Involvement of the Ets-1 Gene in Overexpression of Matrilysin in Human Hepatocellular Carcinoma

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ABSTRACT

Although matrix metalloproteinases (MMPs) are thought to be involved in the invasion and metastasis of a variety of malignant tumors, including human hepatocellular carcinoma (HCC), the mechanisms for the expression of MMPs in HCC are not known. To understand the mechanism(s) of MMP expression, the expression of matrilysin (MMP-7) and several genes of the Ets transcription factor family was investigated in human HCC and hepatoma-derived cell lines. The role of Ets-1 gene expression in HCC was also studied. Analysis by semiquantitative reverse transcription-PCR revealed that MMP-7 and Ets-1 are upregulated and closely associated in HCC. To clarify the role of Ets-1, hepatoma cells were transduced with human Ets-1 or targeted with the Ets-1-specific antisense oligonucleotides. Cells stably transduced with the Ets-1 gene showed increased MMP-7 expression compared to parental and mock-transfected cells. Cells targeted with Ets-1-specific antisense oligonucleotides showed reduced expression of MMP-7. Cotransfection of cells with a MMP-7 promoter-reporter gene plasmid and an Ets-1 expression vector yielded an increase in MMP-7 promoter activity in an Ets-1-responsive element-dependent manner. Taken together, these data suggested that the Ets-1 oncogene is up-regulated and involved in the overexpression of MMP-7 in human HCC and may contribute to the progression of HCC.

INTRODUCTION

HCC3 is one of the most common malignant tumors in Asian countries, including Japan. HCC frequently shows early invasion into blood vessels as well as intrahepatic metastases and later shows extrahepatic metastases (1, 2). The process of invasion and metastasis of cancer cells involves several important steps: (a) detachment of the tumor cell from the primary site; (b) invasion into blood vessels with degradation of the ECM; (c) adhesion to blood vessels at a distant site; and (d) invasion into the distant organ (reviewed in Ref. 3). Although many factors have been reported to be involved in these processes, the molecular mechanisms responsible for the progression of HCC are unclear. An understanding of these molecular mechanisms may provide important information for developing treatment strategies to improve the prognosis of HCC.

Most HCCs develop in fibrotic livers affected by chronic viral hepatitis and liver cirrhosis, and HCC is usually surrounded by a fibrous capsule from an early stage (1, 2). Therefore, degradation of the surrounding ECM seems to be an important step in tumor invasion and metastasis. MMPs are thought to play a crucial role in this step in many malignant tumors (3). Although MMPs are overexpressed in various human malignant tumors (3–6), the expression mechanism of MMPs in HCC is poorly understood. Recently, we and others have reported that several MMPs, including MMP-7 (7, 8), gelatinase [MMP-9 (9, 10)], and membrane-type MMP-1 [MMP-14 (8, 11)], are overexpressed in HCC tissue and are involved in the progression of HCC. Among these MMPs, MMP-7, which is also known as pump-1, small uterine metalloproteinase, or matrin, is a member of the stromelysin subclass of the MMP family and has characteristics distinct from other members of the MMP family (12). MMP-7 has also been reported to be involved in the invasion and metastasis of a variety of malignant neoplasms, including breast, colon, stomach, lung, skin, and prostate cancers (reviewed in Ref. 12).

Constitutive MMP-7 expression is found in the glandular epithelium of several tissues, and human MMP-7 expression appears to be more widespread in the epithelial cells (13, 14), whereas other MMPs are expressed mainly in stromal cells (4). Ozaki et al. (7) and Yamamoto et al. (8) have reported increased expression of MMP-7 in HCC, suggesting that MMP-7 may be involved in the progression of HCC. However, to our knowledge, the mechanism of MMP-7 overexpression in HCC has not been investigated.

