

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 07-034188- -00002-0000

Fecha: 2008-06-13 17:55:19 Dep. 2020 NUECREACIONE
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AS: 103.309

Señores

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

DIVISION DE NUEVAS CREACIONES

E. S. D.

REF.: Expediente No. 07 034.188 - Solicitud de Patente FASE NACIONAL

Corresp. a la Solicitud Internacional PCT/US2005/036300.

a nombre de: **GENENTECH, INC.**

Titulada: MODULADORES DE ACTIVADOR DE FACTOR DE CRECIMIENTO
HEPATOCÍTICO.

JUAN PABLO CADENA SARMIENTO, vecino de Bogotá, portador de la tarjeta profesional No. 57492 del Consejo Superior de la Judicatura, obrando como apoderado de la Sociedad **GENENTECH, INC.**, según lo acredita el documento de poder presentado en esa División, de acuerdo con el Artículo 44 de la Decisión 486 de la Comisión de la Comunidad Andina, me permito solicitar que se examine si la invención contenida en la solicitud en referencia es patentable.

De acuerdo con lo anterior, me permito anexar el recibo oficial de caja No. 08-6,299 por valor de \$420.000.

De la Señora Jefe atentamente,

JUAN PABLO CADENA SARMIENTO

C.C. No. 80.410.337 de Usaquén

T.P. No. 57492 del C.S.J.

Cra 7 No. 71-21 Torre B, Piso 4

Bogotá, 13 de junio de 2008

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FECHA : ENERO 23 DE 2008

***** CONSIGNACION *****

DEPOSITANTE	TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PGO	Ir. PAGO
BRIGARD E CASTRO LUNA	CONSIGNACION	BANCO DE BOGOTA	000754197	120206	23/01/2008	31,370,000.00

***** CONCEPTO *****

N. CONCEPTO	CONCEPTO	TOTAL CONCEPTO
1 50005 01 01 DOLIDIFLOCS	85 EXAMEN DE PATENTABILIDAD DE INVEN	420,000.00
	TOTAL :	\$ 420,000.00

CON. CUATROCIENTOS VEINTE MIL PESOS

RESPONSABLE : _____

RECIBO DE DINERO APLICADO AL EXPEDIENTE No. _____

DIRECCIÓN DE NUEVAS CREACIONES

RADICACIÓN EXPEDIENTE No. PCT 07-34188

EL EXTRACTO DE LA PRESENTE SOLICITUD DE **PATENTE DE INVENCION**

FUE PUBLICADO EN LA GACETA DE PROPIEDAD INDUSTRIAL No **583** DE

FECHA **18 DE DICIEMBRE DE 2007** EL TÉRMINO HÁBIL SEÑALADO POR LA

LEY EXPIRA EL **14 DE MARZO DE 2008**

NOTA: Dentro del plazo de los seis (6) meses siguientes a partir de la fecha de publicación en la gaceta de Propiedad Industrial, deberá solicitar y cancelar el examen de patentabilidad so pena de que la solicitud caiga en abandono, de acuerdo con lo establecido en el Artículo 44 de la Decisión 486/2000.

SE PRESENTARON OPOSICIONES POR PARTE DE TERCEROS.

SI _____

NO _____ **x** _____

Activation of Hepatocyte Growth Factor/Scatter Factor in Colorectal Carcinoma¹

Hiroaki Kataoka,² Ryouichi Hamasuna, Hiroshi Itoh, Naomi Kitamura, and Masashi Kono

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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. Immunoblot 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 morphogenic 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 tumorigenesis, 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 inactive zymogen (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 lumen (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

Received 2/14/00; accepted 8/29/00.

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¹Supported in part by Grants-in-Aid for Scientific Research (C) 11670221 (to H. I.) and 12670209 (to H. K.) from the Ministry of Education, Science, Sports and Culture, Japan, and by grants from the Sagawa Cancer Research Foundation (to H. I.).

