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Invasiveness of Hepatocellular Carcinoma Cell Lines: Contribution of Membrane-Type 1 Matrix Metalloproteinase[1]

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Abstract

Intrahepatic metastasis is one of the malignant features of hepatocellular carcinoma (HCC). Matrix metalloproteoinases (MMPs) and urokinase-type plasminogen activator (u-PA)/plasmin, are known to be associated with the invasive properties of various types of tumor cells. In this study, we examined which proteinases play a role in the metastatic invasion of human HCC cell lines. JHH-5 and JHH-6 cells constitutively expressed mRNAs for both membrane-type 1 matrix metalloproteinase (MT1-MMP) and u-PA and invaded through reconstituted MATRIGEL *in vitro*, whereas JHH-7 cells expressed u-PA mRNA but not MT1-MMP and did not invade. However, hepatocyte growth factor (HGF) induced MT1-MMP expression on the surface of JHH-7 cells and markedly increased invasiveness of JHH-7 in a concentration-dependent manner. Moreover, cleavage activity for pro-MMP-2 was induced in HGF-treated JHH-7 cells. MMP inhibitor, rather than serine proteinase inhibitor, potently inhibited HCC cell invasion. Intrahepatic injection of HCC cell lines into athymic nude mice caused visible intrahepatic metastases *in vivo*. Moreover, JHH-7 tumors showed expression of MT1-MMP mRNA, while *in vitro* cultured JHH-7 cells did not. These findings suggest that MT1-MMP plays an important role in the invasive properties of HCC cells, and that HGF modifies the invasive properties of noninvasive HCC cells.

Keywords: hepatocellular carcinoma, invasion, intrahepatic metastasis, MT1-MMP, HGF.

Introduction

The prognosis of patients with hepatocellular carcinoma (HCC) has been generally improved by refined early detection, surgery, and introduction of percutaneous ethanol infusion therapy and transcatheter arterial embolization [1]. However, the prognosis of patients who have recurrence of multiple hepatic tumors is relatively poor [2,3]. Likewise, multicentric origin, intrahepatic metastasis has been considered to be one of the common modalities in multiple recurrence because tumor invasion into the portal vein system has been frequently observed in HCC [4]. The invasive ability of HCC cells can be characterized by the infiltration of tumor cells into the fibrous tumor capsule. In addition, approximately 90% of HCC cases are related to chronic liver disease in which the extracellular matrix (ECM) is markedly increased [5]. Thus, degradation of the ECM is thought to be a crucial step in intrahepatic metastasis of HCC cells [6].

Matrix metalloproteinases (MMPs) play an important role in the invasion of various types of tumor cells by degrading the extracellular matrix. In addition, plasminogen activators, especially urokinase-type plasminogen activator (u-PA) contributes to the fibrinolytic degradation and the activation of some growth factors by activation of plasminogen to plasmin [7,8]. In the case of human HCC, several investigators have suggested that expressions of matrix-degrading enzymes were correlated with intrahepatic metastasis [6,9–12]. However, little information is available about which enzymes are involved in the invasiveness of HCC cells. Therefore, the relationships between the expression patterns of matrix-degrading enzymes and the invasiveness of HCC cells should be investigated for our understanding of the invasive properties of HCC cells.

We recently reported that hepatocyte growth factor (HGF) induced invasive ability in a noninvasive HCC cell line [13]. HGF shows pleiotropic activities such as mitogenic [14], motogenic [15], and angiogenic [16] activities for various types of cells, and acts through a specific membrane receptor encoded by the c-*met* proto-oncogene [17]. It also induces the migration and invasion of various types of cancer cells, including those from colon, stomach, lung, gallbladder and prostate, and HCC [18–23]. The plasma level of HGF has been reported to be elevated in the patients with hepatic diseases [24] and after hepatectomy [25]. A significant

Abbreviations: HCC, hepatocellular carcinoma; MMP, matrix metalloproteinase; HGF, hepatocyte growth factor; ECM, extracellular matrix; u-PA, urokinase-type plasminogen activator.