Growth factors, inflammatory cytokines, and other environmental factors such as contact with the ECM are reported to influence the expression of MMPs (3, 15, 16). Several nuclear factors acting on MMP gene promoter regions have been reported to be involved in the regulation of MMP gene expression (15, 16). Ets domain transcription factors are a family of genes encoding a conserved Ets domain originally identified as v-ets in E26 avian erythroblastosis virus, which has been reported to be involved in the invasion and metastasis of human malignant tumors (reviewed in Refs. 17 and 18). Ets transcription factors bind to a GGA(A/T) consensus sequence called the Ets binding site or PEA3 element through an Ets domain consisting of about 80 amino acids in the COOH-terminal region (17, 18). Many MMP genes have conserved cis-acting elements including the PEA3 element adjacent to the AP-1 binding site in their promoter regions (15, 16). The regulatory role of Ets1 has been shown in several MMPs, including collagenase-1 (19) and stromelysin-1 (20).

Because the mechanism of MMP-7 expression and the role of Ets domain binding factors in HCC are not known, we investigated the expression and correlation of MMP-7 and several Ets family genes in human HCC tissue and hepatoma cell lines. In this report, we show a close association between Ets-1 and MMP-7 gene expression. To understand the role of Ets-1 in the expression of MMP-7 in HCC, a human hepatoma cell line was transduced with Ets-1 or targeted with an antisense oligonucleotide against Ets-1, and MMP-7 expression was analyzed.

MATERIALS AND METHODS

Human Liver Samples. Sixteen specimens of HCC and corresponding uninvolved liver tissue were examined. The specimens were obtained by surgical resection for treatment of HCC in eight patients or by autopsy performed within 3 h after death in eight patients. All procedures were performed with informed consent at the hospital of Saga Medical School. Clinical features of the patients studied are summarized in Table 1. The histological grading of HCC was based on Edmondson’s grading system (21),
and clinical stage was classified according to the tumor-node-metastasis (TNM) classification system of the Union International Contre Cancer (22).

**Cells Lines.** Human hepatoma cell lines HepG2, Huh7, PLC/PRF/5, HLF, and HLE were obtained from the Japanese Cancer Research Resources Bank (Osaka, Japan). Cells were cultured and maintained in DMEM (Life Technologies, Inc., Gaithersburg, MD) containing 10% FCS (Life Technologies, Inc.).

**RNA Extraction.** Total RNA was extracted from liver specimens and cultured human hepatoma cell lines using the guanidinium thiocyanate-phenol method with minor modifications (23). The concentration of RNA was determined spectrophotometrically, and the integrity of all samples used was confirmed by visualizing 28S and 18S rRNA bands under UV light after gel electrophoresis.

**Oligonucleotides.** The sequences of oligonucleotide primers used in RT-PCR for each gene and the expected sizes of their RT-PCR products are as follows: (a) MMP-7 (24), 5'-GGATCCATTTCATTGCTGGTCACCTACAGGATCGTA-3' (sense primer) and 5'-CAATTCCTTATATTCCTTGTA-3' (antisense primer), 492 bp; (b) Ets-1 (25), 5'-GGATCCATTTCATTGCTGGTCACCTACAGGATCGTA-3' (sense primer) and 5'-CAATTCCTTATATTCCTTGTA-3' (antisense primer), 274 bp; (c) E1AF (26), 5'-GGATCCATTTCATTGCTGGTCACCTACAGGATCGTA-3' (sense primer) and 5'-CAATTCCTTATATTCCTTGTA-3' (antisense primer), 584 bp; (d) E1AF (26), 5'-GGATCCATTTCATTGCTGGTCACCTACAGGATCGTA-3' (sense primer) and 5'-CAATTCCTTATATTCCTTGTA-3' (antisense primer), 231 bp.