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³The abbreviations used are: HGF, hepatocyte growth factor; SF, scatter factor; scHGF, single-chain HGF (latent form); HGFA, HGF activator; HAI-1, HGFA inhibitor type 1; uPA, urokinase-type plasminogen activator; T:N, tumor:normal ratio; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; VEGF, vascular endothelial growth factor; CHO, Chinese hamster ovary; SFCM, serum-free culture conditioned medium; RT-PCR, reverse transcription-PCR; G3PDH, glyceraldehyde 3-phosphate dehydrogenase.

in other tumors: such a study would have potentially great importance. Given the fact that the activation of scHGF 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 scHGF 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 extrahepatic 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 scHGF-processing activity in colorectal carcinoma cells and to test the potential role of HGFA in the scHGF 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 HGF-A. Among the antibodies obtained, anti-HGFA antibody A-1 was suitable for immunohistochemistry. Another anti-HGFA monoclonal antibody, PI-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 Kunitz domain of HAI-1 protein. Antihuman HGF/SF 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% H₂O₂ 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 washed in PBS and incubated with Envision labeled polymer reagents (Dako, Carpinteria, CA) for 30 min at 37°C. The reaction was revealed with nickel and cobalt-3,3'-diaminobenzidine (ImmunoPure Metal Enhanced DAB Substrate kit; Pierce, Rockford, IL) and counterstained with Mayer's hematoxylin. All colon pathology was assessed independently by two pathologists. Diagnoses and terminology were those of the World Health Organization (36). Eight cases of hyperplastic polyp, 57 cases of low-grade (mildly or moderately dysplastic) adenoma, 14 cases of high-grade (severely dysplastic) adenoma, 63 cases of primary colorectal adenocarcinoma, and 15 cases of metastatic lesion in the liver were used. Epithelial cell or tumor cell staining was graded on a scale of 0 to 4 as follows: 0, negative; 1+, <20% of cells staining; 2+, 20–50%; 3+, 50–80%; and 4+, 80–100%.

Tissue Collection, RNA Extraction, RT-PCR, and RNA Blot Analysis. Total cellular RNA was extracted by Trizol reagent (Life Technologies, Inc., Gaithersburg, MD). For RNA extraction, all tissues were received fresh from

the operating room. In each case, tissue samples were taken from the primary tumor and corresponding normal mucosa. Sections taken adjacent to the tissue used for RNA extraction were assessed for degree of histopathological assessment. Methods for RT-PCR analyses of HGFA, *c-met*, and G3PDH mRNAs have been described previously (37). Primer sequences are as follows: *c-met* forward, ATTCATCAGGCTGTGAAGCG; *c-met* reverse, CAGAGAGGCATTGACTGCAG. HGFA forward, AGGACACAAGTGCCAGATTG; HGFA reverse, GTTGATCCAGTCCACATAGT. The expected product sizes are 381, 334, and 300 bp for *c-met*, HGFA, and G3PDH, respectively. RT-PCR for HGFA and *c-met* was carried out by running for 22 and 32 cycles at 2-cycle intervals. The products emerged between 24 and 26 cycles (*c-met*) and between 26 and 28 cycles (HGFA), increased exponentially with cycles up to 30–34, and then reached a plateau. Thus, PCR amplification was set at 28 cycles, because *c-met* PCR products and HGFA PCR products almost linearly increased in proportion to the number of cycles between 24 and 30 cycles (*c-met*) and between 26 and 32 cycles (HGFA). For G3PDH, 23 cycles of amplification were used as in a similar experiment, at 95°C for 30 s, 58°C for 60 s, and 72°C for 90 s using HotStarTaq polymerase (Qiagen, Tokyo, Japan), and followed by incubation at 72°C for 7 min. The conditions of G3PDH amplifications were same as above, except for 20–26 cycles at 2-cycle intervals. The products were electrophoresed on 2% agarose gels and stained with ethidium bromide, and the band intensity was measured with NIH Image software. For semiquantification of the RT-PCR, the reaction cycle/PCR products of each reaction mixture were plotted on semilogarithmic graphs, and PCR cycles in which the products linearly increased in proportion to the number of cycles were determined. The intensity of the product was normalized through that of the corresponding G3PDH product. For RNA blot analysis, 30 µg of total RNA were applied to each well and electrophoresed through formaldehyde-containing 1% agarose gel. The products of electrophoresis were transferred onto Hybond-N⁺ nylon membrane (Amersham, Buckinghamshire, United Kingdom), and RNA was UV-cross-linked onto the membrane. Hybridization was performed in mixed solution of 50% formamide, 5× Denhardt's solution, 25 mM phosphate buffer (pH 6.5), 0.1% SDS, 100 µg/ml sonicated and heat-denatured salmon sperm DNA, and 5× SSC at 42°C for 16 h. The blots were washed as follows: three times in 1× SSC-0.1% SDS for 15 min 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 *NaeI*-*XhoI* 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, proteinase 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 polyclonal antibody (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 pCInco 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 Activation Activity. Five μ l of recombinant scHGF ($1.6 \mu\text{g}/\mu\text{l}$) were mixed with 20 μ l 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 immunoaffinity column. 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 μ l of recombinant M_r 34,000 HGFA ($0.3 \text{ ng}/\mu\text{l}$) 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_r 40,000 secreted form). Total reaction volume was 45 μ l 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% gradient gel) under reducing conditions. Proteins in the gel were stained with 2.5% Coomassie Brilliant Blue in water:methanol:acetic acid solution (6:3:1, v/v). Recombinant scHGF and M_r 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 $\mu\text{g}/\text{ml}$), control mouse IgG (PI-4, 20 $\mu\text{g}/\text{ml}$), 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 sandwich ELISA with a human VEGF EIA kit (Immunobiological Laboratories Co., Gunma, Japan).

