Paradoxically enhanced immunoreactivity of hepatocyte growth factor activator inhibitor type 1 (HAI-1) in cancer cells at the invasion front

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We have previously demonstrated significantly decreased immunoreactivity of hepatocyte growth factor activator inhibitor type 1 (HAI-1), an integral membrane protein that exhibits potent inhibitory activity against hepatocyte growth factor activator (HGFA) and matriptase, in colorectal adenocarcinomas. In this report, we describe further detailed analysis of HAI-1 expression in colorectal adenocarcinoma by using three kinds of anti-HAI-1 antibodies, each of which recognizes a distinct epitope of the HAI-1 molecule, and also by *in-situ* hybridization for HAI-1 mRNA. The results indicated that the decreased immunoreactivity of HAI-1 in colorectal carcinoma cells is largely a result of enhanced ectodomain shedding of HAI-1 in these cells. In contrast, immunoreactivity of mature membrane-form HAI-1 was paradoxically enhanced in cancer cells at the invasion front, showing intense cell-stroma interactions and/or sprouting invasion. This finding indicates that these invading cells showed decreased ectodomain shedding of HAI-1 and consequently might require the existence of the membrane-form HAI-1. Of particular interest was the observation of a possible inverse correlation between paradoxical up-regulation of membrane-form HAI-1 expression and membrane-associated E-cadherin in these cells. These membrane-form HAI-1-positive sprouting cancer cells were also negative for MIB-1 immunohistochemically, indicating a low-proliferating population. All these results suggest that HAI-1 may mediate diverse functions in regard to the progression of colorectal carcinomas, and the immunoreactivity of membrane-form HAI-1 may serve as a marker of invading cancer cells. (Cancer Sci 2004; 95: 728–735)

Hepatocyte growth factor activator inhibitor type 1 (HAI-1) is a recently identified Kunitz-type serine proteinase inhibitor.[1)] HAI-1 has a unique molecular structure consisting of two extracellular Kunitz-type serine proteinase inhibitor domains (bikunin), an extracellular LDL receptor class A domain, a transmembrane domain, and a short intracellular domain at the carboxyl-terminal end. Thus, this inhibitor is a type-1 transmembrane protein that functions on the cellular surface. It should be noted that the membrane-form HAI-1 can be secreted by ectodomain shedding, resulting in secreted-form HAI-1.[1, 2)] Two important proteinases have been identified as the target enzymes of HAI-1, these being hepatocyte growth factor activator (HGFA) and matriptase. In addition to HGFA and matriptase, HAI-1 also inhibits other serine proteinases such as plasmin and trypsin.[2)] HGFA is a factor XII-like serine proteinase that specifically converts a single-chain inactive form of hepatocyte growth factor/scatter factor (HGF/SF) to an active two-chain form in response to tissue injury.[3, 4)] Since HGF/SF is a potent mediator of invasive growth of cancer cells via its high-affinity receptor, MET receptor tyrosine kinase, HGFA might be involved in the malignant behavior of tumors.[2, 5, 6)] Matriptase is also a cognate proteinase of HAI-1 and is identical to membrane-type serine protease 1.[2, 7, 8)] This proteinase is a type-2 transmembrane protein having an extracellular catalytic domain. Like HGFA, matriptase also activates HGF/SF.[9, 10)] Thus, HAI-1 would be a critical regulatory molecule in the pericellular activation of HGF/SF.[2)] In addition to HGF/SF, matriptase also shows processing activities towards pro-urokinase-type plasminogen activator and protease activated receptor 2.[9, 11)] Extracellular matrices, such as gelatin and fibronectin are also sensitive to matriptase, and enhanced expression of matriptase has been reported in several types of carcinoma cells.[12)] Thus, cellular matriptase could have important roles in invasion and metastasis of cancer cells.[13)]

Recent studies have suggested that membrane-form HAI-1 has diverse roles in the pericellular activities of HGFA and matriptase, acting not only as an inhibitor of both enzymes but also as a reservoir of active HGFA,[2, 14)] and an essential cofactor in the activation of pro-matriptase[15)] on the cellular surface. These observations indicate the existence of complex interactions between HAI-1 and target proteinases, which eventually regulate the enzymatic activities positively or negatively.[2)] For example, the binding between membrane-form HAI-1 and active HGFA on the cellular surface is reversible, and the active HGFA is released from the secreted-form HAI-1 (sHAI-1) after regulated shedding of membrane-form HAI-1/HGFA complex, paradoxically ensuring concentrated HGFA activity in the pericellular microenvironment.[14)] This regulated shedding of membrane-form HAI-1 rapidly occurs when the cellular protein kinase C activity is up-regulated or the cells are stimulated by inflammatory cytokines.[14)] Therefore, the function of HAI-1 is highly situational, depending on the presence of other constituents in the intra- or extracellular milieu.

Since HAI-1 could be a key cellular surface regulator of several proteinases, such as HGFA, matriptase, plasmin and trypsin, it is of interest to study the expression of this inhibitor in human cancers. Previously, we have reported that membrane-form HAI-1 is expressed on the basolateral surface of the epithelial cells,[16)] and the expression of membrane-form HAI-1 is down-regulated in colon carcinoma cells relative to the adjacent reactive normal epithelial cells and adenoma cells.[5)] Here we describe a detailed analysis of the expression pattern of HAI-1 in colorectal adenocarcinomas by using antibodies that recognize different epitopes of HAI-1 and also by *in-situ* hybridization analysis.

