Activation of Hepatocyte Growth Factor/Scatter Factor in Colorectal Carcinoma

Hiroaki Kataoka,1 Ryuichi Hamasuna, Hiroshi Itoh, NaomI Kitamura, and Masashi Koono

The Second Department of Pathology, Miyazaki Medical College, Miyazaki 889-1692 [H. K., R. H., H. I., M. K.], and Department of Biological Sciences, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, Yokohama 226-8026 [N. K.], Japan

ABSTRACT

Activation of hepatocyte growth factor/scatter factor (HGF/SF) in the extracellular milieu is a critical limiting step in the HGF/SF-induced signaling pathway mediated by Met receptor tyrosine kinase, which has potentially important roles in tumor biology and progression. However, little is known concerning the regulation of HGF/SF activation in tumors. Immunohistological analysis revealed that the activation of HGF/SF was enhanced significantly in colorectal carcinoma tissues compared with the corresponding normal mucosa. Serum-free conditioned media of cultured human colorectal carcinoma cell lines contained HGF/SF-activating activity, and the addition of a single-chain precursor form of HGF/SF to the serum-free culture of these cells resulted in HGF/SF-dependent modulation of cellular phenotypes, such as increased scattering and enhanced secretion of vascular endothelial growth factor. This processing activity was enhanced by thrombin treatment but was inhibited significantly by a neutralizing antibody against HGF activator (HGFA), a factor XIIa-like serine proteinase believed to be expressed mainly in the liver. The activity was also inhibited by recombinant HGFA inhibitor type 1 (HAI-1). The presence of HGFA mRNA and secretion of HGFA protein were confirmed in the cell lines. Therefore, extrahepatic expression of HGFA in the colorectal carcinoma cells could be responsible for the single-chain HGF/SF-processing activity of the cells. We examined the expression of HGFA and HAI-1 in human colorectal mucosa and adenoma-carcinoma sequence. Immunohistochemically, HGFA was stained weakly in the normal enterocytes, and immunoreactivity was increased modestly in the neoplastic differentiation. The subcellular localization of HGFA immunoreactivity was altered in carcinoma cells showing basal or cell-stroma interface staining patterns, compared with normal and adenoma cells with a supranuclear or apical staining pattern. In contrast to HGFA, the expression of HAI-1 decreased significantly in carcinoma cells relative to the adjacent normal or adenoma cells, indicating that the net balance between HGFA and HAI-1 shifts in favor of HGFA in carcinomas. In fact, pro-HGFA and the active form of HGFA proteins increased in carcinoma tissue compared with the corresponding normal mucosa. It was concluded that HGFA is expressed in colorectal mucosa and tumors and could be involved in the activation of HGF/SF in colorectal carcinomas. Therefore, the balance between HGFA and HAI-1 could play an important role in the regulation of HGF/SF activity in colorectal carcinomas.

INTRODUCTION

HGF3 is a pleiotropic factor initially identified as a growth factor for hepatocytes (1–3), and it is indistinguishable from SF, a motility factor (4–6). It has mitogenic, motogenic, and morphogenetic functions in various types of cells through its high-affinity receptor tyrosine kinase, Met, that is encoded by the c-met proto-oncogene (7, 8). A number of recent studies have proved that HGF/SF and Met have important roles in tumorogenesis, invasiveness of tumor cells, differentiation, and tumor angiogenesis (9–14). HGF/SF is a mesenchymally derived heparin-binding glycoprotein secreted as an inactive precursor. Normally, it remains in this precursor form, which probably is associated with the extracellular matrix in producing tissues (15). To exhibit its biological function, the extracellular proteolytic conversion of scHGF to the two-chain heterodimeric active form is essential (15, 16). Therefore, this activation process is a critical event in regulating the HGF/SF activity in vivo. Although attention has been focused on the roles of HGF/SF on tumor progression, the molecular mechanism underlying HGF/SF activation in tumor tissue remains undefined. Plasminogen activators, particularly uPA, have been shown to activate scHGF and have been thought to be major cellular activators of scHGF (17–19). However, the activity of uPA on scHGF activation is very weak and slow in vitro (20, 21), and this activity is controlled by a stoichiometric reaction that is capable of yielding and releasing an active two-chain form of HGF/SF that is only approximately one-half the molecular amount of active uPA, regardless of the amounts of scHGF present (22).

Recently, a novel factor XIIa-like serine proteinase having an efficient scHGF-activating activity was identified and designated as HGFA (23, 24). This enzyme is reported to be secreted by the liver as an inactivezymogen (pro-HGFA), circulating in the blood in this form (21, 24, 25). It is activated by limited proteolysis brought about by thrombin in injured tissue, and it then activates scHGF very efficiently as a typical catalyst (25–27). In fact, the activation of scHGF in an injured rat liver was abrogated by treatment with the anti-HGFA antibody, indicating that HGFA may be involved crucially in the activation of scHGF in vivo (25). It is important to note that the activity of HGFA is not inhibited by major plasma proteinase inhibitors, and that HGFA is in fact active in serum (21). HAI-1 was identified as a potent endogenous inhibitor of HGFA (28). Mature HAI-1 has two well-defined Kunitz-type serine proteinase inhibitor domains. The first domain appears to be responsible for the inhibition of HGFA (28). This inhibitor has a presumed transmembrane domain in the COOH-terminal region, suggesting that HAI-1 is an integral membrane serine proteinase inhibitor. In fact, we reported recently that the HAI-1 protein is present on the cellular basolateral surface and is expressed predominantly in columnar epithelial cells covering the mucosal surface and duct lumens (29). Its expression is up-regulated in response to tissue injury and regeneration (29). We also reported that HAI-1 mRNA was present in gastrointestinal mucosa and carcinoma; the level is lower in carcinoma tissue than in the corresponding normal mucosa (30).