Data Analysis. Statistical parameters were ascertained with 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_r 96,000 pro-HGFA and a very low level of M_r 32,000 active form under the reducing condition. This

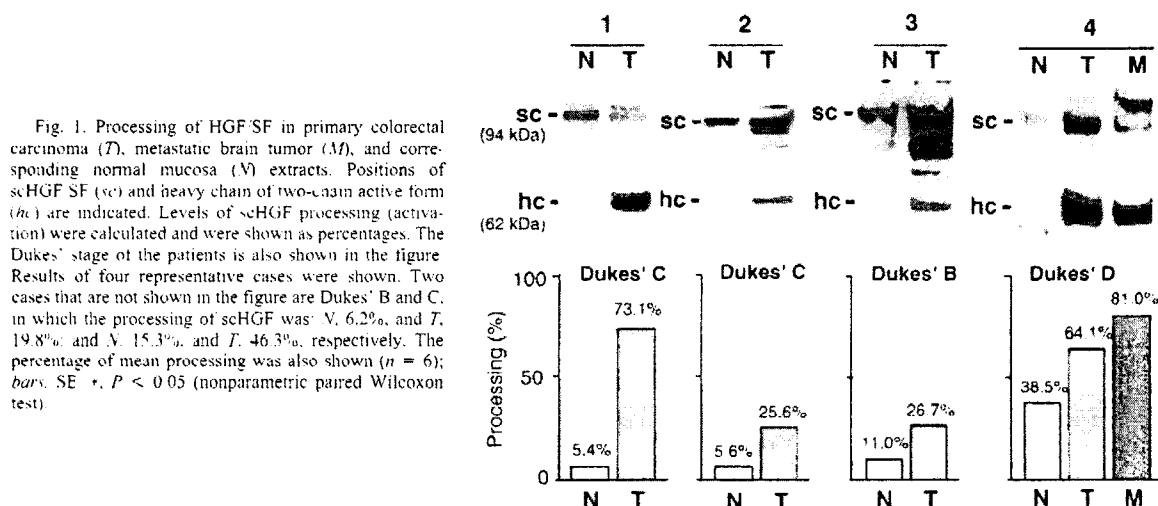
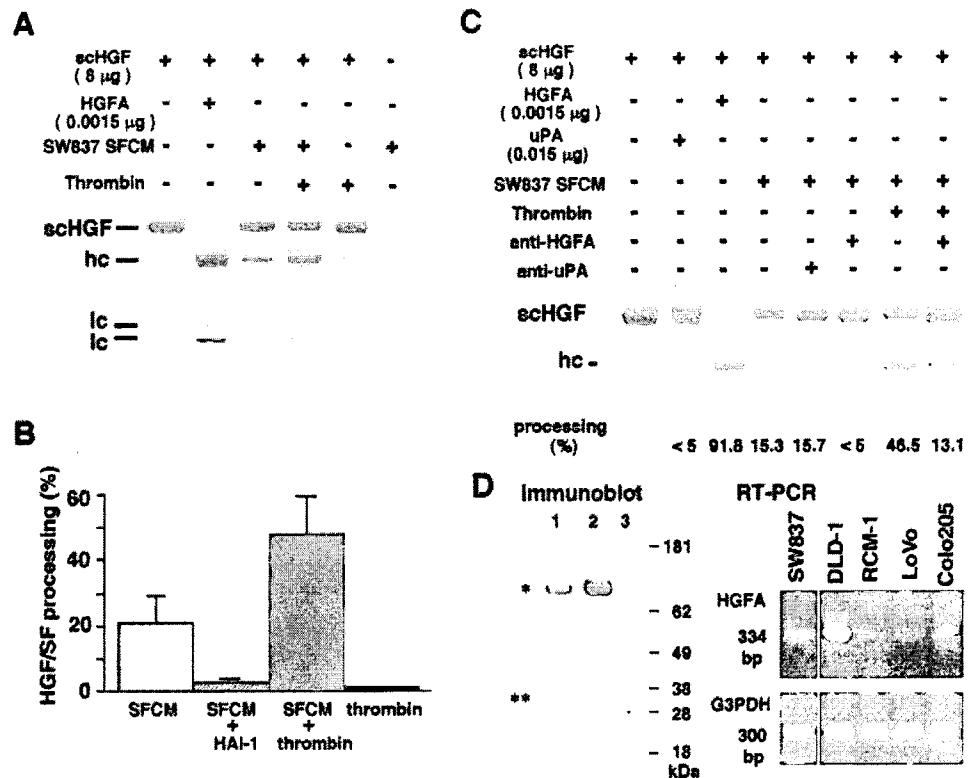