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Abbreviations: HAI-1, hepatocyte growth factor activator inhibitor type 1; HGF, hepatocyte growth factor; HGFA, hepatocyte growth factor activator; SF, scatter factor; sHAI-1, secreted-form HAI-1; KD1, first Kunitz domain; KD2, second Kunitz domain.

Materials and Methods

Antibodies. Three different primary antibodies against human HAI-1, namely C76-18, 1N7 and a-C, were used in this study (Fig. 1). C76-18 and 1N7 are mouse monoclonal antibodies that recognize the regions around the 1st Kunitz domain (KD1) and 2nd Kunitz domain (KD2) of HAI-1, respectively.[1, 5, 14] The antibody a-C is a rabbit polycolonal antibody prepared by immunization with a synthetic peptide, corresponding to a part of the carboxyl terminus of the intra-cytoplasmic domain of HAI-1 (Pro^{484}–Leu^{507}). After immunization, the IgG was prepared from the rabbit serum, followed by immunopurification on an antigen-peptide affinity column. Anti-human E-cadherin (M106, TaKaRa Shuzo, Otsu, Japan), anti-human β-catenin rabbit polyclonal antibody (Sigma, Steinheim, Germany) and Ki-67 (clone MIB-1 M7240, DAKO, Glostrup, Denmark) monoclonal antibodies were also used.

Comparable *in-situ* hybridization and immunohistochemical analyses of HAI-1. Small tissue fragments of cancer tissue were obtained from resected colorectal carcinoma tissues at surgery. For the preparation of serial frozen sections, the tissues were embedded in OCT compound (Sakura Fintek, Inc., Torrance, CA) and snap-frozen with liquid nitrogen. Then, 4 μm thick serial sections were prepared, fixed in freshly prepared 4% paraformaldehyde in phosphate-buffered saline (PBS) for 16 h. The frozen sections were air-dried, rinsed in nuclease-free water and used for the subsequent *in-situ* hybridization (ISH) study and immunohistochemical study in order to determine the localization and expression of HAI-1 mRNA and protein.

For the ISH study, a 483-bp cDNA fragment of HAI-1 corresponding to bases 294–776 of the human HAI-1 cDNA sequence was generated by means of the polymerase chain reaction (PCR) for the preparation of hybridization probe. The PCR product was subcloned into pBluescript II SK(+) (Stratagene, La jolla, CA), and *in vitro* transcription to generate digoxigenin-labeled probes was carried out according to the manufacturer's instructions (Roche Diagnostics GmbH, Penzberg, Germany). The same amount of each antisense or sense probe was used for hybridization, in which the sense probe was used as a negative control. The ISH reaction was performed using fully automated ISH apparatus (Ventana HX System Discovery and RiboMap System, Ventana, Yokohama, Japan), according to the manufacturer's instruction. Briefly, after the pre-treatment step (fixation, acid treatment and conditioning without protease treatment), the sections were subjected to hybridization using 1 ng per slide of digoxigenin-labeled probe at 65°C for 6 h. After the hybridization, signals were detected with biotin-labeled anti-DIG antibody. The reaction was detected with a BlueMap Kit and counterstaining was done with nuclear fast red.

Serial sections to the above ISH specimen were also used for the immunohistochemical analysis for HAI-1 protein, by using three kinds of primary antibodies, these being C76-18 (20 μg/ml), 1N7 (10 μg/ml) and a-C (1 μg/ml). For C76-18, the sections were fixed with cold acetone instead of paraformaldehyde. The sections were treated with 3% H_2O_2 in methanol for 10 min and washed in PBS twice, followed by blocking in 3% bovine serum albumin (BSA) and 10% goat serum in PBS for 1 h at room temperature (RT). For the cases of 1N7 and a-C, an additional antigen retrieval step (autoclaving in 10 m*M* citrate buffer, pH 6.0 for 5 min) was added prior to the blocking step. Then the section was incubated with each primary antibody for 16 h at 4°C. Negative controls consisted of omission of the primary antibodies and as a positive control, a mucosal tissue in which their expressions were confirmed previously was used. The sections were then washed in PBS and incubated with Envision-labeled polymer reagent (DAKO) for 30 min at 37°C. The reaction was revealed with nickel, cobalt-3,3′-diaminobenzidine (ImmunoPure Metal Enhanced DAB Substrate Kit, Pierce, Rockford, IL) and counterstained with Mayer's hematoxylin. The protocol was approved by the Ethical Board of the Faculty of Medicine, University of Miyazaki.

Immunohistochemistry for paraffin-embedded tissue sections. Formalin-fixed, paraffin-embedded tissue specimens prepared according to the routine procedure from surgically resected 88 colorectal adenocarcinoma tissues at our institution between 1994 and 2003 were used for the immunodetection of HAI-1 (1N7 antibody), E-cadherin and MIB-1. Selected samples (12 cases) were also immunostained with a-C antibody, and some samples were used for the detection of β-catenin. Ages ranged from 38 to 91 years (mean 64.2 years). There were 32 women and 56 men. The distribution according to the TNM classification was as follows: 11 T1, 20 T2, 31 T3 and 26 T4 cases. Forty of the patients (45.5%) had lymph node metastases (N1, N2). Twenty patients (22.7%) presented with distant metastases in the liver or lungs. In addition, 11 cases of early gastric adenocarcinoma and 6 cases of invasive pancreatic ductal adenocarcinoma were also immunostained. 3–5 μm thick sections were dewaxed in xylene and rehydrated in decreasing ethanol solutions and water. After antigen retrieval (autoclaving in 10 m*M* citrate buffer, pH 6.0 for 5 min), 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 and 10% goat serum in PBS for 1 h at RT. Then the sections were incubated with the primary antibodies against HAI-1 (1N7, 10 μg/ml or a-C, 1 μg/ml) for 16 h at 4°C. In several cases, serial sections were similutaneously stained for E-cadherin (1:200 dilution), β-catenin (1:750 dilution) and MIB-1 (1:50 dilution). Negative controls consisted of omission of the primary antibodies and as a positive control, a mucosal tissue in which their expression had previously been confirmed, was used. For the absorption test, the antibody was pre-treated with a 10-fold excess (weight/volume) of recombinant HAI-1 (for 1N7) or antigen peptide (for a-C). The sections were then washed in PBS and incubated with Envision-labeled polymer reagent (DAKO, Carpinteria, CA) for 30 min at 37°C. The reaction was revealed as described above.