Colorectal cancer is one of the most common types of cancer diagnosed and the second leading cause of cancer death in the United States, and its incidence is increasing in Japan. The c-met proto-oncogene is overexpressed in colorectal carcinomas (31, 32), and HGF/SF stimulates the growth and motility of the colorectal carcinoma cells in vitro (33, 34). Moreover, serum HGF/SF level is correlated with disease progression in patients with colorectal carcinoma, and the amounts of HGF/SF increase in the tumor tissue (35). However, essentially nothing is known concerning the mechanism and regulation of HGF/SF activation in colorectal carcinomas, as well as...
in other tumors; such a study would have potentially great importance. Given the fact that the activation of sHGF by uPA is controlled by a stoichiometric reaction, the coexistence of another mechanism may be suggested in colorectal carcinomas in which secreted and stored extracellular sHGF is processed efficiently enzymatically. In this context, it can be hypothesized that HGFA and HAI-1 may have roles in the regulation of HGF/SF activation in colorectal tumors. However, the extrapeptic expression of HGFA in colorectal tumors has not yet been determined, and little is known about HAI-1 expression during the course of colorectal tumor progression. Moreover, it has not yet been established whether activation of HGF/SF is in fact enhanced in the colorectal cancer tissue in vivo compared with normal mucosa. The principal purposes of the present study were to examine an existence of sHGF-processing activity in colorectal carcinoma cells and to test the potential role of HGFA in the sHGF activation of the cells. Furthermore, we evaluated the comparative expression pattern of HGFA and HAI-1 in the human colorectal adenoma-carcinoma sequence.

MATERIALS AND METHODS

Preparation of Antibodies and Cell Culture. To obtain antihuman HGFA antibody, a BALB/c mouse was immunized with purified HGFA protein as described (21). Monoclonal antibodies were generated according to established procedures, and the resulting hybridoma supernatants were screened by enzyme immunoassay. Briefly, the mouse spleen cells were removed and fused with P3U1 myeloma. Hybridomas were screened for secretion of antibodies against HGFA. Among the antibodies obtained, anti-HGFA antibody A-1 was suitable for immunohistochemistry. Another anti-HGFA monoclonal antibody, P1-4, was used for the neutralizing assay described below (21). Preparation and specificity of antihuman HAI-1 antibody 1N7 has been described previously (29). The epitope of this antibody was around the second Kunzit domain of HAI-1 protein. Antihuman HGFA/SM monoclonal antibody (1H56), which recognizes the heavy chain of HGF/SF, was kindly provided by Dr. H. Tsubouchi (Second Department of Internal Medicine, Miyazaki Medical College, Miyazaki, Japan). Antihuman uPA neutralizing antibody (PGM2001) was obtained from MONOSAN (Uden, the Netherlands).

Human colorectal carcinoma cell lines used were SW837, DLD-1, RCM-1, LoVo, and Colo 205. SW837 and Colo 205 were obtained from Dainihon Seiyaku (Osaka, Japan), DLD-1 and LoVo were obtained from RIKEN cell bank (Tsukuba, Japan), and RCM-1 was established in our laboratory.

Immunohistochemistry. The method used for immunohistochemistry was described previously (29). Formalin-fixed, paraffin-embedded tissue specimens were prepared according to the routine procedure. Sections were dewaxed in xylene and rehydrated in decreasing ethanol solutions and water. After antigen retrieval [5 min of autoclave in 10 mM citrate buffer (pH 6.0)], the sections were treated with 3% H.O, in PBS for 10 min, washed in PBS twice, and then blocked in 3% BSA in PBS for 1 h at room temperature. Then the sections were incubated with the primary antibody (10 μg/ml) in PBS containing 1% BSA for 16 h at 4°C. Negative controls consisted of omission of the primary antibody. For adsorption test, each antibody was pretreated with a 10-fold excess of recombinant HGFA or HAI-1. The sections were then blocked in 3% BSA in PBS for 1 h at room temperature, and twice in the same solution for 20 min at 65°C. The membranes were autoradiographed with Kodak XR-5 film at −80°C for 6 or 18 h. The Naed-Xhol fragment (1221 bp) from human HAI-1 cDNA was used as a probe. For internal control of loading, the blots were hybridized subsequently to the G3PDH probe (Clontech, Palo Alto, CA). The probes were radiolabeled by random priming with [32P]CTP. For quantification of the RNA blot analysis, the radioactivity of mRNA signals for HAI-1 was measured directly by a bioimaging analyzer (FUJIX BAS2000 system; Fuji Photo Film, Tokyo, Japan), and normalized by division through those of the corresponding G3PDH mRNA signals. To assess the level of indicated mRNA in tumor, a ratio of the T to that of the corresponding N was calculated, and expressed as the T/N ratio.

Immunoblot Analysis. To prepare a tissue extract specimen, fresh human colon carcinoma tissue and corresponding normal mucosa tissue (160 mg of each), which were obtained from surgically resected colon of a colon cancer patient, and normal liver tissue obtained at autopsy were frozen immediately in liquid nitrogen, crushed, and homogenized in an extraction buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, protease inhibitor mixture (Complete; Boehringer Mannheim, Germany), and 0.01% CHAPS, without (for HGF/SF) or with (for HGFA and HAI-1) 0.2% Triton X-100, followed by centrifugation (25,000 × g for 15 min). For the detection of HGF/SF, the resultant supernatants were mixed with heparin-Sepharose beads (Pharmacia, Uppsala, Sweden) in the presence of 100 μM nafamostat mesilate (Banyu Seiyaku, Tokyo, Japan). After the Sepharose beads had been washed three times with PBS, bound HGF/SF was eluted with SDS-PAGE sample buffer and boiled for 3 min. Protein extraction from the cultured cells was done according to the method described previously (29). Each sample was separated by SDS-PAGE (4–12% gradient gel); Novex, San Diego, CA) under reducing conditions and processed for immunoblot analysis as described previously (29). Primary antibodies used were anti-HGF/SF (1H56) monoclonal antibody (0.5 μg/ml), anti-HGFA (A1) monoclonal antibody (1 μg/ml), anti-HAI-1 (1N7) monoclonal antibody (1 μg/ml), and anti-Met (C-28) rabbit IgG (0.5 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA). After incubation with peroxidase-conjugated secondary antibodies, the labeled proteins were visualized with a chemiluminescence reagent (New England Nu-
clear-Life Science, Boston, MA). For positive controls of HGFA and HAI-1, SFCM of CHO cells transfected with HGFA expression vector and cell extract of CHO transfected with HAI-1 expression vector, respectively, were used. The vectors were constructed by using pCIneo plasmid (Promega Corp., Madison, WI) and the whole coding region of HGFA and HAI-1 cDNA (24, 28). To detect phosphorylated Met, peroxidase-conjugated antiphosphotyrosine monoclonal antibody (PY-20) was used according to the instruction of the manufacturer (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom).