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correlation between the expression of c-MET protein and intrahepatic metastasis was reported, and the 5-year survival rate was significantly worse in the patients with high levels of c-MET [26].

In the present study, we investigated which matrix-degrading enzymes contribute to the invasiveness and intrahepatic metastasis of human HCC cell lines, and also examined the effect of HGF on the expression of matrix-degrading enzymes in a noninvasive HCC cell line.

Materials and Methods

Cell Lines

Human HCC cell lines JHH-5, JHH-6 and JHH-7 [27] were maintained in ASF-104 serum-free medium (Ajinomoto Co., Ltd., Tokyo, Japan) in a humidified atmosphere of 5% CO_2/95% air at 37°C.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) for Human Matrix Metalloproteinases MMPs, Urokinase-Type Plasminogen Activator (u-PA) and its Receptor (u-PAR)

Isolation of total RNA and RT-PCR were performed as described previously [13]. Briefly, total RNAs of *in vitro* HCC cell lines and the *in vivo* JHH-7 tumor were isolated and reverse-transcribed at 42°C for 45 minutes to maximize cDNA synthesis, and reverse-transcription was terminated by heating at 99°C for 5 minutes. The PCR was performed using cDNA (100 ng) templates and specific oligonucleotide primers. Annealing was carried out at 48°C for u-PA and u-PAR, at 56°C for MMP-3 and GAPDH, at 58°C for MMP-1 and MMP-7, at 60°C for MT1-MMP, MMP-2 and MMP-9, for 60 seconds each. The sequences of the primers were as follows: MT1-MMP sense and antisense, 5′-CCCTATGCCTACATCCGTGA-3′ and 5′-TCCATCCATCACTTGGTTAT-3′ [28]; MMP-1 sense and antisense, 5′-GAGCAGATGTGGACCAT-3′ and 5′-ACCGGACTTCATCTCTGTCG-3′ (GenBank, X54925); MMP-2 sense and antisense, 5′-CCACGTGACAAGCCCATGGGGCCCC-3′ and 5′-GGAGCCTAGCCAGTCGGATTTGATG-3′ [29]; MMP-3 sense and antisense, 5′-GCAGAAGTTCCTTGGATTGG-3′ and 5′-TATCATCTTGAGACAGGCGG-3′ (GenBank, J03209); MMP-7 sense and antisense, 5′-AACAATTGTCTC-TGGACGGC-3′ and 5′-ATGGAGTGGAGGAAC-AGTGC-3′ (GenBank, X07819); MMP-9 sense and antisense, 5′-AACGCTATGGTTACACTCGG-3′ and 5′-AACTGGATGACGATGTCTGC-3′ (GenBank, J05070); u-PA sense and antisense, 5′-AGAATTCACCACCATCGAGA-3′ and 5′-ATCAGCTTCACAACAGTCAT-3′ [30]; u-PAR sense and antisense, 5′-TTACCTCGAATGCATTTCCT-3′ and 5′-TTGCACAGCCTCTTACCATA-3′ [31] and GAPDH sense and antisense, 5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′ and 5′- CATGTG-GGCCATGAGGTC-CACCAC-3′ [13]. All of these primers were verified to work in these conditions. The PCR products were electrophoresed on 1.5% agarose gels, and detected by ethidium bromide staining.

In Vitro Invasion Assay for Human HCC Cell Lines

The invasive ability of HCC cell lines was determined as described previously [32]. Briefly, filters of the assay chamber were coated with 1 μg/10 μl of fibronectin on the lower surface and 10 μg/50 μL of MATRIGEL (Collaborative Biochemical Products Inc., MA) on the upper surface and dried. HCC cells (1×10^5/chamber) were added to the upper compartment of the reconstituted chamber, and incubated in the absence or presence of recombinant human HGF (Collaborative Biochemical Products), added into both compartments. To determine the contribution of MMPs or u-PA in invasiveness of HCC cells, MMP inhibitor (BB2516 [33,34], synthesized) or serine proteinase inhibitor (Pefabloc; Merck Co., Ltd.) were added to both compartment of assay chamber. HGF was then added to the JHH-7 assay chamber. After 24 hours incubation, cells that had invaded through the MATRIGEL (Collaborative Biochemical Products) and the filter to the lower surface were detected using crystal violet staining. Each assay was performed in quadruplicate.