Immunoblot analysis. A human colorectal carcinoma cell line WiDr was obtained from Dainippon Seiyaku (Osaka, Japan), and was maintained in a mixture of RPMI 1640 and Ham's F-12 (1:1) supplemented with 10% fetal bovine serum. Subconfluent WiDr cells on 100-mm dishes were washed three times with PBS and immediately scraped into 2 ml of 10% trichroloacetic acid on ice. The precipitated proteins were harvested by centrifugation (14,000 rpm, 3 min) and the pellet was extracted with 200 μl of 7 *M* urea/2% Triton X-100/5% 2-mercaptoethanol, followed by centrifugation (14,000 rpm, 3 min). To prepare tis-

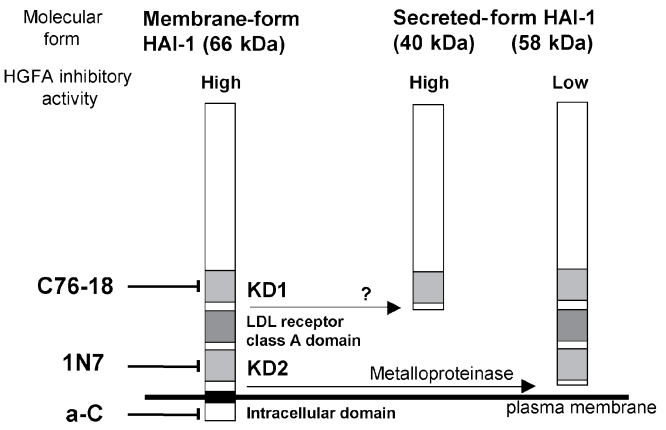

Fig. 1. Schematic representation of the molecular structure of HAI-1 and epitopes of the antibodies (C76-18, 1N7, a-C) used in this study.

sue extract, fresh human colon carcinoma tissue and corresponding normal mucosa tissue (150 mg of each), which were obtained from a surgically resected colon of a colon cancer patient, were immediately frozen in liquid nitrogen, crushed and homogenized in an extraction buffer containing 50 m*M* Tris-HCl (pH 7.5), 150 m*M* NaCl, 5 m*M* EDTA, protease inhibitor cocktail tablets ("Complete mini," Roche Diagnostics GmbH), and 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (Sigma), followed by centrifugation (14,000 rpm, 3 min), and the resultant supernatants were collected. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed under reducing conditions using a 4–12% gradient gel. After electrophoresis, the proteins were transferred onto Immobilon membrane (Millipore; Bedford, MA). Nonspecific binding was blocked with 5% nonfat dry milk in 50 m*M* Tris-HCl (pH 7.5), 150 m*M* NaCl, 0.05% Tween 20 (TBS-T), and the membrane was incubated with C76-18, 1N7 or a-C (1 μg/ml in TBS-T containing 1% BSA) at 4°C overnight, followed by washing in TBS-T four times and incubation with peroxidase-conjugated swine anti-mouse (for C76-18 and 1N7) or anti-rabbit (for a-C) immunoglobulin IgG (Bio-Rad, Hercules, CA), diluted 1:5000 in TBS-T with 1% BSA for 1 h at RT. The labeled proteins were visualized with a chemiluminescence reagent (NEN Life Science, Boston, MA).

Statistics. All statistical analyses were performed using the Statview 4.0 program (Brainpower, Inc., Calabasas, CA). Kruskal-Wallis non-parametric test or Mann-Whitney *U* test was used. The criterion for significance was set at $P<0.05$.

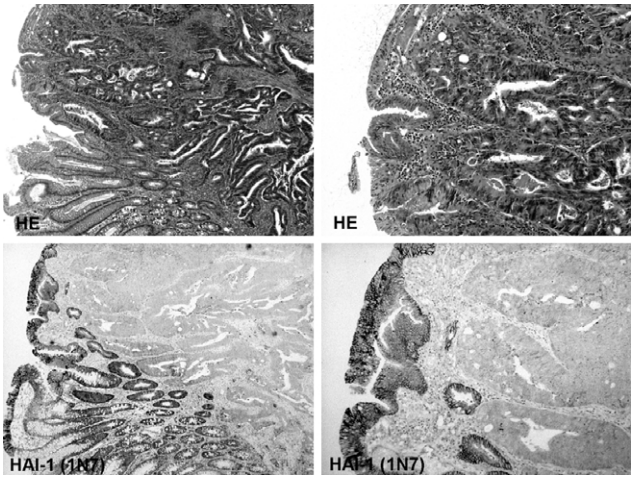

Fig. 2. Immunohistochemical stainings of membrane-form HAI-1 protein in colon adenocarcinomas at ×40 (left) and ×100 (right) original magnification. The antibody used was 1N7 that recognizes the 2nd Kunitz domain. Note that the immunoreactivity is clearly decreased in the cancer cells compared with the adjacent non-neoplastic epithelium.