**Assay for HGF/SF Activity**. Five µl of recombinant scHGF (1.6 µg/µl) were mixed with 20 µl of concentrated SFCM of cultured colon carcinoma cells in PBS containing 0.05% CHAPS and incubated at 37°C for 12 h. To avoid the contamination of fetal bovine serum-derived HGFA, colon carcinoma cells were maintained in a mixture of RPMI 1640:Ham’s F-12 medium (1:1) supplemented with HGFA-depleted fetal bovine serum (5%) before starting the serum-free culture. The HGFA-depleted serum was prepared by using anti-HGFA immunoadsorbent columns. Subconfluent cells were then washed three times with SFCM and cultured in SFCM for 24 h. SFCM was collected and concentrated by ultrafiltration (5- or 10-fold). In the concentrated sample, 0.05% CHAPS was added and used for the assay. In an indicated assay, SFCM was pretreated with 1 unit of thrombin (Sigma Chemical Co., St. Louis, MO) for 1 h at 37°C. For positive control of pro-HGF/SF activation, 5 µl of recombinant M, 34,000 HGFA (0.3 ng/µl) were mixed with scHGF and incubated. For neutralizing study, the sample was preincubated at 37°C for 1 h with 1 µg of antibody, 10 ng of nafamostat mesilate, or 400 ng of recombinant HAI-1 (M, 40,000 secreted form). Total reaction volume was 45 µl for each assay. After the incubation of scHGF with each sample, HGF/SF was bound to heparin-Sepharose beads. After the Sepharose beads had been washed three times with PBS, HGF/SF was eluted with SDS-PAGE sample buffer, boiled for 3 min, and then separated by SDS-PAGE (4–12% gel) under reducing conditions. Proteins in the gel were stained with Coomassie Brilliant Blue in water:methanol:acetic acid solution (6:3:1, v/v). Recombinant scHGF and M, 34,000 two-chain form of HGFA were prepared as described (23, 24). uPA was kindly provided by Dr. Etsuo Yoshida (Second Department of Physiology, Miyazaki Medical College, Miyazaki, Japan). To study cellular activation of scHGF and subsequent cellular response, SW837 and DLD-1 cells were cultured in serum-free condition as described above and treated with 50 ng/ml scHGF with or without thrombin (4 units/ml), anti-HGFA antibody (20 µg/ml), control mouse IgG (PI-1, 20 µg/ml), or nafamostat mesilate (250 ng/ml). Assay for scHGF-processing activity in tissue extracts was done according to the method described previously (25).

**Measurement of VEGF**. Amounts of VEGF protein in the cultured conditioned media were measured by using ELISA with a human VEDF EIA kit (Immunobiological Laboratories Co., Gunma, Japan).

**Data Analysis**. Statistical parameters were ascertained using the Statview 4.0 program (Brainpower, Inc., Calabasas, CA). Kruskal-Wallis nonparametric one-way ANOVA tests or Fisher’s protected the least significant difference tests were used to compare between groups; the difference between means was tested with the Mann-Whitney U test or nonparametric paired Wilcoxon test. Significance was set at P < 0.05.

**RESULTS**

**Enhanced Production and Activation of HGF/SF in Colorectal Carcinoma Tissues**. Expression and molecular forms of HGF/SF protein were analyzed using extracts of colon carcinoma and the corresponding normal mucosa tissues. Six cases of primary colon carcinoma and a metastatic brain lesion were analyzed. As shown in Fig. 1, HGF/SF present in normal mucosa tissue distant from the tumor was primarily scHGF. In contrast, a significant proportion of scHGF had been converted to the two-chain active form in the corresponding carcinoma tissue. In accordance with the previous reports (12, 35), total amounts of HGF/SF protein were increased apparently in the carcinoma tissues. These findings indicate that the activation process of HGF/SF and synthesis of HGF/SF are up-regulated significantly in colorectal carcinomas.

**Presence of scHGF-processing Activity in SFCM of Colon Carcinoma Cell Lines and Its Identification as HGFA**. We examined whether cultured human colorectal carcinoma cells produce scHGF-processing activity. Concentrated SFCM of SW837 cells contained scHGF-processing activity, and the pattern of processing was the same as that for recombinant HGFA (Fig. 2A). Interestingly, this processing activity was enhanced significantly when SFCM was pretreated with thrombin (Fig. 2, A and B). However, the same concentration of thrombin itself did not activate scHGF (Fig. 2, A and B). Because pro-HGFA is activated specifically by thrombin (26), these observations suggested that the processing activity in SFCM may represent HGFA produced by the carcinoma cells. Indeed, the activity was inhibited significantly by recombinant HAI-1, a specific endogenous inhibitor for HGFA (Fig. 2B). A subsequent neutralizing assay using specific antibody for HGFA confirmed that HGFA is, in fact, responsible for the scHGF-processing activity present in SFCM (Fig. 2C). On the other hand, the activity was not inhibited by anti-uPA neutralizing antibody. As reported previously (22–24), in contrast to uPA, the reaction of recombinant HGFA-induced scHGF/SF activation was typical catalytic reaction in which virtually all of the substrate was processed (Fig. 2, A and C). Then the presence of HGFA mRNA and proteins was confirmed by RT-PCR study and immunoblot analysis, respectively, in SW837 cells (Fig. 2D). SFCM of SW837 cells, indeed, contained M, 96,000 pro-HGFA and a very low level of M, 32,000 active form under the reducing condition. This
HGFA mRNA with sensitive RT-PCR study (Fig. 2). GFA lines examined (DLD-1, RCM-1, LoVo, and Colo205) also expressed specific phenomenon, because four other colorectal carcinoma cell also detected. The expression of HGFA was not seen to HGFA but not to uPA (21, 38; Fig. 3). A synthetic serine proteinase inhibitor having potent inhibitory activity to scHGF was suppressed by anti-HGFA neutralizing antibody or by nafamostat mesilate, enhanced markedly by thrombin treatment and was inhibited significantly by expression vector, and RT-PCR analysis for HGFA mRNA in five human colorectal carcinoma cell lines were shown. For the immunoblot, 0.8 ml (SW837) and 0.4 ml (CHO) of SFCM were concentrated and analyzed. + and ++, positions of M, 96,000 proform and M, 32,000 active form of HGFA proteins, respectively. In the RT-PCR study, 30 cycles of amplification for HGFA and 24 cycles for G3PDH were applied.

