitis, although normal hepatocytes did not express HAI-1 (9, 11). HAI-1 was also upregulated in the regenerative colon epithelium of acetic acid-induced mouse colitis models (46). These findings suggest that the expression of HAI-1 may be involved in tissue injury or regeneration. However, in the current study, reactive expression of HAI-1 was not observed in hepatocytes of chronic hepatitis and cirrhosis. Further investigations should be performed to clarify the involvement of HAI-1 in tissue injury or regeneration.

In conclusion, we demonstrated that the expression of HAI-1 was detected specifically in HCC tissues but not in non-neoplastic hepatocytes, whereas HGFA was expressed in both HCC and nontumorous liver tissues. Furthermore, we showed that the inhibition of HAI-1 expression in human hepatoma cells induced a decrease in cell growth. These findings suggest the possibility that HAI-1 plays an important role in the progression of HCC. This is the first report describing the expression of HGFA and HAI-1 in the liver tissues of various chronic liver diseases, including HCC, and further experimentation will be necessary to clarify the involvement of HAI-1 in various aspects of tumor biology.

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REFERENCES

1. Miyazawa, K., Shimomura, T., Naka, D., and Kitamura, N. (1994) Proteolytic activation of hepatocyte growth factor in response to tissue injury. *J. Biol. Chem.* **269,** 8966–8970.
2. Shimomura, T., Miyazawa, K., Komiyama, Y., Hiraoka, H., Naka, D., Morimoto, Y., and Kitamura, N. (1995) Activation of hepatocyte growth factor by two homologous proteases, blood-coagulation factor XIIa and hepatocyte growth factor activator. *Eur. J. Biochem.* **229,** 257–261.
3. Miyazawa, K., Shimomura, T., and Kitamura, N. (1996) Activation of hepatocyte growth factor in the injured tissues is mediated by hepatocyte growth factor activator. *J. Biol. Chem.* **271,** 3615–3618.
4. Naldini, L., Tamagnone, L., Vigna, E., Sachs, M., Hartmann, G., Birchmeier, W., Daikuhara, Y., Tsubouchi, H., Blasi, F., and Comoglio, P. M. (1992) Extracellular proteolytic cleavage by urokinase is required for activation of hepatocyte growth factor/scatter factor. *EMBO J.* **11,** 4825–4833.
5. Mars, W. M., Zarnengar, R., and Michalopoulos, G. K. (1993) Activation of hepatocyte growth factor by the plasminogen activators uPA and tPA. *Am. J. Pathol.* **143,** 949–958.
6. Shimomura, T., Kondo, J., Ochiai, M., Naka, D., Miyazawa, K., Morimoto, Y., and Kitamura, N. (1993) Activation of the zymogen of hepatocyte growth factor activator by thrombin. *J. Biol. Chem.* **268,** 22927–22932.
7. Miyazawa, K., Shimomura, T., Kitamura, A., Kondo, J., Morimoto, Y., and Kitamura, N. (1993) Molecular cloning and sequence analysis of the cDNA for a human serine protease responsible for activation of hepatocyte growth factor, structural similarity of the protease precursor to blood coagulation factor XII. *J. Biol. Chem.* **268,** 10024–10028.
8. Okajima, A., Miyazawa, K., Naitoh, Y., Inoue, K., and Kitamura, N. (1997) Induction of hepatocyte growth factor activator messenger RNA in the liver following tissue injury and acute inflammation. *Hepatology* **25,** 97–102.
9. Shimomura, T., Denda, K., Kitamura, A., Kawaguchi, T., Kito, M., Kondo, J., Kagaya, S., Qin, L., Takata, H., Miyazawa, K., and Kitamura, N. (1997) Hepatocyte growth factor activator inhibitor, a novel Kunitz- type serine protease inhibitor. *J. Biol. Chem.* **272,** 6370–6376.
10. Kawaguchi, T., Qin, L, Shimomura, T., Kondo, K., Matsumoto, K., Denda, K., and Kitamura, N. (1997) Purification and cloning of hepatocyte growth factor activator inhibitor type 2, a Kunitz-type serine protease inhibitor. *J. Biol. Chem.* **272,** 27558–27564.
11. Kataoka, H., Suganuma, T., Shimomura, T., Itoh, H., Kitamura, N., Nabeshima, K., and Koono, M. (1999) Distribution of hepatocyte growth factor activator inhibitor type 1(HAI-1) in human tissues: Cellular surface localization of HAI-1 in simple columnar epithelium and its modulated expression in injured and regenerative tissues. *J. Histochem. Cytochem.* **47,** 673–682.
12. Jeffers, M., Rong, S., and Vande Woude, G. F. (1996) Enhanced tumorigenicity and invasion-metastasis by hepatocyte growth factor/scatter factor-met signaling in human cells concomitant with induction of the urokinase proteolysis network. *Mol. Cell. Biol.* **16,** 1115–1125.
13. Kamiyama, T., Une, Y., Uchino, J., and Hamada, J. (1998) Hepatocyte growth factor enhances the invasion activity of human hepatocellular carcinoma cell lines. *Int. J. Oncol.* **12,** 655–659.
14. Monvoisin, A, Neaud, V., De Ledinghen, V., Dubuisson, L., Balabaud, C., Bioulac-Sage, P., Desmouliere, A., and Rosenbaum, J. (1999) Direct evidence that hepatocyte growth factor-induced invasion of hepatocellular carcinoma cells is mediated by urokinase. *J. Hepatol.* **30,** 511–518.
15. Sakata, H., Takayama, H., Sharp, R., Rubin, J. S., Merlino, G., and LaRochelle, W. J. (1996) Hepatocyte growth factor/scatter factor overexpression induces growth, abnormal development, and tumor formation in transgenic mouse livers. *Cell Growth Differ.* **7,** 1513–1523.
16. Bell, A., Chen, Q., DeFrances, M. C., Michalopoulos, G. K., and Zarnegar, R. (1999) The five amino acid-deleted isoform of hepatocyte growth factor promotes carcinogenesis in transgenic mice. *Oncogene* **18,** 887–895.
17. Takagi, H., Sharp, R., Hammermeister, C., Goodrow, T., Bradley, M. O., Fausto, N., and Merlino, G. (1992) Molecular and