Results

Decreased immunoreactivity of membrane-form HAI-1 in colorectal adenocarcinoma cells. Initially, HAI-1 reactivity was characterized by immunohistochemistry in paraffin-embedded colorectal carcinoma samples by using 1N7 monoclonal antibody that recognized the region around the 2nd Kunitz domain (KD2) of the extracellular part of HAI-1 (Fig. 1). The normal epithelium adjacent to the cancer tissue consistently showed up-regulated immunostaining of membrane-form HAI-1 (Fig. 2). Since the immunoreactivity in these epithelial cells near the cancer cells was apparently stronger than that in normal epithelium apart from the cancerous portion, it is possible that membrane-form HAI-1 is up-regulated in reactive/regenerative epithelial cells. On the other hand, the cancer cells were less stained for HAI-1 than the adjacent normal epithelium or adenoma cells concomitantly present in the cancer tissue (Fig. 2), suggesting down-regulation of membrane-form HAI-1 expression in the cancer cells. Decreased immunoreactivity of membrane-form HAI-1 in cancer cells was observed in 67% of the cases immunostained with 1N7. This finding may be compatible with the previous results of RNA blot analysis, in which the cancer tissues showed a modest (30%) reduction of HAI-1 mRNA level compared with normal counterparts.[17] However,

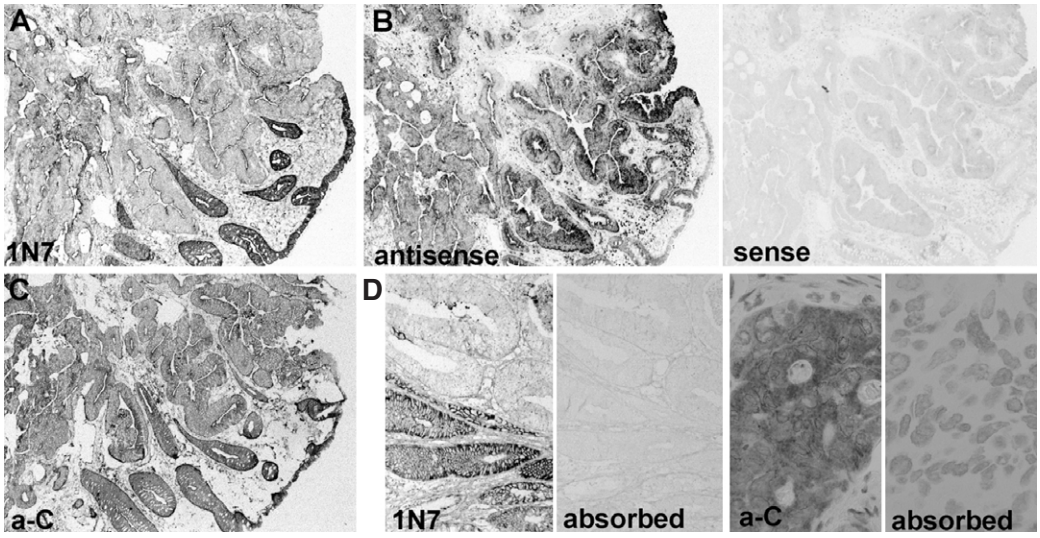

Fig. 3. Comparative analysis of HAI-1 protein and mRNA in colon adenocarcinoma by using serial frozen sections. (A) Immunohistochemical staining of membrane-form HAI-1 protein by using 1N7 at ×40 original magnification. The cancer cells were less stained compared with adjacent normal epithelium. (B) *In-situ* hybridization analysis of HAI-1 mRNA at ×40 original magnification. The cancer cells showed distinct HAI-1 mRNA. Sense probe was used as a negative control. (C) Immunohistochemical staining of HAI-1 protein by using a-C that recognizes intracytoplasmic domain of HAI-1 at ×40 original maginification. As in the *in-situ* hybridization study, the cancer cells were positively stained. (D) Absorption tests for the specificity of antibodies at ×100 original maginification for 1N7 and ×400 original maginification for a-C. Tenfold excess amounts of each antigen were used for the absorption.