Cellular HGFA Is Involved Crucially in Cellular Scattering and VEGF Secretion Induced by scHGF in Vitro. We next examined whether scHGF can, in fact, induce the alteration of the phenotypes of colon carcinoma cells via activation by HGFA in situ. In this study, we checked the effects of scHGF on cellular scattering and secretion of VEGF of DLD-1 and SW837 cells. Both cell lines expressed Met protein, the specific receptor for HGF/SF (Fig. 3C). The two-chain active form of HGF/SF induced enhanced secretion of DLD-1 cells. SW837 cells did not show apparent scattering response and migrated as coherent cell sheets. The scattering response could be reproduced by scHGF treatment in DLD-1 accompanying tyrosine phosphorylation of Met, particularly in the presence of thrombin, and the induction of scattering by scHGF was suppressed by anti-HGFA antibody (Fig. 3D) or by nafamostat mesilate (not shown). On the other hand, the two-chain active form of HGF/SF induced enhanced VEGF secretion in both DLD-1 and SW837 cell lines (Fig. 3E). This VEGF-inducing effect of HGF/SF was also observed in other colorectal carcinoma cell lines showing 1.6–2.5-fold increase in VEGF secretion after HGF/SF (20 ng/ml) treatment. As shown in the Fig. 3E, scHGF also showed the VEGF-inducing effects, although the extent of stimulation was lower than that of two-chain active form. However, the addition of thrombin into the culture significantly enhanced the scHGF-induced VEGF up-regulation. The effects of scHGF was inhibited significantly, although not completely, by the presence of anti-HGFA antibody (up to 72 and 67% inhibition in DLD-1 and SW837, respectively) or nafamostat mesilate (80% inhibition). The failure of complete inhibition of scHGF-induced VEGF up-regulation in this study may be because of the minor contamination, which represents ~5% of total protein, of the two-chain active form in the scHGF sample as observed in SDS-PAGE analysis. In addition, thrombin itself also showed weak stimulatory effect. These results indicated that HGFA is a major activator of scHGF of these colon carcinoma cells in vitro, and they support the hypothesis that cellular HGFA is involved in the processing of scHGF in the extracellular milieu of colorectal carcinomas.

Comparative Analysis of Expression of mRNAs for HGFA, HAI-1, and c-met in Colorectal Carcinoma and Corresponding Normal Colon Mucosa Tissues. To examine the in vivo relevance of the above observations in vitro, expression of HGFA and HAI-1 was analyzed in vivo in colorectal mucosa. Total RNAs extracted from colon carcinoma and corresponding normal colon mucosa tissue were used to determine the expression of specific mRNAs for HGFA and HAI-1, as well as for c-met. Fig. 4 shows a representative result of analyses. There were low but distinct levels of mRNA for HGFA in the colorectal mucosa. The expression was conserved or was enhanced modestly in the neoplastic colorectal mucosa, although the difference was not statistically significant (mean T:N ± SE, 1.6 ± 0.5). In accordance to a number of previous studies (12, 31, 32), there existed a tendency for the expression of c-met mRNAs to be more enhanced in carcinoma tissues than in the corresponding normal counterparts (mean T:N ± SE, 2.3 ± 0.5; P < 0.05). Next, expression of HAI-1, a potent inhibitor of HGFA, was analyzed by RNA blot analysis. The level of HAI-1 mRNA was lower in carcinoma tissue than in its corresponding normal counterpart (mean T:N ± SE, 0.58 ± 0.1; P < 0.01).
Fig. 3. Involvement of HGFA in scHGF-induced cellular responses. A, presence of HGFA activity in SFCM of DLD-1 cells. B, presence of pro-HGFA protein in SFCM of DLD-1 cells and its activation upon thrombin treatment. C, expression of functional M, 145,000 (β2+) form of Met in DLD-1 and SW837 cells. D, effect of scHGF/SF on cellular scattering of DLD-1. Cultured DLD-1 cells were treated without (a) or with (b and c) 20 ng/ml scHGF/SF in the presence of 4 units/ml of thrombin and cultured for 24 h. In b and c, 20 μg/ml of mouse nonspecific IgG and anti-HGFA IgG, respectively, were also added in the medium. Tyrosine phosphorylation of Met protein 3 h after the treatment in which Lanes 1, 2, and 3 correspond to a, b, and c, respectively. E, effects of scHGF/SF on VEGF production of DLD-1 and SW837 cells. The cells were treated with or without scHGF (50 ng/ml), two-chain HGF/SF (20 ng/ml), thrombin (4 units/ml), anti-HGFA antibody (20 μg/ml), nonspecific mouse IgG (20 μg/ml), or nafamostat mesilate (250 ng/ml), as indicated in the figure. *, P < 0.001; one-way ANOVA, Fisher’s protected the least significant difference test. Bars, SE.