Detection of MT1-MMP Expression Induced by Recombinant Human HGF

To detect the increase of MT1-MMP mRNA, JHH-7 cells were incubated with HGF (10 ng/ml) for 4, 8, 12, or 24 hours, and RT-PCR was performed as described above. The relative abundance of MT1-MMP was expressed as the ratio of the signal intensity relative to that of GAPDH, as determined by using a Master Scan Gel Analysis System (Scanalytics, Billeica, MA). To detect the increase of MT1-MMP at the protein level, JHH-7 cells (2×10^6/flask) were incubated with HGF (10 ng/ml) for 24 hours. The cells were harvested and incubated with rabbit anti–MT-MMP (CHEMICON International Inc., CA) antibody followed by FITC-conjugated goat anti–rabbit IgG antibody (Cedarlane Lab. Ltd., Ontario, Canada). Fluorescence intensity was measured using a FACScaliber flow cytometer (Becton Dickinson Immunocytometry Systems, NJ) set to count 20,000 cells per sample. The data were collected and analyzed using a Macintosh Power PC computer equipped with CELLQuest research software (Becton Dickinson Immunocytometry Systems, NJ).

Assay for Pro-MMP-2 Activation

JHH-7 cells (1×10^5 cells/well) were seeded into 24-well plates and incubated for 24 hours. After 24 hours incubation, the medium was replaced with fresh medium in the presence or absence of HGF (10 ng/ml), and then incubation was continued for a further 24 hours. JHH-7 cell lysate was extracted as previously described [13]. The reaction mixture (total volume: 100 μl), containing 10 μg of cell lysate, 20 mU of pro-MMP-2 (Yagai Co. Ltd., Yamagata, Japan) and reaction buffer (50 mM Tris–HCl, 5 mM $CaCl_2$, 1 mM $ZnCl_2$, 0.05% NaN_3) was incubated for 1 hour at 37°C. Gelatin zymography was performed using 20-μl aliquots of reaction mixture as described [35].

Mice

Specific-pathogen-free female BALB/cA athymic nude mice, 6 weeks old, were obtained from Japan Crea (Tokyo, Japan). The animals were housed under laminar air-flow conditions and maintained in a 12-hour lighting cycle at a temperature of 22 to 25°C. The care of the animals was conducted in accordance with the standards established by the Guidelines for the Care and Use of Laboratory Animals of Toyama Medical and Pharmaceutical University.

Production of Intrahepatic Metastasis

HCC cells (1×10^6/20 µl per mouse) and 20 µg of MATRIGEL (Collaborative Biochemical Products) were implanted into the left lobe of the liver of mice under anesthesia. To evaluate the intrahepatic growth and formation of intrahepatic metastasis, the mice were sacrificed on day 35, and then measurements of the liver weight and observation of visible metastasis were performed.

After the evaluation, part of the JHH-7 tumor section was fixed with formalin and stained with hematoxylin and eosin for histopathological analysis. Another part of the tumor was used for isolation of total RNA.

Statistical Analysis

Dunnett's *t*-test for the invasion assay (Figures 3 and 4) was performed as a two-sided test to decrease the multiplicity in comparisons of treated groups with untreated controls [36].

Results

Expression of mRNAs for Human MMPs and u-PA in Cultured HCC Cell Lines

The RT-PCR study revealed that JHH-5 and JHH-6 cells constitutively expressed mRNA for MT1-MMP (Figure 1). JHH-6 cells also expressed mRNAs for MMP-2 and MMP-3. JHH-7 cells expressed mRNA for MMP-3. No expressions of mRNAs for MMP-1, MMP-7 or MMP-9 were detected in the three cell lines. The expression of the mRNAs for u-PA and its receptor were detected among three cell lines.