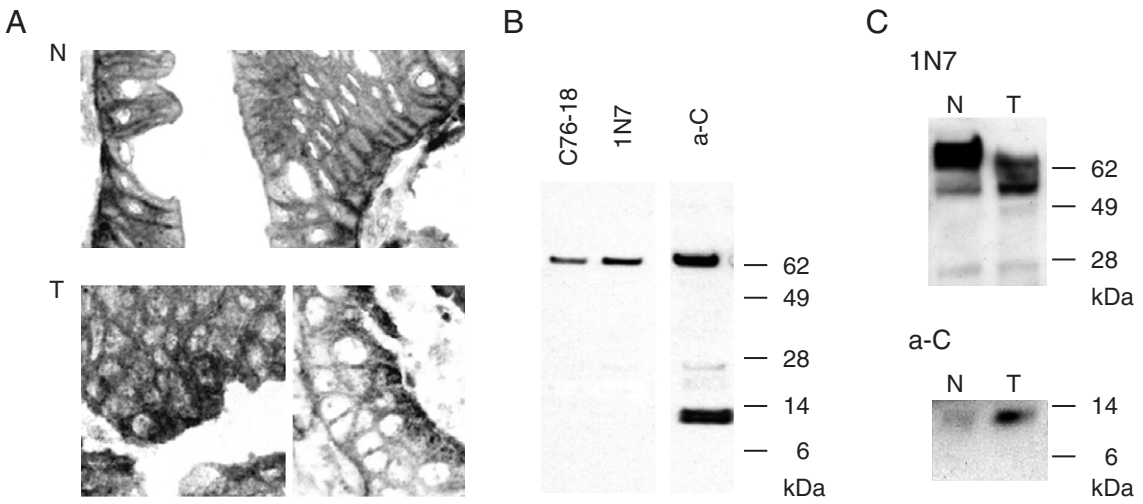

Fig. 4. (A) Immunohistochemical staining of HAI-1 by a-C antibody at ×400 original magnification. A cellular surface staining pattern was observed in normal epithelium (N), whereas the cancer cells showed a predominantly intracytoplasmic immunostaining pattern (T). (B) Immunoblot analysis of cellular extract of cultured colon cancer cells. Subconfluent WiDr cells were washed with PBS and extracted for the immunoblot analyses. C76-18 and 1N7 recognized 66-kDa membrane-form HAI-1, whereas a-C recognized both membrane-form HAI-1 and carboxyl-terminal fragment of HAI-1. (C) Immunoblot analysis of tissue extracts of colon cancer (T) and corresponding normal mucosa (N). With 1N7 antibody, decreased membrane-form HAI-1 and increased secreted-form HAI-1 (58 kDa) were observed in the cancer tissue. With a-C antibody, carboxyl-terminal fragment of HAI-1, generated by the ectodomain shedding of HAI-1, was increased in the cancer tissue.

the reduced immunoreactivity of the membrane-form HAI-1 in cancer cells was frequently very significant (Fig. 2), and it appeared that there might be a discrepancy between the extent of the reduction of immunoreactivity and the reduction of mRNA level observed in the previous RNA blot analysis. To clarify this point, we performed both immunohistochemical staining and *in-situ* hybridization by using serial sections in order to compare the level of HAI-1 mRNA and immunoreactivity in the cancer tissue. Rather surprisingly, the level of mRNA was not decreased, though the immunoreactivity of the membrane-form HAI-1 was significantly reduced (Fig. 3, A and B). The immunoreactivity was also significantly reduced as detected with C76-18 monoclonal antibody that recognizes the region around the 1st Kunitz domain (KD1) of HAI-1 (data not shown).

Enhanced ectodomain shedding of HAI-1 in cancer cells. One possible explanation for the discrepancy between immunohistochemical analysis and *in-situ* hybridization analysis might be that the decreased immunoreactivity was caused by enhanced ectodomain shedding of HAI-1, because the antibodies used for the above immunohistochemical study recognize the extracellular domain of this protein. Therefore, we had performed an immunohistochemical study by using the antibody (a-C) that recognized the intracytoplasmic domain of HAI-1. As expected, the staining pattern of HAI-1 by using a-C was similar to that observed in the *in-situ* hybridization study, showing distinct immunoreactivity in both cancer cells and adjacent non-neoplastic epithelium (Fig. 3C). The specificity of immunoreactivity was further verified by an absorption test (Fig. 3D). Of particular interest was the observation that, at higher magnification, the immunolocalization appeared to be different between the cancer cells and normal epithelial cells. In the normal cells, the lateral (or basolateral) surface of the cells was predominantly stained with a-C, showing a similar immunostaining pattern to 1N7 or C76-18 antibody (Fig. 4A). In contrast, cancer cells showed predominantly intracytoplasmic staining pattern with a-C (Fig. 4A). Taking the above results together with the fact that the same cancer cells were poorly immunostained with 1N7 and C76-18 that recognized the extracellular domain of HAI-1 protein (Fig. 1), it is reasonable to postulate that the shedding of the extracellular portion of HAI-1 is significantly up-regulated in cancer cells, resulting in a significantly reduced signal when stained with 1N7 and C76-18 antibodies. Indeed, immunoblot analyses revealed that C76-18 and 1N7 recognized only the membrane-form HAI-1 (Fig. 4B) and shedding of the extracellular domain occurred in cancer cells, resulting in the enhanced signal of carboxyl-terminal fragment of HAI-1 recognized by a-C antibody (Fig. 4, B and C). After the ectodomain shedding, endocytosis of the remaining carboxyl-terminal part of HAI-1 may occur, since the immunostaining pattern of a-C was largely intracytoplasmic in the cancer cells.

Preserved expression of membrane-form HAI-1 protein in cancer cells at the invasion front. In contrast to the main part of the tumor tissue, we observed a paradoxical up-regulation of membrane-form HAI-1 immunoreactivity in cancer cells at the invasion front showing extensive cell-stroma interactions (Fig. 5). It should be noted that, in addition to the cancer cells, the stromal (myo)fibroblasts in the cancer tissue were frequently positive for HAI-1, whereas fibroblasts in the normal tissue are largely negative. Mesothelial cells and endothelial cells of the lymphatics were also positively stained (data not shown). Decreased immunoreactivity of membrane-form HAI-1 was observed in 67% (59 cases) of colorectal adenocarcinoma cases (88 cases) examined. Among the 59 cases, 53% (31 cases) showed paradoxically up-regulated immunoreactivity of membrane-form HAI-1 in the cancer cells at invasion front (Table 1). In addition, among the 59 cases, 12 cases were randomly selected and immunostained with a-C antibody. In all cases, both cancer cells and adjacent non-neoplastic epithelial cells were stained positively (100%). At the invasion front, 8 cases showed a cellular surface staining pattern even with a-C antibody, and these cases also showed up-regulated 1N7 immunoreactivity at the invasion front.

Preliminary analysis of clinicopathological significance of the paradoxical up-regulation of membrane-form HAI-1 expression was performed. The cases with paradoxical HAI-1 up-regulation tended to show a higher incidence of distant metastasis (8/31: 25.8%) than those without HAI-1 up-regulation (4/26: 15.4%). However, the difference was not statistically significant. No apparent correlation was observed in the other clinicopathological parameters.