Fig. 1. Processing of HGF/SF in primary colorectal carcinoma (T), metastatic brain tumor (M), and corresponding normal mucosa (N) extracts. Positions of scHGF/SF (sc) and heavy chain of two-chain active form (hc) are indicated. Levels of scHGF processing (activation) were calculated and were shown as percentages. The Duke's stage of the patients is also shown in the figure. Results of four representative cases were shown. Two cases that are not shown in the figure are Duke's B and C, in which the processing of scHGF was: N, 6.2%, and T, 19.8%; and N, 15.3%, and T, 46.3%, respectively. The percentage of mean processing was also shown ($n = 6$); bars, SE. *, $P < 0.05$ (nonparametric paired Wilcoxon test).

Fig. 2. Activation of scHGF by SFCM of SW837 cells. *A*, SFCM of SW837 cells contains scHGF-processing activity similar to recombinant HGFA. This activity was enhanced by thrombin pretreatment. *hc*, heavy chain of active two-chain form HGF/SF. *lc*, light chain. *B*, quantification of scHGF-processing activity of SFCM and effects of recombinant HAI-1 or thrombin pretreatment. *Bars*, SE. *C*, neutralizing study for scHGF/SF-processing activity of SFCM using anti-HGFA (1 μ g) or anti-uPA (1 μ g) and comparative study for the effects of HGFA and uPA on scHGF processing. *D*, expression of HGFA in colon carcinoma cell lines. Immunoblot for HGFA protein present in SFCM of SW837 cells (*Lane 1*) and of CHO cells transfected with (*Lane 2*) or without (*Lane 3*) HGFA 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_r 96,000 proform and M_r 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.