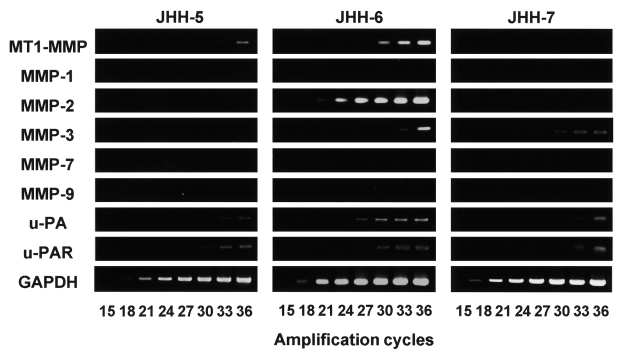

Figure 1. *Expression patterns of mRNAs for MMPs and u-PA in cultured HCC cell lines. Total RNAs of three HCC cell lines: JHH-5, JHH-6 and JHH-7 were extracted and reverse transcribed to cDNA. The PCR products for MT1-MMP, MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, u-PA, u-PAR and GAPDH (550, 646, 480, 566, 920, 361, 474, 455, and 983 bp, respectively) were amplified for the indicated numbers of cycles (ranging from 15 to 36) and electrophoresed on 1.5% agarose gels.*

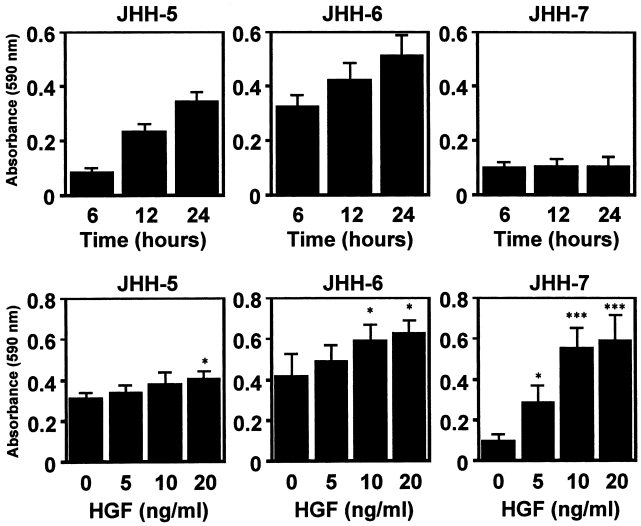

Figure 2. *Invasive ability of HCC cell lines. Each cell line (1×10^5/well) seeded into the Matrigel-embedded invasion chamber was incubated for 6, 12 or 24 hours (upper panels). The invasive ability of HCC cell lines by treatment of various concentrations of HGF was examined (lower panels; 1×10^5/well, 24 hours incubation). The invaded cells were detected by the crystal violet staining method. The results represent the mean±SD of quadruplicate cultures. *P<0.05; ***P<0.001 (two-sided Dunnett's t-test).*

Invasive Ability of Human HCC Cell Lines with or without HGF

The invasive ability of HCC cell lines was demonstrated using the MATRIGEL-invasion assay. JHH-5 and JHH-6