We then examined the immunostaining pattern of membrane-

Table 1. Immunoreactivity of membrane-form HAI-1 (antibody 1N7) in colorectal carcinomas (n=88)

	Well[1)]	Mod.[1)]	Poor[1)]	Muc.[1)]	Total
Preserved expression[2)]	6	16	6	1	29
Decreased expression					
in whole area	7	16	4	1	28
with upregulation at invasion front[3)]	14	14	1	2	31

Normal epithelium adjacent to the cancer tissue was consistently positive, and was used as an internal positive control.
1) Histology of the tumor. Well, well differentiated adenocarcinoma; Mod., moderately differentiated; Poor, poorly differentiated; Muc., mucinous.
2) >25% of cancer cells are positive for membrane-form HAI-1.
3) >50% of cancer cells are strongly positive for membrane-form HAI-1 at the invasion front.

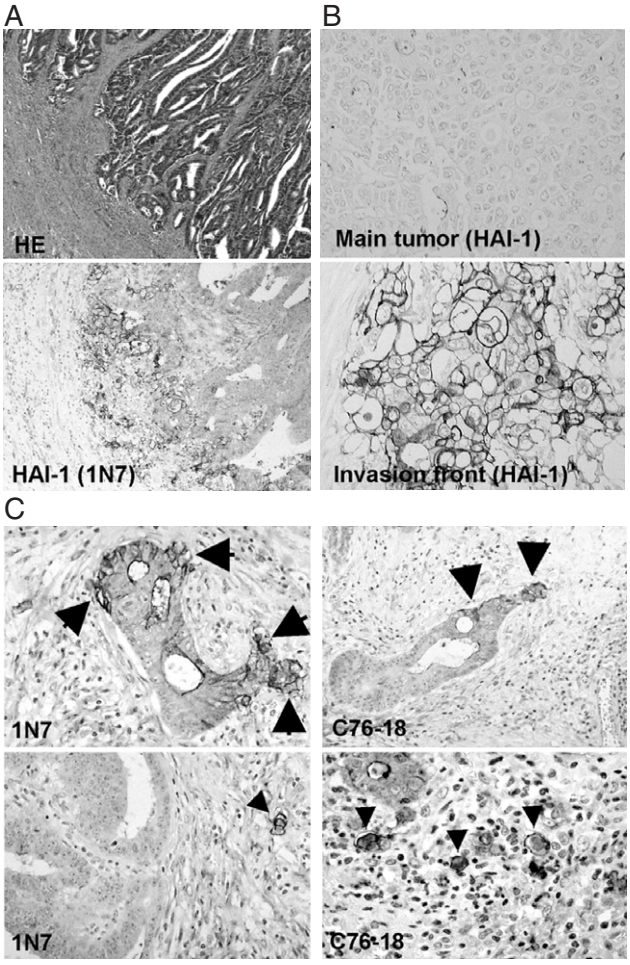

Fig. 5. Paradoxically up-regulated immunoreactivity of membrane-form HAI-1 in cancer cells at the invasion front. (A) Low magnification (×40 original magnification) of well differentiated colon adenocarcinoma tissue. The membrane-form HAI-1 immunoreactivity is predominantly observed at the invasion front. (B) Immnohistochemistry of membrane-form HAI-1 (1N7) in moderately differentiated adenocarcinoma tissue at ×200 original magnification. Intense immunoreactivity (1N7 and C76-18) of membrane-form HAI-1 is observed only at the invasion front. (C) Enhanced immunoreactivity of membrane-form HAI-1 in colon cancer cells showing sprouting invasion (arrows) and also in the isolated cells showing stromal invasion (arrowhead), at ×200 original magnification.

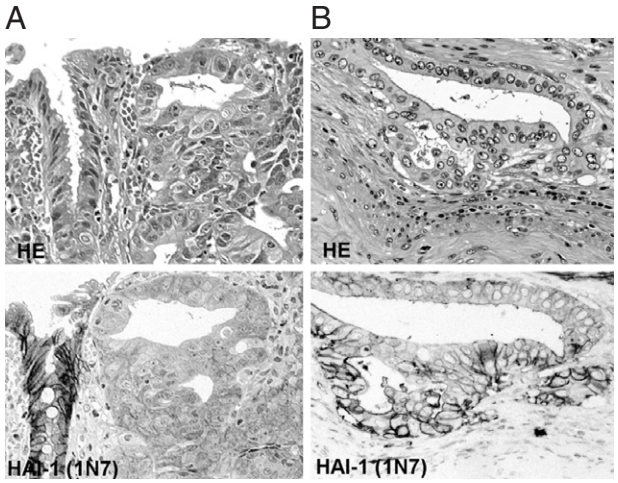

Fig. 6. (A) Decreased immunoreactivity of membrane-form HAI-1 in early gastric cancer cells at ×200 original magnification. (B) Enhanced expression of membrane-form HAI-1 in the invasive or pro-invasive pancreatic ductal carcinoma cells at ×200 original magnification.

form HAI-1 in adenocarcinomas of other organs, such as gastric adenocarcinoma and pancreatic ductal adenocarcinoma. As seen with colorectal adenocarcinoma, the immunoreactivity (1N7 antibody) of membrane-form HAI-1 was decreased in the gastric cancer cells compared with adjacent normal or hyperplastic epithelium (Fig. 6A). Among 11 cases of early gastric carcinomas immunostained, all showed decreased immunoreactivity of membrane-form HAI-1 relative to the adjacent normal epithelium, and 6 cases (55%) showed the paradoxical up-regulation of membrane-form HAI-1 at the invasion front. Six cases of invasive pancreatic adenocarcinoma were also immunostained with 1N7 antibody. In 3 cases, invading cancer cells tended to show more intense immunoreactivity of the membrane-form HAI-1 (Fig. 6B).