Fig. 4. Comparative results of mRNAs expression of HGFA, c-met, and HAI-1 in colorectal carcinomas and corresponding normal mucosa. HGFA and c-met were analyzed by RT-PCR study (28 cycles of amplification, as mentioned in “Materials and Methods”), and HAI-1 was by RNA blot analysis. The signals obtained were normalized by the corresponding G3PDH signal, and ratio of T.N (T/N ratio) was calculated. RT-PCR for G3PDH was 23 cycles of amplification. Cases 3, 4, and 9 correspond to 1, 2, and 3 in Fig. 1, respectively.

Immunohistochemical Study for HGFA in Normal and Neoplastic Colorectal Mucosa. A previous RNA blot analysis revealed that HGFA mRNA was most abundant in the liver (24). To confirm the specificity of the anti-HGFA monoclonal antibody A-1, normal liver tissue was stained with the antibody. Hepatocytes were stained strongly with the antibody (Fig. 5, A and B). Specificity of the antibody was further verified by immunoblot analysis of the liver tissue extracts (Fig. 5B). Then normal human colon mucosa tissue was stained with the antibody. In normal colon mucosa, HGFA immunoreactivity was observed in the enterocytes (Fig. 5C), although it was much weaker than that in hepatocytes. In addition, macrophages in the stroma tissue and serous fluid in the blood vessels were also stained (data not shown). The latter immunoreactivity may represent zymogen of HGFA produced by the liver and present in the plasma.

In colorectal adenoma and carcinoma tissues, there was consistently positive immunoreactivity to HGFA in the neoplastic epithelial cells, and the immunoreactivity appeared to be enhanced more in neoplastic cells than in the adjacent normal epithelium. Interestingly, although the levels of HGFA expression were relatively consistent in adenomas and carcinomas (Table 1), the subcellular localization of HGFA immunoreactivity was altered significantly in the carcinoma cells. In normal cells, the positive immunoreactivity for HGFA was localized beneath the apical membrane or in supranuclear region. This pattern was preserved relatively well in adenoma cells (Fig. 5, C and D), whereas in carcinoma cells the immunoreactivity frequently localized to the basal membrane or cell-stroma interface (Fig. 5, E–H). This basal pattern of HGFA immunoreactivity was observed predominantly in 52% (29 of 56) of total carcinoma cases stained for HGFA (Table 2). On the other hand, none of hyperplastic polyps or low-grade adenomas showed the predominant basal staining pattern of HGFA, and this basal pattern was correlated with the progression of adenoma-carcinoma sequence (P < 0.01, Kruskal-Wallis test). There was a tendency for carcinoma cases with liver metastases and metastatic liver tumor to exhibit the basal immunostain pattern of HGFA more frequently (Table 2).

HAI-1 Immunoreactivity in the Normal Colorectal Epithelium-Adenoma-Carcinoma Sequence. With evidence that HGFA expression is relatively consistent in the adenoma-carcinoma sequence of the colorectum, we then asked whether HAI-1, an endogenous inhibitor of HGFA, was down-regulated along with the progression of adenoma-carcinoma sequence. In normal colon mucosa, HAI-1 protein was expressed predominantly on the basolateral surface of epithelial cells covering the mucosal surface, whereas deep crypt epithelium was stained only faintly (Fig. 6A, a). This immunoreactivity was much more evident in surface
absorptive cells than in goblet cells (Fig. 6A, b). Detaching or shedding nests of somewhat degenerative epithelial cells were stained more strongly (Fig. 6A, b). The immunoreactivity appeared to increase in hyperplastic polyp (not shown), in which not only the surface epithelium but also deep glandular epithelial cells were stained. In the low-grade adenoma epithelium, HAI-1 immunoreactivity apparently was enhanced compared with the corresponding normal mucosa (Fig. 6A, c). Intracellular immunolocalization of HAI-1 in adenoma cells was similar to that in normal epithelial cells, showing a predominant basolateral cellular surface stain (Fig. 6A, c). In contrast, the immunoreactivity was reduced more significantly in carcinoma cells than in the adjacent normal epithelium (Fig. 6A, d–f). Interestingly, normal epithelial cells adjacent to the cancer cells or left behind in cancer tissue expressed a significantly higher level of cell surface HAI-1 (Fig. 6B).

To address the question of whether relative hyper- or hypopexpression of HAI-1 exists in colorectal adenomas and carcinomas, several cases of carcinoma in adenoma were investigated. We also studied multiple adenomas and carcinomas in a single patient with familial adenomatous polyposis. In these cases, it is possible to compare the immunoreactivity of HAI-1 in a single specimen in which adenoma and carcinoma are present with the same fixation and staining conditions. Clearly, HAI-1 immunolabeling was reduced in carcinomas compared with adenomas (Fig. 6C). The reduced expression of HAI-1

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* Determined by estimating the percentage of cells that was positive for HGFA: 0, negative; 1+, <20%; 2+, 20–50%; 3+, 50–80%; and 4+, >80%.

* n, number of cases stained.

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Fig. 5. Representative immunohistochemical analysis of HGFA in human liver (A and B), colorectal normal mucosa (C), adenoma (D), carcinomas (E–G), and lymph node metastasis (H). An immunoblot for HGFA in the extract of liver tissue using the same antibody (A1) is also shown in B in which a single major band of M, 96,000 pro-HGFA was observed. Note that HGFA reactivity in carcinoma cells frequently shows basal distribution. Hematoxylin counterstain. Bars, 100 μm.
in carcinomas was further confirmed by immunoblot analysis (Fig. 6D). The band corresponding to the M₆ 66,000 membrane-bound form of HAI-1 (29) clearly was reduced in carcinoma tissue compared with corresponding normal and adenoma tissues. Semiquantitative analysis of HAI-1 immunoreactivity indicated that the immunoreactivity was correlated inversely with the progression of the adenoma-carcinoma sequence (P < 0.001 Kruskal-Wallis test; Table 3). However, an apparent relationship was not observed between HAI-1 immunoreactivity and Dukes’ stage of the cancer.