genetic analysis of liver oncogenesis in transforming growth factor α transgenic mice. *Cancer Res.* **52,** 5171–5177.

18. Burr, A. W., Hillan, K. J., McLaughlin, K. E., Ferrier, R., Chapman, C., Mathew, J., and Burt, A. D. (1996) Hepatocyte growth factor levels in liver and serum increase during chemical hepatocarcinogenesis. *Hepatology* **24,** 1282–1287.
19. Shiota, G., Rhoads, D. B., Wang, T. C., Nakamura, T., and Schmidt, E. V. (1992) Hepatocyte growth factor inhibits growth of hepatocellular carcinoma cells. *Proc. Natl. Acad. Sci. USA* **89,** 373–377.
20. Arakaki, N., Kazi, J. A., Kazihara, T., Ohnishi, T., and Daikuhara, Y. (1998) Hepatocyte growth factor/scatter factor activates the apoptosis signaling pathway by increasing caspase-3 activity in sarcoma 180 cell. *Biochem. Biophys. Res. Commun.* **245,** 211–215.
21. Gohda, E., Okauchi, H., Iwano, M., and Yamamoto, I. (1998) Induction of apoptosis by hepatocyte growth factor/scatter factor and its augmentation by phorbol esters in Meth A cells. *Biochem. Biophys. Res. Commun.* **245,** 278–283.
22. Santoni-Rugiu, E., Preisegger, K. H., Kiss, A., Audolfsson, T., Shiota, G., Schmidt, E. V., and Thorgeirsson, S. S. (1996) Inhibition of neoplastic development in the liver by hepatocyte growth factor in a transgenic mouse model. *Proc. Natl. Acad. Sci. USA* **93,** 9577–9582.
23. Liu, M. L., Mars, W. M., and Michalopoulos, G. K. (1995) Hepatocyte growth factor inhibits cell proliferation *in vivo* of rat hepatocellular carcinomas induced by diethylnitrosamine. *Carcinogenesis* **16,** 841–843.
24. Koshikawa, N., Yasumitsu, H., Umeda, M., and Miyazaki, K. (1992) Multiple secretion of matrix serine proteinases by human gastric carcinoma cell lines. *Cancer Res.* **52,** 5046–5053.
25. Andreasen, P. A., Kjoller, L., Christensen, L., and Duffy, M. J. (1997) The urokinase-type plasminogen activator system in cancer metastasis: A review. *Int. J. Cancer* **72,** 1–22.
26. Shiota, G., Okano, J., Kawasaki, H., Kawamoto, T., and Nakamura, T. (1995) Serum hepatocyte growth factor levels in liver diseases: Clinical implications. *Hepatology* **21,** 106–112.
27. Suzuki, K., Hayashi, N., Yamada, Y., Yoshihara, H., Miyamoto, Y., Ito, Y., Ito, T., Katayama, K., Sasaki, Y., Ito, A., Kisida, Y., Kashiwagi, T., Fusamoto, H., and Kamada, T. (1994) Expression of the c-met protooncogene in human hepatocellular carcinoma. *Hepatology* **20,** 1231–1236.
28. Boix, L., Rosa, J. L., Ventura, F., Castells, A., Bruix, J., Rodes, J., and Bartrons, R. (1994) c-met mRNA overexpression in human hepatocellular carcinoma. *Hepatology* **19,** 88–91.
29. D'Errico, A., Fiorentino, M., Ponzetto, A., Daikuhara, Y., Tsubouchi, H., Brechot, C., Scoazec, J. Y., and Grigioni, W. F. (1996) Liver hepatocyte growth factor does not always correlate with hepatocellular proliferation in human liver lesions: Its specific receptor c-met does. *Hepatology* **24,** 60–64.
30. Ueki, T., Fujimoto, J., Suzuki, T., Yamamoto, H., and Okamoto, E. (1997) Expression of hepatocyte growth factor and its receptor c-met proto-oncogene in hepatocellular carcinoma. *Hepatology* **25,** 862–866.
31. Kataoka, H., Uchino, H., Denda, K., Kitamura, N., Itoh, H., Tsubouchi, H., Nabeshima, K., and Koono, M. (1998) Evaluation of hepatocyte growth factor activator inhibitor expression in normal and malignant colonic mucosa. *Cancer Lett.* **128,** 219–227.
32. Shimomura, T., Ochiai, M., Kondo, J., and Morimoto, Y. (1992) A novel protease obtained from FBS-containing culture supernatant, that processes single chain form hepatocyte growth factor to two chain form in serum-free culture. *Cytotechnology* **8,** 219–229.