Reciprocal immunostain pattern of membrane-form HAI-1 and E-cadherin in colon cancer cells at the invasion front. Next, we had performed immunostaining for E-cadherin. Interestingly, in E-cadherin-positive carcinomas, there was a fairly consistent reciprocal correlation of immunohistochemical staining pattern between membranous E-cadherin and membrane-form HAI-1. The cancer cells at the invasion front with up-regulated membrane-form HAI-1 showed significantly decreased membrane-associated E-cadherin. As shown in Fig. 7, the sprouting cancer cells often showed decreased membrane staining of E-cadherin, whereas the same cells showed up-regulated immunoreactivity of membrane-form HAI-1. These cells are also showed decreased MIB-1 labeling index (Fig. 8), indicating a low proliferation activity.

Discussion

In this study, we found that the immunoreactivity of mature membrane-form HAI-1 is significantly down-regulated in colorectal carcinoma cells relative to the adjacent normal epithelium, and this down-regulation appears to be a result of

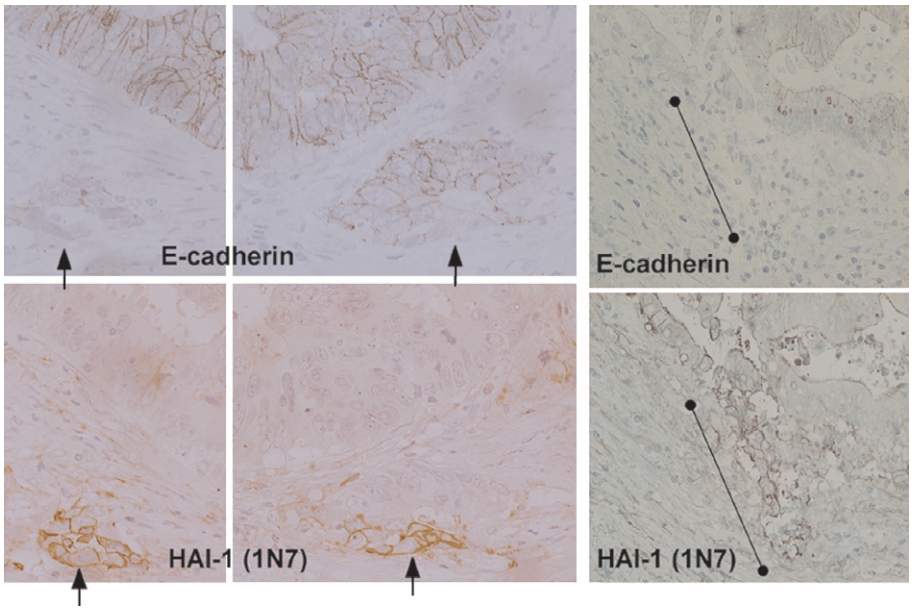

Fig. 7. Reciprocal expression pattern of membrane-form HAI-1 and E-cadherin at ×200 original magnification. Cancer cells with enhanced HAI-1 immunoreactivity (arrows and bars) show decreased membrane-associated E-cadherin.

enhanced ectodomain shedding, rather than decreased mRNA level. At present, two molecular forms of secreted-form HAI-1 (40-kDa sHAI-1 and 58-kDa sHAI-1) have been reported, both of which are generated by proteolytic ectodomain cleavage of the membrane-form HAI-1. The 40-kDa sHAI-1 consists only of KD1 whereas 58-kDa sHAI-1 contains both KD1 and KD2. Although KD1 is responsible for the inhibition of HGFA, it is known that the affinity of 58-kDa sHAI-1 to active HGFA is very low compared with that of the 40-kDa sHAI-1.[14, 18, 19] In this study, since the immunoreactivity of the membrane-form HAI-1 was significantly decreased in cancer cells stained with 1N7 that recognizes KD2, the shedding may occur via a proteolytic cleavage at the juxtamembrane region of the extracellular domain of membrane-form HAI-1, generating the low-affinity 58-kDa sHAI-1 (Fig. 1). Thus it can be hypothesized that, in cancer tissue, HGFA activity is enhanced in the pericellular microenvironment of the cancer cells. In fact, increased amounts of active HGFA and enhanced processing of HGF/SF have been reported in cancer tissues.[5, 20]

In contrast to the above observations, the membrane-form HAI-1 was retained on the cellular surface of cancer cells that showed sprouting invasion or intense cell-host interactions in the invasion field. This finding may suggest that the membrane-form HAI-1 modulates tumor-host interaction in favor of the cancer cells in the invasion field. The role of HAI-1 in these invasive cells is poorly understood. A previous report indicated that the membrane-form HAI-1 could be a multifunctional molecule for the activity of its target proteinases, HGFA and matriptase.[2] As regards HGFA activity, the membrane-form HAI-1 has diverse roles, acting as a specific inhibitor of mature HGFA at the cell surface and also as a reservoir of mature HGFA. The interaction between HAI-1 and HGFA is reversible, and after regulated ectodomain shedding of the HAI-1-HGFA complex by a certain metalloproteinase, the bound HGFA could be released from the secreted low-affinity 58-kDa form of sHAI-1, resulting in a concentrated pericellular activity of HGFA.[14] Such regulated shedding occurs in response to protein kinase C activation or by inflammatory cytokines,[14] both of which would be expected to be present in the invasion field of cancer tissue eventually. The membrane-form HAI-1 also plays a complex role in the activity of matriptase. Recent study by