**RT-PCR.** cDNA was synthesized from 1 μg of total RNA with or without reverse transcriptase (Life Technologies, Inc.) with random primers (Takara, Kyoto, Japan). The reaction mixture was then subjected to PCR amplification with specific primers by using AmpliTaq Gold DNA polymerase (Perkin-Elmer, Branchburg, NJ). The RT mixture was amplified for 40 cycles consisting of heat denaturation (94°C for 1 min), annealing (55°C for 1 min), and extension (72°C for 2 min). PCR products were electrophoresed on 2% agarose gels stained with ethidium bromide and visualized under UV light. The sequence of each RT-PCR product was confirmed by DNA sequencing with specific primers by using AmpliTaq Gold DNA polymerase (Perkin-Elmer, Branchburg, NJ).

**CAT Assay.** The human MMP-7 promoter-CAT plasmids, p933CAT, p295CAT, and p95CAT (22), were a generous gift from Dr. Lynn Matrisian (Vanderbilt University School of Medicine, Nashville, TN). These plasmids were transiently transfected into HepG2 cells with or without the human Ets-1 expression vector under the control of a CMV promoter by using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer’s instructions, with minor modifications. Cells were transfected with 4 μg of plasmid containing the CAT reporter gene together with 0, 2, 4, or 8 μg of the Ets-1 expression vector driven by the CMV promoter and 1 μg of pSVβ-gal.

**Table 1 Characteristics of patients with chronic liver disease and HCC**

<table>
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<tr>
<th>Case no.</th>
<th>Age (yr)</th>
<th>Gender</th>
<th>HBsAg*</th>
<th>Anti-HCV</th>
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* HBsAg, hepatitis B surface antigen; CH, chronic hepatitis; LC, liver cirrhosis; HCV, hepatitis C virus.
(Promega, Madison, WI), a β-galactosidase expression vector to normalize transfection efficiency. After 48 h of culture, cells were washed with PBS and lysed with 900 μl of lysis buffer (Promega) according to the manufacturer’s instructions. CAT activity was visualized by thin-layer chromatographic separation of [14C]chloramphenicol from its acetylated derivatives exposed to X-ray film (Fuji). In each experiment, the CAT assay was repeated independently three or four times.

**Oligonucleotide Transfection.** Antisense or sense phosphorothioate oligonucleotides corresponding to the Ets-1 gene and incorporating an initiation codon were purchased from Hokkaido System Bioscience (Sapporo, Japan). The sequences were 5’-ACCATGAGCGGCCGTGATCTCA-3’ (sense) and 5’-TGAGATGACGCGCCCTTCACTGAT-3’ (antisense) (29). The oligonucleotides were purified by high-pressure liquid chromatography. The human hepatoma-derived cell line, HLE, was treated with oligonucleotides using LipofectAMINE (Life Technologies, Inc.) as described previously (29). After 24 h of incubation, cells were harvested and subjected to RNA analysis by RT-PCR.

**Statistical Analysis.** The Dunnet test was used for nonparametric multiple comparisons of RT-PCR products between the chronically diseased liver tissues and HCCs. Data are expressed as the mean ± SE. The relationships between parameters were estimated using linear regression analysis. P < 0.05 were considered statistically significant.

**RESULTS**

**Detection and Confirmation of RT-PCR Products.** To confirm that the RT-PCR products were RNA dependent, PCR was performed with or without RT (Fig. 1A, Lanes 1 and 2 of each RT-PCR product, respectively). All samples without RT showed no PCR product, demonstrating that these products were RNA dependent. These RT-PCR products were sequenced directly and confirmed to be identical to the sequences reported previously (data not shown).