33. Gossen, M., and Bujard, H. (1992) Tight control of gene expression in mammalian cells by tetracycline-responsive promotors. *Proc. Natl. Acad. Sci. USA* **89,** 5547–5551.
34. Kataoka, H., Hamasuna, R., Itoh, H., Kitamura, N., and Koono, M. (2000) Activation of hepatocyte growth factor/scatter factor in human colorectal carcinomas. *Cancer Res.* **60,** 6148–6159.
35. Mimori, K., Mori, M., Shiraishi, T., Fujie, T., Baba, K., Haraguchi, M., Abe, R., Ueo, H., and Akiyoshi, T. (1997) Clinical significance of tissue inhibitor of metalloproteinase expression in gastric carcinoma. *Br. J. Cancer* **76,** 531–536.
36. Yamamoto, H., Itoh, F., Adachi, Y., Fukushima, H., Itoh, H., Sasaki, S., Hinoda, Y., and Imai, K. (1999) Messenger RNA expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases in human hepatocellular carcinoma. *Jpn. J. Clin. Oncol.* **29,** 58–62.
37. Takahara, T., Furui, K., Funaki, J., Nakayama, Y., Itoh, H., Miyabayashi, C., Sato, H., Seiki, M., Ooshima, A., and Watanabe, A. (1995) Increased expression of matrix metalloproteinase-II in experimental liver fibrosis in rats. *Hepatology* **21,** 787–795.
38. Yata, Y., Takahara, T., Furui, K., Zhang, L. P., Jin, B., and Watanabe, A. (1999) Spatial distribution of tissue inhibitor of metalloproteinase-1 mRNA in chronic liver disease. *J. Hepatol.* **30,** 425–432.
39. Grondahl-Hansen, J., Christensen, I. J., Rosenquist, C., Brunner, N., Mouridsen, H. T., Dano, K., and Blichert-Toft, M. (1993) High levels of urokinase-type plasminogen activator and its inhibitor PAI-1 in cytosolic extracts of breast carcinomas are associated with poor prognosis. *Cancer Res.* **53,** 2513–2521.
40. Bouchet, C., Spyratos, F., Martin, P. M., Hacene, K., Gentile, A., and Oglobine, J. (1994) Prognostic value of plasminogen activator (uPA) and plasminogen activator inhibitors PAI-1 and PAI-2 in breast carcinomas. *Br. J. Cancer* **69,** 398–405.
41. Liu, G., Shuman, M. A., and Cohen, R. L. Co-expression of urokinase, urokinase receptor and PAI-1 is necessary for optimum invasiveness of cultured lung cancer cells. *Int. J. Cancer* **60,** 501–506.
42. Bajou, K., Noel, A., Gerard, R. D., Masson, V., Brunner, N., Holst-Hansen, C., Skobe, M., Fusenig, N. E., Carmeliet, P., Collen, D., and Foidart, J. M. (1998) Absence of host plasminogen activator inhibitor 1 prevents cancer invasion and vascularization. *Nat. Med.* **4,** 923–928.
43. Van Nostrand, W. E., Wagner, S. L., Suzuki, M., Choi, B. H., Farrow, J. S., Geddes, J. W., Cotman, C. W., and Cunningham, D. D. (1989) Protease nexin-II, a potent antichymotrypsin, shows identity to amyloid β-protein precursor. *Nature* **341,** 546–549.
44. Seguchi, K., Kataoka, H., Uchino, H., Nabeshima, K., and Koono, M. (1999) Secretion of protease nexin-II/amyloid beta protein precursor by human colorectal carcinoma cells and its modulation by cytokines/growth factors and protease inhibitors. *Biol. Chem.* **380,** 473–483.
45. Kataoka, H., Shimomura, T., Kawaguchi, T., Hamasuna, R., Itoh, H., Kitamura, N., Miyazawa, K., and Koono, M. (2000) Hepatocyte growth factor activator inhibitor type 1 (HAI-1) is a specific cell surface binding protein of hepatocyte growth factor activator (HGFA) and regulates HGFA activity in pericellular microenvironment. *J. Biol. Chem.* **275,** 40453–40462.
46. Itoh, H., Kataoka, H., Tomita, M., Hamasuna, R., Nawa, Y., Kitamura, N., and Koono, M. (2000) Upregulation of HGF activator inhibitor type 1 but not type 2 along with regeneration of intestinal mucosa. *Am. J. Physiol. Gastrointest. Liver Physiol.* **278,** G635–G643.