active form migrated on the gel as a M_r 34,000 protein under nonreducing conditions (not shown). Faint bands of M_r 66,000, M_r 50,000–60,000, and M_r 41,000 species immunoreactive to the antibody were also detected. The expression of HGFA was not SW837 cell line-specific phenomenon, because four other colorectal carcinoma cell lines examined (DLD-1, RCM-1, LoVo, and Colo205) also expressed HGFA mRNA with sensitive RT-PCR study (Fig. 2D). Of the cell lines, DLD-1 showed the highest mRNA level of HGFA, and SFCM of DLD-1 also contained scHGF-processing activity. This activity was enhanced markedly by thrombin treatment and was inhibited significantly by anti-HGFA neutralizing antibody or by nafamostat mesilate, a synthetic serine proteinase inhibitor having potent inhibitory activity to HGFA but not to uPA (21, 38; Fig. 3A). The presence of pro-HGFA in SFCM of DLD-1 and its activation upon thrombin treatment were confirmed by immunoblot analysis (Fig. 3B).

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 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 scattering 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 $\pm$ 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 $\pm$ 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 $\pm$ SE, 0.58 ± 0.1 ; $P < 0.01$).

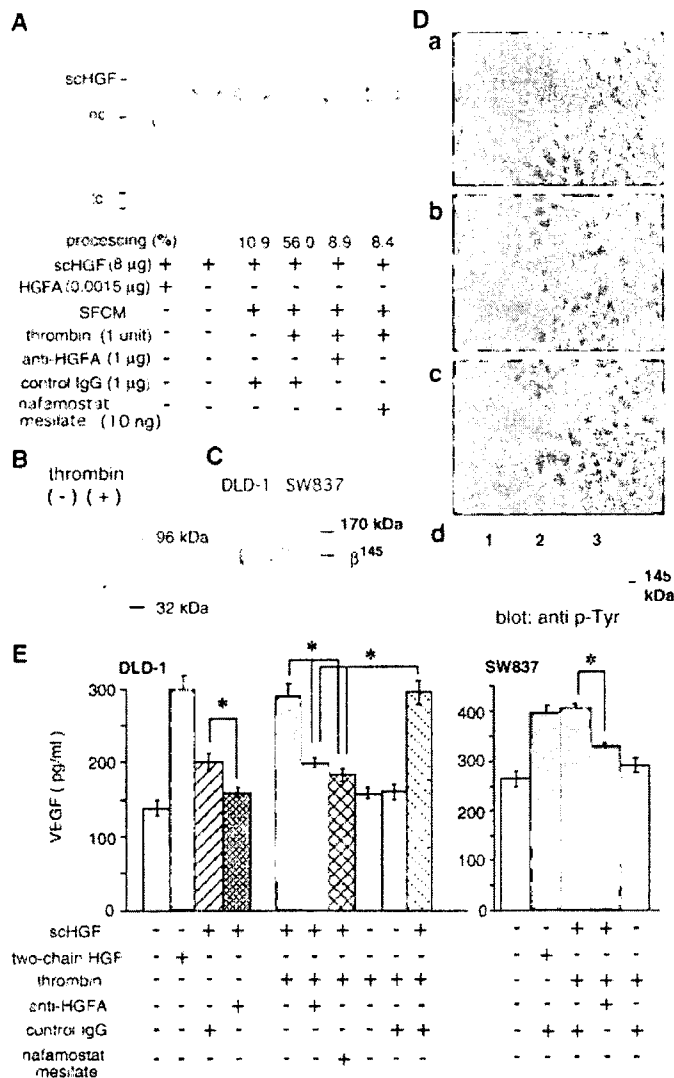


Fig. 3 Involvement of HGFA in scHGF-induced cellular responses. *A*, presence of HGFA activity in SFCM of DLD-1 cells. *B*, presence of pro-HGFA protein in SFCM of DLD-1 cells and its activation upon thrombin treatment. *C*, expression of functional M_r 145,000 (β^{45}) form of Met in DLD-1 and SW837 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 μ g/ml of mouse nonspecific IgG and anti-HGFA IgG, respectively, were also added in the medium. *E*, 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 μ g/ml), nonspecific mouse IgG (20 μ g/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.