**Semiquantitation of RT-PCR Products.** We confirmed the validity of semiquantitative RT-PCR by showing that the intensity of RT-PCR products was dependent on both the amount of RNA present and the number of PCR cycles. First, we performed RT from serially diluted RNA samples and subsequently subjected the samples to PCR for 35 cycles. The signal intensity of RT-PCR products increased exponentially as the amount of RNA increased (Fig. 1B, left). Second, we performed RT-PCR using 1 μg of total RNA while changing the number of PCR cycles from 15 to 40. The signals of the RT-PCR products increased exponentially as the number of PCR cycles increased, although the signals showed a tendency to reach a plateau at more than 35 cycles (Fig. 1B, right). We also performed RT-PCR using 0.1 μg of RNA while changing the number of PCR cycles, and the signals also increased exponentially, although they were slightly less sensitive compared with those from RT-PCR using 1 μg of RNA (data not shown). After establishing these conditions, we evaluated mRNA levels of MMP-7, Ets-1, Ets-2, E1AF, and GAPDH in human liver tissue using 0.5 μg of total RNA, which was chosen as a value between 0.1 and 1 μg, and 30 cycles of PCR.

**Expression of MMP-7 and Ets Family Genes in Human HCC and Hepatoma Cell Lines.** Representative expressions of MMP-7, Ets-1, Ets-2, and E1AF, and GAPDH detected by semiquantitative RT-PCR in noncancerous human liver tissue (white square) and HCC tissue (white triangle) are shown in Fig. 2. The quantified levels of mRNA expression of these genes are summarized in Fig. 3. In noncancerous liver tissue with chronic liver disease, MMP-7 mRNA was weakly detected in most cases. Two Ets family genes, Ets-1 and Ets-2, were expressed in all noncancerous liver tissue, although E1AF was detected only weakly in 5 of 16 cases. HCC tissue demonstrated increased MMP-7 expression compared with corresponding noncancerous liver tissue in 14 of

**Fig. 2.** Representative RT-PCR of MMP-7, Ets family genes (Ets-1, Ets-2, and E1AF), and GAPDH detected in noncancerous liver tissue (Lanes N) and HCC tissue (Lanes T). Lane P, positive control for RT-PCR; Lane C, negative control for PCR without RT; Lane M, DNA size marker.

**Fig. 3.** Expression of MMP-7, Ets family genes (Ets-1, Ets-2, and E1AF), and GAPDH in human liver tissue using 0.5 μg of total RNA, which was chosen as a value between 0.1 and 1 μg, and 30 cycles of PCR.
To examine the role of Ets-1 in MMP-7 expression, HepG2 cells were transduced with Ets-1 and most of the HepG2 clones showed increased expression of MMP-7 mRNA, although parental and mock-transfected HepG2 cells did not show increased expression of MMP-7.

Regulation of MMP-7 Promoter Activity by Ets-1. Serially 5'-deleted MMP-7 promoter-CAT plasmids p933CAT, p295CAT, and p95CAT (24) were used to transduce HepG2 cells with or without a full-length human Ets-1 expression construct. p933CAT and p295CAT contain an Ets-1 binding site, but p95CAT does not (Fig. 6A). Cotransfection of the Ets-1 expression vector stimulated p933CAT and p295CAT activity as the amount of Ets-1 increased; p95CAT, which lacks an Ets-1 binding site, did not show any significant change (Fig. 6, B and C). Therefore, MMP-7 promoter activity seems to be regulated by Ets-1.

Effect of Ets-1 Antisense Oligonucleotides on Ets-1 and MMP-7 mRNA Levels. To confirm that Ets-1 could stimulate expression of MMP-7 mRNA, the human hepatoma-derived cell line HLE, which expresses Ets-1 and MMP-7 mRNA, was transduced with 0–5 μM sense or antisense oligonucleotide against Ets-1. Ets-1 and MMP-7 mRNA expression was analyzed by semiquantitative RT-PCR. As shown in Fig. 7, Ets-1 mRNA was down-regulated according to the increase in antisense oligonucleotide concentration, although sense oligonucleotides did not show any effect on the Ets-1 mRNA level. MMP-7 mRNA was also down-regulated in HLE cells treated with antisense oligonucleotides but was not down-regulated in HLE cells treated with sense oligonucleotides. GAPDH did not show any change in HLE cells treated with sense or antisense oligonucleotide.