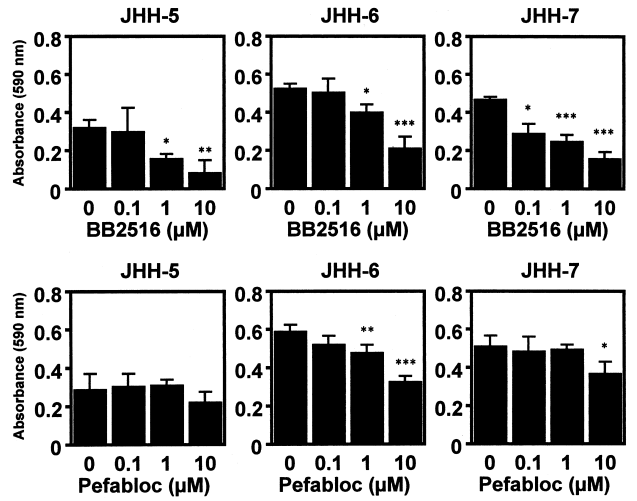

Figure 3. *Effect of an MMP inhibitor and a serine proteinase inhibitor on the invasive ability of HCC cell lines. Each cell line (1×10^5/well) was seeded into the Matrigel-embedded invasion chamber and incubated with or without various concentrations of BB2516 or Pefabloc for 24 hours. The JHH-7 cell line was treated with 10 ng/ml HGF. Invaded cells were detected by the crystal violet staining method. The results represent the mean±SD of quadruplicate cultures. *P<0.05; **P<0.01; ***P<0.001 (two-sided Dunnett's t-test).*

cells time-dependently invaded through the reconstituted extracellular matrix (ECM), whereas JHH-7 cells hardly invaded (Figure 2, *upper panels*). Since we previously found that HGF induced the invasive ability of JHH-7 cells [13], we next examined the effect of HGF on the invasion of HCC cell lines. HGF caused a concentration-dependent increase of HCC cell invasion (Figure 2, *lower panels*). HGF slightly enhanced the invasion of JHH-5 and JHH-6 cells (1.3 and 1.6 time-fold increases at 20 ng/ml HGF, respectively). On the other hand, the invasion of JHH-7 cells was markedly enhanced by HGF (six time-fold increase at 20 ng/ml HGF). This finding indicates that HGF might induce the expression of some matrix-degrading enzymes to promote the invasiveness of HCC cell lines.

Relationship between Matrix-Degrading Enzymes and Invasive Properties of HCC Cell Lines

The inhibition assay for tumor invasion was performed using an MMP inhibitor (BB2516) and a serine proteinase inhibitor (Pefabloc; Merck) to determine which matrix-degrading enzymes are involved in the invasive property of HCC cells. BB2516 significantly inhibited the invasion of the three cell lines in a concentration-dependent manner (Figure 3, *upper panels*). In contrast, Pefabloc showed no or a slight inhibition of the invasion of HCC cell lines, including JHH-6 cells, which expressed the high level of u-PA mRNA (Figure 3, *lower panels*). Therefore, it is considered that the MMP system rather than the serine proteinase system is mainly involved in the invasive potential of HCC cells.

HGF-Induced Expression of MT1-MMP in JHH-7 Cells

As shown in Figure 4, treatment of JHH-7 cells with HGF markedly induced the expression of both the MT1-MMP mRNA and protein. In addition, the cleavage activity of the cytosomal fraction of HGF-treated JHH-7 cells for pro-MMP-2 was detected by gelatin zymography. (Figure 5). The expression of the mRNAs for u-PA and MMP-2 was slightly enhanced by HGF, although the expression of other matrix-degrading enzymes, including MMP-1, MMP-3, MMP-7 and MMP-9 was not affected (data not shown).

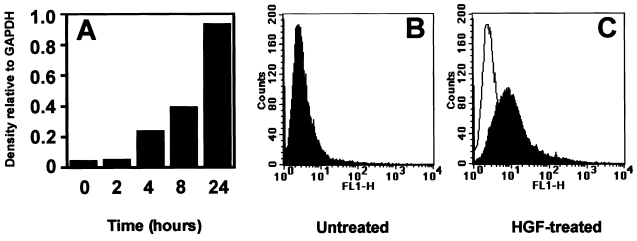

Figure 4. *Induction of the expression of MT1-MMP by HGF in JHH-7 cells. (A), RT-PCR was performed with total RNA isolated from the JHH-7 cells, which had been treated with HGF (10 ng/ml) for 24 hours. Thirty-six and 21 amplification cycles were used with human MT1-MMP and GAPDH primers, respectively. The abundance of MT1-MMP relative to that of GAPDH was determined by using a Master Scan Gel Analysis System. Flow-cytometric analysis was performed using untreated (B) and HGF-treated (C) JHH-7 cells. The cells were incubated with anti-MT1-MMP rabbit antibody followed by FITC-conjugated anti-rabbit IgG to analyze the fluorescense intensity.*