Presence of Active Form of HGFA in Colon Carcinoma Tissue.

The expressions of HGFA and HAI-1 were compared in serial sections of colon carcinoma adjacent to normal mucosa in which down-regulation of HAI-1 was more evident in carcinoma cells than in neighboring normal epithelia. As expected, the patterns of HGFA and HAI-1 expression were reciprocal (Fig. 7). To confirm that HGFA and its active form were, in fact, present in carcinoma tissue, immunoblot analysis was performed (Fig. 8A). The amounts of pro-HGFA as well as the M₆ 32,000 active form of HGFA were increased in carcinoma tissue and its metastatic lesion compared with the corresponding normal mucosa distant from the carcinoma lesion. The presence of HGFA-dependent scHGF-processing activity was further confirmed in the carcinoma tissue extracts (Fig. 8B). Therefore, the net balance between HGFA and HAI-1 appears to be shifted in favor of HGFA activity in carcinoma tissues.

DISCUSSION

Extracellular activation of a scHGF is a limiting step in the Met signaling pathway believed to be involved in various aspects of tumor biology in vivo, such as growth, differentiation, invasion, and tumor angiogenesis (7–14). However, to date little attention has been focused on the activation process of HGF/SF in tumors. uPA was shown to activate scHGF (17–19, 22), and given the fact that the uPA/uPA receptor system is up-regulated in many tumors (39, 40), it has been suggested that uPA may have an important role in the activation of scHGF in tumors. However, the processing of scHGF by uPA is weak and slow, and it is controlled by a stoichiometric reaction rather than a catalytic reaction (20–22). Thus, the activation of scHGF caused by uPA would be restricted in the limited environments in which a high concentration of active uPA is available, and it remains to be clarified whether this level of activation is enough to affect the behavior of the tumor as well as the neangiogenesis in tumor tissue. On the other hand, HGFA acts as a typical catalyst for scHGF (23, 24) and would, thus, activate scHGF secreted and stored in the extracellular spaces of tumor tissue very efficiently. In this study, we demonstrated for the first time, to our knowledge, that human colon carcinoma cells express functionally active HGFA, and that cellular HGFA could be involved in the activation of scHGF and the subsequent cellular response induced by the activated HGF/SF in colon carcinomas. The observation that a significant proportion of scHGF was processed to the two-chain active form in colorectal carcinoma tissues in vivo may also support the involvement of the enzymatic activation by HGFA in the activation of scHGF that occurred in tumor tissue.

It is worth noting that HGFA is secreted as an inactive zymogen (pro-HGFA) in the liver (24). This is also the case in the colon carcinoma cells as shown in this study. Thrombin generated in an injured tissue has been considered to be an important activator of pro-HGFA (26). Indeed, HGFA activities of colon carcinoma cells, as well as in SFCM, were enhanced significantly by thrombin treatment. Because thrombin did not alter the expression level of HGFA mRNA (data not shown), the effects of thrombin would be mediated by its pro-HGFA-activating activity. Therefore, the present study indicates a crucial role of blood coagulation system in HGF/SF activation in tumors. In cancer tissue, increased procoagulant activity has been reported accompanying pericellular fibrin generation, indicating thrombin generation in tumor tissues (41). We have reported that colorectal carcinoma cells express tissue factor, a major initiator of the coagulation cascade in vivo (42). Moreover, a highly metastatic subline of a rectal carcinoma cell line exhibited higher tissue factor activity than its parent line (42). Thus, it is reasonable to speculate that thrombin is generated frequently in the pericellular microenvironment of colon carcinoma cells via the aberrant activation of a coagulation cascade, and the generated thrombin would activate cellular pro-HGFA, eventually resulting in the generation of active HGF/SF in vivo. In fact, the active M₆ 32,000 form (M₆ 34,000 in nonreducing conditions) of HGFA was detectable in carcinoma tissues. Moreover, the current observation that HGF/SF enhances VEGF production in colon carcinoma cells may provide an amplifying circuit for the activation of scHGF by HGFA, because VEGF also enhances the permeability of blood vessels in tumor tissue (hence its other name, “vascular permeability factor”), resulting in elevated concentrations of prothrombin and pro-HGFA derived from plasma in the interstitial fluid of the tumor (41). The plasma-derived HGFA would also contribute to the activation of scHGF upon activation by thrombin. Indeed, immunoblot analysis showed an increase in the level of pro-active forms of HGFA at the protein level in cancer tissue (Fig. 8A), whereas this increase was not apparent by RT-PCR (Fig. 4), suggesting the contribution of plasma-derived HGFA in cancer tissue as a result of enhanced vascular permeability.

HGFA immunoreactivity was observed consistently in normal and neoplastic colorectal epithelia, and the level of expression tended to increase in the neoplastic cells compared with the normal counterparts. The expression of HGFA mRNA in colorectal tissues was focused on the activation process of HGF/SF in tumors. uPA was shown for the first time, to our knowledge, that human colon carcinoma cells express functionally active HGFA, and that cellular HGFA could be involved in the activation of scHGF and the subsequent cellular