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. 5*B*). Then normal human colon mucosa tissue was stained with the antibody. In normal colon mucosa, HGFA immunoreactivity was observed in the enterocytes (Fig. 5*C*), 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. 6.4, *a*). This immunoreactivity was much more evident in surface

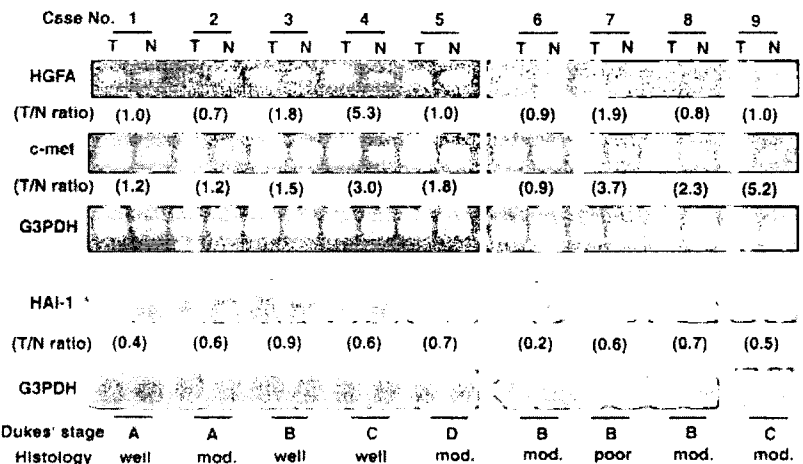
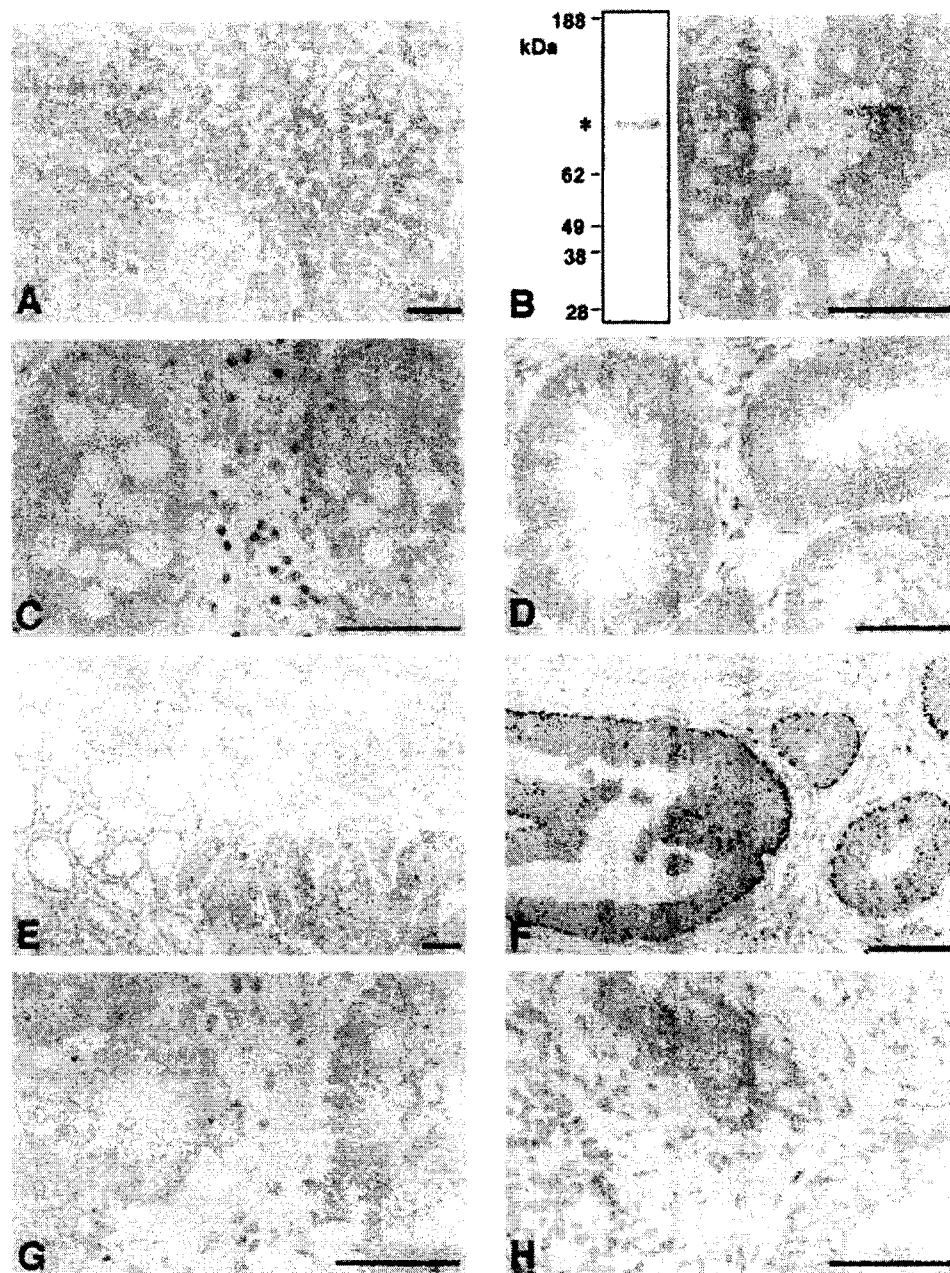