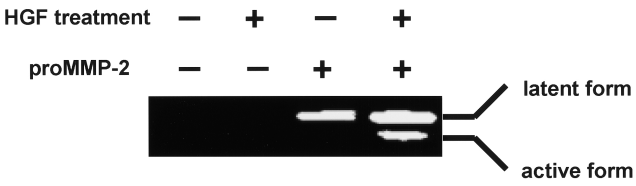

Figure 5. *Cleavage activity of HGF-treated JHH-7 cells for proMMP-2. Lysates of untreated and HGF-treated JHH-7 cells were incubated with or without proMMP-2 for 1 hour and then analyzed by gelatin zymography.*

Intrahepatic Metastasis of HCC Cell Lines

The formation of intrahepatic metastasis by HCC cells was examined in athymic nude mice. Implantation of three cell lines admixed with MATRIGEL into the left lobe of the liver resulted in formation of tumor nodules in the liver and in an increase in the weight of the liver on day 35 (Figure 6); however, the spreading properties of each cell line were different. On day 35 after the implantation, JHH-5 and JHH-7 cells formed a large tumor nodule at the implanted site and smaller nodular intrahepatic metastases (Figure 7, *A* and *C*). In contrast, the implanted tumor nodule of JHH-6 was unclear, and disseminated tumor spread was observed in the liver (Figure 7*B*). Implantation of all tumors admixed with MATRIGEL formed a solitary tumor mass in the liver on day 1 after the implantation (Figure 7*D*: only a liver implanted with JHH-7 is shown). The incidence of intrahepatic metastases is summarized in Table 1. Histopathological examination suggested that the *in vivo* JHH-7 tumor was hemangiopoietic and had the clinical characteristics of HCC (Figure 7, *E* and *F*). Moreover, microsatellite nodules were also detected in the sections of the right lobe, although no visible nodules were seen (Figure 7*G*).

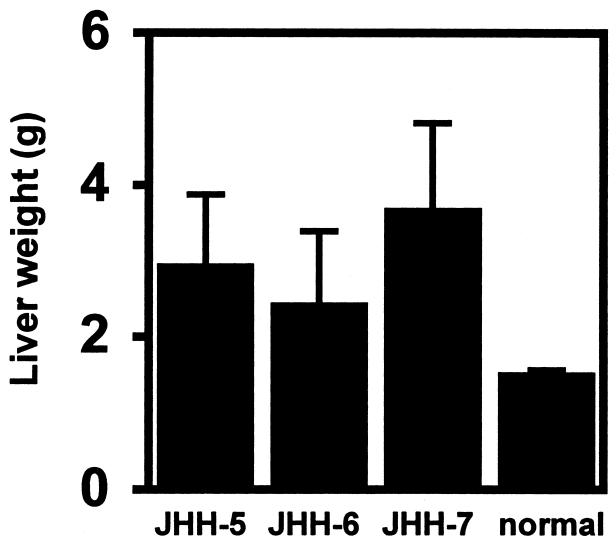

Figure 6. *Liver weight of the mice implanted with human HCC tumor. Each cell line admixed with Matrigel was implanted into the left lobe of the liver of KSN mice. The mice were sacrificed and formation of intrahepatic tumors was examined on day 35 after the implantation. The number of livers examined from JHH-5-, JHH-6- and JHH-7-implanted mice were four, five and five, respectively. The number of livers examined from normal mice was four.*