DISCUSSION

To date, nearly 20 members of the MMP gene family have been identified (30). Some MMPs, including MMP-7, have been shown to be overexpressed in many tumors, including HCC. MMPs may be involved in the process of invasion and metastasis (3–12, 15, 16). The mechanisms by which MMPs are overexpressed in tumors are unclear. In this study, we demonstrated that MMP-7 and Ets-1 are overexpressed in human HCC tissue and that Ets-1 plays a crucial role in the expression of MMP-7 in HCC cells. Therefore, Ets-1 may be involved in the overexpression of MMP-7 in human HCC.

In this study, 14 of 16 cases examined showed increased expression of MMP-7 in HCC tissue compared with adjacent uninvolved tissue. Eleven of 14 cases that showed overexpression of MMP-7 in HCC had advanced-stage HCC (TNM stage III or IV), suggesting an

Table 2 Correlation between expression of MMP-7 mRNA and Ets transcription factors, Ets-1, Ets-2, and E1AF mRNA

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<tr>
<td>Ets-1</td>
<td>0.757</td>
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<tr>
<td>Ets-2</td>
<td>0.177</td>
<td>0.4538</td>
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<tr>
<td>E1AF</td>
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Data were analyzed using linear regression analysis.
association between MMP-7 expression and the progression of HCC. These results are consistent with previous reports describing other malignant tumors in which MMP-7 expression closely correlated with malignant potential (31–36). MMP-7 is the smallest known MMP and has a wide variety of substrates, including fibronectin, laminin, collagen type IV, gelatin, and proteoglycans (12). One unique aspect of MMP-7 is its tendency to localize in epithelial cells, whereas other MMPs are localized mainly in stromal fibroblasts (13, 14). Another interesting feature of MMP-7 in HCC is its ability to activate other MMPs. Usually, MMPs are secreted in zymogen form and activated by proteolytic cleavage. MMP-9, which has recently been reported to be overexpressed in HCC tissue (38), has been shown to be activated by MMP-7 (39). Our study showed that cultured HCC cells express MMP-7 to a varying degree. Overexpression of MMP-7 in HCC cells could participate in the proteolytic cascade and may activate gelatinases, which would enhance invasiveness and the metastatic potential of tumor cells. These processes would occur independently of any direct contribution by MMP-7 itself to the pathogenesis of invasion and metastasis.

Recent data obtained from knockout mice have begun to provide in vivo evidence of the role of MMPs, including the role of MMP-7 in tumor development. Wilson et al. (40) reported that mice lacking MMP-7 showed reduced intestinal tumorigenesis. Moreover, More recently, activation of $M_{72,000}$ gelatinase-deficient mice showed reduced angiogenesis and tumor progression (41), and mice lacking stromelysin-3 showed reduced tumorigenesis induced by chemical carcinogens (42). Indeed, the relationship between MMP-7 expression and HCC recurrence after tumor resection has been reported in a clinical study (8). Therefore, MMP-7 might be involved in early-stage carcinogenesis by altering the integrity of the ECM and thereby modulating cellular dedifferentiation. Recently, direct evidence that MMPs play an important role in tumor invasion and progression has been accumulating. For example, it has been shown that MMP activity is required for increased motility of epithelial cells (43) and for growth of metastasized tumor cells (44). MMPs have also been shown to play an essential role in angiogenesis and tumor cell invasation (45, 46). Therefore, control of MMP expression and activity may be an effective strategy for the prevention and treatment of cancer.