### Table 2 Frequency of basal localization of HGFA

<table>
<thead>
<tr>
<th>Histology</th>
<th>0–20% (0)</th>
<th>20–50% (1+)</th>
<th>&gt;50% (2+)</th>
<th>Total positivity (%)</th>
<th>Mean rating ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyperplastic polyp</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0/7 (0%)</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Low-grade adenoma</td>
<td>31</td>
<td>0</td>
<td>0</td>
<td>0/31 (0%)</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>High-grade adenoma</td>
<td>6</td>
<td>1</td>
<td>1</td>
<td>2/8 (25%)</td>
<td>0.38 ± 0.26</td>
</tr>
<tr>
<td>Carcinoma (primary)</td>
<td>22</td>
<td>11</td>
<td>8</td>
<td>19/41 (46%)</td>
<td>0.66 ± 0.12</td>
</tr>
<tr>
<td>Metastatic carcinoma (liver)</td>
<td>5</td>
<td>6</td>
<td>4</td>
<td>10/15 (67%)</td>
<td>0.93 ± 0.21</td>
</tr>
<tr>
<td>Primary carcinoma</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without liver metastasis</td>
<td>18</td>
<td>5</td>
<td>4</td>
<td>9/27 (33%)</td>
<td>0.48 ± 0.15</td>
</tr>
<tr>
<td>With liver metastasis</td>
<td>4</td>
<td>6</td>
<td>4</td>
<td>10/14 (71%)</td>
<td>1.00 ± 0.21*</td>
</tr>
</tbody>
</table>

*The frequency of basal localization or cell-stromal interface staining pattern of HGFA was graded on a scale of 0–2 as follows: 0 (negative); 0–20% of HGFA-positive carcinoma cells show basal or cell-stroma interface staining pattern; 1+, 20–50% of cells; and 2+, >50% of cells show the basal pattern.

*The differences between the groups were statistically significant (P < 0.01) with the Kurskal-Wallis nonparametric one-way ANOVA test.

*P = 0.05 compared with nonmetastatic case. Mann-Whitney U test.
Fig. 6. Immunohistochemical detection of HAI-1. A, immunohistochemistry of normal colon (a and b), low-grade tubular adenoma (c), and carcinomas (e and f) of colon. Hematoxylin counterstain. A photo of H&E stain corresponding to e is shown in d. Bar, 100 μm. B, up-regulation of HAI-1 in normal epithelium left behind in cancer tissue. In contrast, carcinoma cells are stained only faintly. Hematoxylin counterstain. Bar, 100 μm. C, HAI-1 expression in adenocarcinoma arising within adenoma (top four photos) and adenoma and invasive carcinoma present in a single tissue specimen (bottom two photos). In the former case, adenoma cells are stained more eosinophilic with H&E stain, whereas carcinoma cells have clearer cytoplasm. HAI-1 immunostain clearly highlighted the adenoma cells because of significant reduction of the immunoreactivity in carcinoma cells (top four photos). Bottom two photos, HAI-1 immunostain of low-grade adenoma and invasive carcinoma in a single specimen of a familial adenomatous polyposis case. Apparently, HAI-1 immunoreactivity is reduced in carcinoma compared with adenoma. Bar, 100 μm. D, immunoblot analysis of HAI-1 in tissue extracts of carcinoma (C), adenoma (A), and corresponding normal (N) mucosa using 1N7 antibody. Mr 66,000 of transmembrane form of HAI-1 was present in all specimens, and the levels of expression were decreased in carcinoma tissues. Twenty μg of extracted proteins were loaded in each lane. Cell extracts of HAI-1 cDNA-transfected CHO cells and mock-transfected CHO cells were used as positive and negative controls, respectively.
study revealed that the human colorectal epithelium also expresses HGFA. In accordance with the present observation, Matsubara et al. (43) have reported recently that HGFA mRNA is expressed in epithelia expressing c-met mRNA in the fetal rat gastrointestinal tract. Using RNA blot analysis, we have also observed that HGFA mRNA is expressed in the gastrointestinal tract of mouse, particularly in the colon, suggesting the possible important role of HGFA in the gastrointestinal tract (44). Interestingly, the subcellular localization of HGFA often is altered significantly in carcinoma cells showing a predominant basal staining pattern. It may be a result of altered cancer-specific sorting of HGFA or may reflect the binding of activated HGFA on the cellular surface proteoglycan because active HGFA has strong affinity for heparin (25). This abnormal subcellular distribution of HGFA in carcinoma with respect to normal and low-grade adenoma cells may increase the availability of mesenchyme-derived sHGF for the activation by HGFA in carcinoma. In fact, primary carcinomas with distant metastasis (Dukes’ D) and metastatic liver lesions tended to show this basal HGFA staining pattern more frequently than nonmetastatic carcinomas. However, the precise relevance of HGFA activity to the disease process in vivo could not be analyzed in this study. For this purpose, an immunoassay system to quantitate the antigen level of the active form of HGFA and an

### Table 3  HAI-1 protein expression in colorectal adenoma-carcinoma sequence and hyperplastic polyp

<table>
<thead>
<tr>
<th>Lesion stained</th>
<th>Score for immunostain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n^a</td>
</tr>
<tr>
<td>Hyperplastic polyp</td>
<td>8</td>
</tr>
<tr>
<td>Low-grade adenoma</td>
<td>57</td>
</tr>
<tr>
<td>High-grade adenoma</td>
<td>14</td>
</tr>
<tr>
<td>Carcinoma (primary)</td>
<td>63</td>
</tr>
<tr>
<td>Metastasis (liver)</td>
<td>15</td>
</tr>
<tr>
<td>Primary carcinoma</td>
<td></td>
</tr>
<tr>
<td>Without liver metastasis</td>
<td>46</td>
</tr>
<tr>
<td>With liver metastasis</td>
<td>17</td>
</tr>
</tbody>
</table>

^a Determined by estimating the percentage of cells that had membrane staining for HAI-1: 0, negative; 1+, <20%; 2+, 20–50%; 3+, 50–80%; and 4+, >80%.

^b n, number of cases stained.

^c P < 0.001 between the groups. Kurskal-Wallis nonparametric one-way ANOVA test.

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Fig. 7. Representative results of reciprocal expression pattern of HAI-1 (A, b, and B, a and c) and HGFA (A, c, and B, b and d). Both cases (A and B) are Dukes’ A stage. A photo of H&E stain corresponding to A, b and c is shown in A, a. Hematoxylin counterstain. Bar, 100 μm.
were used for the processing assay, and the extent of processing is shown at the bottom.