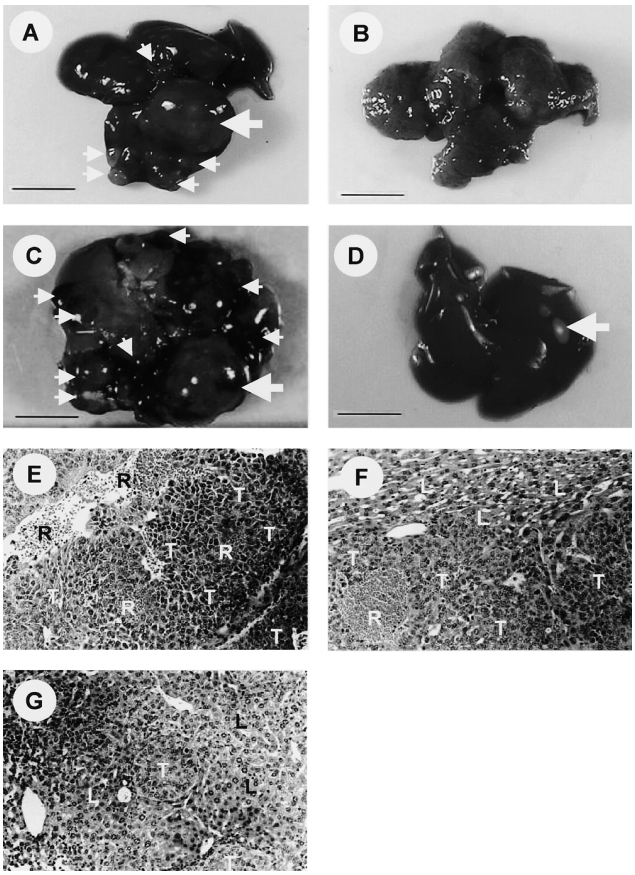

Figure 7. *Macroscopic and microscopic observation of the intrahepatic metastasis produced by orthotopic implantation of JHH-5, JHH-6, and JHH-7 cells. JHH-5 (A), JHH-6 (B) and JHH-7 (C) cells were implanted into the mouse liver, and the mice were sacrificed on day 35 after the implantation. Livers of JHH-7-implanted mice were collected on day 1 after the implantation (D). Large arrow, implanted tumor; small arrows, intrahepatically metastasized tumor. Bar, 1 cm. Hematoxylin and eosin staining of histopathological sections of the implanted JHH-7 tumor (E), metastasized JHH-7 tumor in the right lobe (F) and micro-satellite metastasis of JHH-7 in the right lobe (G). T, tumor tissue; L, liver tissue; R, red blood cells, Magnification: ×200.*

Expression Patterns of mRNAs for Human MMPs and u-PA in JHH-7 Tumor Tissue

Total RNA was isolated from implanted tumor nodules and RT-PCR was performed. As shown in Figure 8, the expression of mRNA for MT1-MMP was detected in JHH-7 tumor tissue. The expression of MMP-2 mRNA was also slightly enhanced. The expression of other MMPs and u-PA were not changed in comparison with their expression in *in vitro* cultured cells. This finding indicates that some endocrine factors including HGF might induce the expression of MT1-MMP, which causes enhanced invasiveness of tumor cells.

Discussion

Invasion of primary tumors into the surrounding tissues is a crucial step in the multistage of metastatic process. Degradation of the ECM by proteolytic enzymes is necessary for invasion and metastasis of tumor cells [37]. MMP families have been shown to play an important role in the process of tumor invasion [38]. Although most MMPs are secreted as latent forms and that are catalyzed to the active form by various serine proteinases, such as trypsin, elastases and plasmin [39], proMMP-2 is specifically catalyzed by MT1-MMP [40]. MT1-MMP also directly digests some ECM components, including type I, II and III collagens, fibronectin, vitronectin and laminin [41]. Recent studies have shown that expression of MT1-MMP, accompanied by activation of MMP-2, was elevated in the liver tissues of patients with cirrhosis or HCC [6].

On the other hand, u-PA is a serine proteinase which converts plasminogen to plasmin, and is also involved in the process of metastasis [42]. In addition to contributing to ECM turn over, u-PA and plasmin regulate proteolytic activation of some growth factors, such as HGF, macrophage-stimulating protein, transforming growth factor-β and basic-fibroblast growth factor, which promote migration and invasion of tumor cells and neovascularization.

We demonstrated here that HCC cell lines, which constitutively expressed mRNAs for MT1-MMP and u-PA,

Table 1. Visible Incidence of Intrahepatic Metastasis Caused by Human HCC Cell Lines in Athymic Nude Mice.