The Ets domain transcription family includes more than 30 members. These genes possess a conserved region, termed the Ets domain, which recognizes and binds to GGA(A/T) purine-rich core sequences called the Ets binding motif or PEA3 element (17, 18). In many MMP genes, including MMP-7, PEA3 elements are identified in the regulatory region. The PEA3 element is often combined with AP-1 binding site, generating a complex (15, 16, 24) that facilitates cooperative interaction between AP-1 and Ets transcription factors. This type of interaction has been reported in collagenase-1 and stromelysin-1 (19, 20). In this study, we investigated the expression and relationship of MMP-7 and three members of the Ets gene family, Ets-1, Ets-2, and E1AF. We demonstrated that MMP-7 and the Ets-1 gene are expressed in a closely associated manner both in vivo in human HCC tissue and in vitro in hepatoma cell lines. The difference between in vivo HCC tissue and in vitro hepatoma cells was the expression of Ets-2 and E1AF. In in vivo HCC tissue, MMP-7 showed a positive correlation with E1AF expression as well as with Ets-1, although in in

**Fig. 6. Transcriptional regulation of the human MMP-7 promoter by Ets-1.** A, schematic representation of constructs containing human MMP-7 promoter ligated to CAT (24). The transcription start site is indicated by bent arrows, and the relative positions of the TATA box, AP-1, PEA3, and transforming growth factor β1 inhibitory elements (TIE) are shown. B, representative results of the CAT assay. Four μg of CAT constructs (p933CAT, p295CAT, and p95CAT) containing various lengths of the human MMP-7 promoter were cotransfected with 0, 2, 4, and 8 μg of human Ets-1 expression vector into HepG2 cells. Ac, acetylated derivatives of chloramphenicol. C, increase in CAT activity by cotransfection with Ets-1. CAT conversion rates were evaluated by densitometric analysis. Values are expressed as the relative increase over basal CAT activity. Data are given as the mean ± SE ($n = 4$).
vitro HCC cells, MMP-7 and Ets-1 expression showed an inverse expression pattern compared with E1A1F and Ets-2 expression. One reason for this discrepancy may be due to the cell type specificity of each Ets family gene expression (18, 25). In vivo HCC tissue, cell type(s) other than HCC cells such as endothelial cells or stromal fibroblasts can also express E1A1F and/or Ets-2 in addition to Ets-1. Therefore, a positive correlation between E1A1F and MMP-7 expression in HCC tissue might be due to the expression of E1A1F in the cell types with the exception of HCC cells. Further study using immunohistochemistry or in situ hybridization is required. Another possibility is due to the interaction between each of the Ets family gene products. Different Ets transcription factors may have different effects on target gene expression by cooperating with or opposing target gene expression (18, 19).

Analysis of MMP-7 expression showed that Ets-1 stimulates MMP-7 promoter activity in a PEA3 element-dependent manner. A hepatoma cell line stably transfected with Ets-1 also showed up-regulation of MMP-7 mRNA. Conversely, inhibition of Ets-1 expression using a specific antisense oligonucleotide showed a dramatic, dose-dependent decrease in MMP-7 transcription, although sense oligonucleotides had no effect. These data strongly suggest that among the Ets domain transcription factors, Ets-1 seems to play an important role in the expression of MMP-7 as a transactivator and is involved in overexpression of MMP-7 in human HCC. Furthermore, clones of HepG2 cells transfected with Ets-1 frequently showed morphological changes and increased scattering compared with the parental hepatoma cell line.1 Interestingly, Ets-1 has also been reported to be associated with the process of invasion and angiogenesis in human carcinomas (47–50). Therefore, overexpression of Ets-1 may contribute to the invasion and metastasis of HCC through MMP-7 overexpression and other unknown mechanisms. Recently, a novel mechanism of MMP-7 activation has also been reported. Crawford et al. (51) showed that β-catenin, a component of the cadherin complex that mediates cell-cell contact, transactivated MMP-7 expression. Therefore, several regulatory mechanisms may be involved in overexpression of the MMP-7 gene in various tumors, and different tumors may use different pathways to increase MMP-7 expression.

In conclusion, MMP-7 is overexpressed in human HCC through increased expression of the Ets domain transcription factor, Ets-1, and may contribute to the progression of HCC. The Ets-1-MMP-7 pathway is a potent therapeutic target in patients with cancer, including those with HCC.

ACKNOWLEDGMENTS

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