HGFA and HAI-1 shifts in favor of HGFA activity in the carcinoma

HAI-1 and HGFA were frequently reciprocal at the normal (or ade-

normal or adenoma epithelium. Therefore, the staining patterns of

reduced significantly in carcinoma cells compared with the adjacent
carcinoma cells. On the other hand, HAI-1 immunoreactivity was

up-regulated in hyperplastic and low-grade

adenoma epithelia and in the injured normal epithelium adjacent to

immunoreactivity was up-regulated in hyperplastic and low-grade

and the stem cells in the crypt were hardly stainable. However, the

activation of HGFA. In a normal colon, HAI-1 was stained on the

level of HAI-1 expression and/or by the change in subcellular local-

relatively consistent, local HGFA activity seems to be regulated by the

given the observation that the level of HGFA mRNA expression is

with the progression of the adenoma-carcinoma sequence. Therefore,

was highly dependent on cellular situations and correlated inversely

in the progression of the adenoma-carcinoma sequence. Therefore,
given the observation that the level of HGFA mRNA expression is
relatively consistent, local HGFA activity seems to be regulated by the
level of HAI-1 expression and/or by the change in subcellular local-
ization of HGFA. In a normal colon, HAI-1 was stained on the
basolateral cellular surface of enterocytes of the mucosal surface
(zone of maturation), and both the cells in the zone of proliferation
and the stem cells in the crypt were hardly stainable. However, the
immunoreactivity was up-regulated in hyperplastic and low-grade
adenoma epithelia and in the injured normal epithelium adjacent to
carcinoma cells. On the other hand, HAI-1 immunoreactivity was
reduced significantly in carcinoma cells compared with the adjacent
normal or adenoma epithelium. Therefore, the staining patterns of
HAI-1 and HGFA were frequently reciprocal at the normal (or ade-

oma)-carcinoma boundaries, suggesting that the net balance between
HGFA and HAI-1 shifts in favor of HGFA activity in the carcinoma
tissues. This shift of proteinase/inhibitor balance would contribute to
an efficient activation of HGF/SF in colorectal carcinomas, which
may result in enhanced cellular growth, migration, and angiogenesis,
because HGF/SF is a potent growth factor for gastrointestinal epithelial
cells (33, 45) as well as for vascular endothelial cells (8, 46) and
stimulates cellular migration and VEGF production of colon carcino-
ma cells. Stimulatory effects of HGF/SF on VEGF production were
also reported in other cell types (47, 48). Moreover, HGF/SF also
stimulates the expression of VEGF receptor in vascular endothelial
cells (48).

It should be noted that normal epithelium left behind in cancer
tissue and exfoliating surface epithelial cells showed significant up-
regulation of HAI-1. Moreover, although the immunoreactivity of
HAI-1 was reduced significantly in cancer cells relative to the adja-
cent normal epithelium, we have observed paradoxical up-regulation
of HAI-1 in certain carcinoma cells showing cell injury, degeneration,
or intense cell-stroma interactions (data not shown). These findings
suggest that the HAI-1 molecule has multiple roles that may be highly
situational, depending on the presence of other constituents in the
intra- or extracellular milieu; some of these roles are possibly inde-
pendent of the HGFA-inhibitory activity of the molecule. HAI-1 has
two Kunitz domains. The first domain appears to be responsible for
the inhibition of HGFA (28), indicating the possible presence of other
target proteinase(s) in which the second Kunitz domain might be
involved predominantly. Because all situations where HAI-1 immu-
noreactivity was up-regulated in vivo appear to be adverse situations
for the cells, one possibility is that HAI-1 may act as a cell survival
factor in adverse and unstable circumstances through its inhibitory
activities against excess pericellular proteinase(s). Alternatively, it
may be somehow involved in a regenerating process in response to
tissue and cellular injury. Indeed, recent observations supporting this
hypothesis indicate that HAI-1 is also up-regulated in severely injured
human kidneys and liver (29) and at the ulcer edge of mouse experi-
mental colitis (49). In this regard, we have observed that the 5’-
regulatory region of the human HAI-1 gene contained consensus
binding sites of early responsive transcription factors in case of tissue
injury, such as heat shock transcription factors, nuclear factor xB, and
Egrs (50). Further study for the proteinase specificity of HAI-1 in the
colorectum and analysis of the regulatory elements of the HAI-1 gene
is required to clarify these phenomena. Recently, a novel serine

Fig. 8. A, immunoblot analysis of HGFA protein in normal mucosa (N), primary carcinoma tissue (T), and metastatic carcinoma tissue (Meta). Immunoblot using antiactin antibody was also shown as internal loading control. The same tissue extracts were processed to enrich HGF/SF using heparin Sepharose, and eluted samples were used to detect the heavy chain of activated HGF/SF (bottom). This case corresponds to case 4 in Fig. 1. B, processing of HGF/SF by tissue extracts of normal mucosa (N) and primary carcinoma (T) of the same case as A. The extracts were pretreated at 4°C overnight with 400 μg/ml anti-HGFA (P1-4) antibody or nonspecific mouse IgG (25). Eight μg of scHGF were used for the processing assay, and the extent of processing is shown at the bottom.
proteinase designated as matritase was identified as a target of HAI-1 in the mammary tissue in vivo (51).

In conclusion, this study provides evidence of the crucial involvement of HGFA in the regulation of HGF/SF activation in colon carcinoma cells and reports the comparative expression patterns of HGFA and HAI-1 in colon mucosa, adenomas, and carcinomas for the first time. In Fig. 9, we propose a hypothetical model for intermolecular interactions in the activation of HGF/SF in colorectal cancer tissue. Considering the important roles of HGF/SF in gastrointestinal mucosa and tumors as well as the fact that the extracellular activation of sHGF is a critical limiting step in inducing the pleiotropic biological effects of HGF/SF through its receptor Met, more studies of HGFA and HAI-1 in the pathophysiology of gastrointestinal tract will be necessary. The activation step of HGF/SF may serve as a promising new target of therapeutic intervention of tumors including colon cancers.

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