	Incidence of intrahepatic metastasis			
	Negative	Left lobe only	Left and right lobes	Left, right and other lobes
JHH-5	1/4	1/4	0/4	2/4
JHH-6	0/5	3/5	0/5	2/5
JHH-7	1/5	0/5	1/5	3/5

Each HCC cell line admixed with MATRIGEL (1×10^6/20 µg per mouse) was implanted into the left lobe of the liver. Thirty-five days after the implantation, the mice were sacrificed and the incidence of visible intrahepatic metastasis was observed.

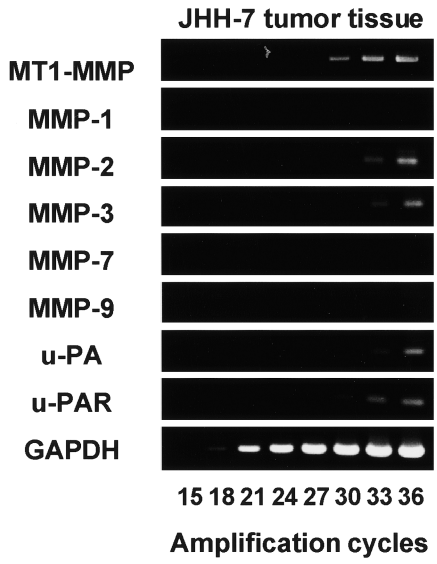

Figure 8. *In vivo expression of mRNAs for matrix-degrading enzymes in JHH-7 tumour. Total RNA was isolated from the implanted tumour tissues on day 35 after the implantation of JHH-7 cells. PCR was performed as described in Materials and Methods.*

showed the ability to invade through reconstituted ECM (Figures 1 and 2). In addition, HGF induced the expression of mRNA and protein for MT1-MMP in noninvasive JHH-7 cells, and consequently led to the promotion of tumor invasion through MATRIGEL which mainly contains laminin and type IV collagen (Figures 2 and 4). Cell lysates of HGF-treated JHH-7 cells cleaved proMMP-2 to the active form (Figure 5). Invasion inhibition assays using an MMP inhibitor and a serine proteinase inhibitor indicated that the MMP system rather than the serine proteinase system, was involved in the invasion of the three HCC cell lines (Figure 3). Thus, the above findings suggest that MT1-MMP plays an important role in the invasive properties of HCC cells due to both direct proteolytic degradation of the ECM and proMMP-2 activation.

To further investigate the process of intrahepatic metastasis, we implanted HCC cell lines admixed with MATRIGEL into the mouse liver to produce a solitary tumor nodule followed by the formation of metastases. All three HCC cell lines caused intrahepatic metastasis in athymic nude mice on day 35 after the implantation (Figures 6 and 7). The incidence of metastasis of JHH-5, JHH-6 and JHH-7 cells was 3/4, 5/5 and 4/5, respectively (Table 1). Although JHH-7 cells did not invade into the ECM *in vitro* or express MT1-MMP, they caused intrahepatc metastasis *in vivo* and expressed mRNA for MT1-MMP in *in vivo* tumor tissues (Figures 7 and 8). These findings suggest that expression of MT1-MMP in the JHH-7 tumor was regulated by host factors which were secreted by nontumoral tissues *in vivo*.

HGF is mainly secreted by nonparenchymal cells in the liver and has various biological activities, such as effects on proliferation, motility and invasion of epithelial cells via the surface receptor encoded by the *c-met* proto-oncogene [17]. HGF/c-MET signals have been reported to play an important role in the promotion of intrahepatic metastasis [16, 23–26]. Although the *in vivo* regulatory mechanism for the expression of MT1-MMP will remains to be analyzed in detail, HGF might be one of the factors responsible for the induction of MT1-MMP in HCC cell lines.

In conclusion, we demonstrated that among matrix-degrading enzymes, MT1-MMP was mainly involved in the invasiveness of HCC cell lines. HGF induced the invasion of a noninvasive HCC cell line through the induction of MT1-MMP expression. These molecules may be involved in the intrahepatic metastasis of HCC.

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