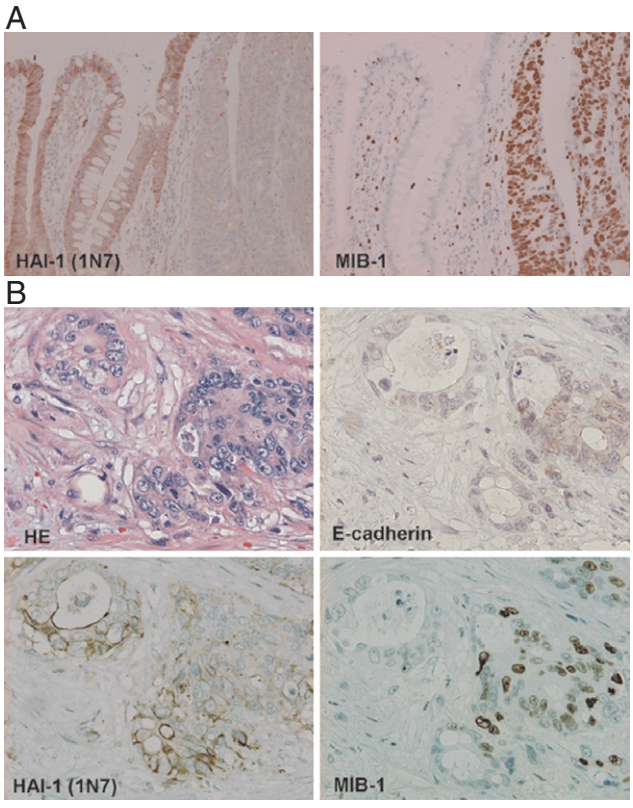

Fig. 8. Reciprocal immunostain pattern of membrane-form HAI-1 and MIB-1 at ×100 original magnification (A) and at ×200 original magnification (B). In the invasion field, the cells with enhanced immunoreactivity for membrane-form HAI-1 show decreased MIB-1 labeling (B).

Oberst *et al.* has indicated that the LDL receptor class A domain of HAI-1 is required in the activation of pro-matriptase on the cellular surface, though HAI-1 potently inhibits matriptase activity.[15] In that study, the LDL receptor class A domain re-

cruited the inhibitor to a matriptase-HAI-1 activation complex, in which HAI-1 may help to activate matriptase prior to acting as an inhibitor.[15] Taking these findings together, it appears that a certain level of HAI-1 expression on the cellular surface is critically required for the optimal activities of HGFA and matriptase. Thus, the existence of membrane-form HAI-1 in the invading cells does not necessarily indicate negative regulation of HGFA and matriptase activities. An alternative and attractive possibility is that HAI-1 may have another undefined function in the survival of cancer cells in the invasion field, which is independent of HGFA and matriptase, directly or indirectly acting for cellular survival. Indeed, membrane-form HAI-1 was also up-regulated in injured and regenerating epithelial tissues[16, 21] and in reactive epithelial cells adjacent to the cancer tissue, as observed in this study. In this regard, the possibly protective effects of this inhibitor on cellular apoptosis and excessive proteinase activities in inflammation as well as on the interactions between the tumor cells and immunological effector cells should be examined.

Another interesting observation in this study was that the membrane-associated E-cadherin and MIB-1 labeling index were significantly reduced in the cancer cells with retained membrane-form HAI-1 at the invasion front. Consequently, these HAI-1-positive cells might show invasive phenotype with low proliferative activity. Indeed, recent studies clearly indicated that invasion is not necessarily analogous with proliferation and the invasion front of human colorectal adenocarcinomas is a region of low proliferation.[22, 23] At present, it remains to be determined whether the inverse correlation between membrane-form HAI-1 immunoreactivity and membrane-associated E-cadherin is simply an epiphenomenon or is a result of complex molecular interactions including HGF/SF-Met signaling. In accordance with a previous report,[23] these invading colon carcinoma cells showed nuclear localization of β-catenin (data not shown). Since HGF/SF promotes tyrosine phosphorylation of β-catenin and impairs association of β-catenin with E-cadherin in human colon carcinoma cells,[24] it can be hypothesized that active HGF/SF signaling is established in these invading cells with intense cell-stroma interactions, and consequently, the existence of membrane-form HAI-1 in the invading cells does not necessarily indicate impaired HGF/SF activation, as discussed above. Also it remains undetermined whether the existence of membrane-form HAI-1 is somehow causative of low proliferating activity of the cells.

In summary, HAI-1 might have a potential to participate in a range of events at the plasma membrane, as HAI-1 can be complexed with not only HGFA, but also other important serine proteinases, such as matriptase, plasmin and trypsin. The biological effects of HAI-1 may be highly situational depending on the target proteinases and the presence of other constituents in the intra- or extracellular milieu. There is clearly a need for further studies on the pathophysiological functions and mechanisms of ectodomain shedding of HAI-1 in order to obtain a better picture of the dynamics occurring on the cancer cell surface. The finding that membrane-form HAI-1 is frequently upregulated in cancer cells showing intense cell-host interaction suggests that this molecule may be a useful molecular marker to detect actively invading cancer cells. Therefore, further detailed clinicopathological analysis using larger series of cancer cases seems worthwhile. In addition, this study indicates that careful interpretation is required in the immunohistochemical study of HAI-1 expression of tumors, as enhanced ectodomain shedding of this protein appears to occur in the tumor cells.

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