Fig. 5. Representative immunohistochemical analysis of HGFA in human liver (A and B), colorectal normal mucosa (C), adenoma (D), carcinomas (E-G), and lymph node metastasis (H). An immunoblot for HGFA in the extract of liver tissue using the same antibody (A1) is also shown in B in which a single major band of M_r 96,000 pro-HGFA was observed. Note that HGFA reactivity in carcinoma cells frequently shows basal distribution. Hematoxylin counterstain. Bars, 100 μ m.



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 immunore-

activity 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 hypoeexpression 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

Table 1. HGFA protein expression in colorectal adenoma-carcinoma sequence and hyperplastic polyp

Lesion stained	n ^a	Score for immunostain ^b						mean score $\pm$ SE
		0	1+	2+	3+	4+		
Hyperplastic polyp	8	1	4	2	1	0	1.4 $\pm$ 0.3	
Low-grade adenoma	31	0	2	11	18	0	2.5 $\pm$ 0.1	
High-grade adenoma	8	1	0	3	4	0	2.3 $\pm$ 0.4	
Carcinoma (primary)	42	2	6	12	15	7	2.5 $\pm$ 0.2	
Metastasis (liver)	16	0	2	7	7	0	2.3 $\pm$ 0.2	

^a 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%.

^b n, number of cases stained.

Table 2 Frequency of basal localization of HGFA

Histology	Frequency of basal localization of HGFA ^a (rating)			Total positivity (%)	Mean rating $\pm$ SE
	0-20% (0)	20-50% (1-)	>50% (2-)		
Hyperplastic polyp	7	0	0	0.7 (0%)	0 $\pm$ 0
Low-grade adenoma	31	0	0	0.31 (0%)	0 $\pm$ 0
High-grade adenoma	6	1	1	2.8 (25%)	0.38 $\pm$ 0.26
Carcinoma (primary)	22	11	8	19.41 (46%)	0.66 $\pm$ 0.12
Metastatic carcinoma (liver)	5	6	4	10.15 (67%)	0.93 $\pm$ 0.21
Primary carcinoma					
Without liver metastasis	18	5	4	9.27 (33%)	0.48 $\pm$ 0.15
With liver metastasis	4	6	4	10.14 (71%)	1.00 $\pm$ 0.21 ^c

^a 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.

^b The differences between the groups were statistically significant ($P < 0.01$) with the Kruskal-Wallis nonparametric one-way ANOVA test.

^c $P = 0.05$ compared with nonmetastatic case, Mann-Whitney U test.

in carcinomas was further confirmed by immunoblot analysis (Fig. 6D). The band corresponding to the M_r 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 M_r 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 neoangiogenesis 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 M_r 32,000 form (M_r 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- and 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 further confirmed by RT-PCR analysis. Although the major source of plasma HGFA protein has been considered to be the liver (24), this

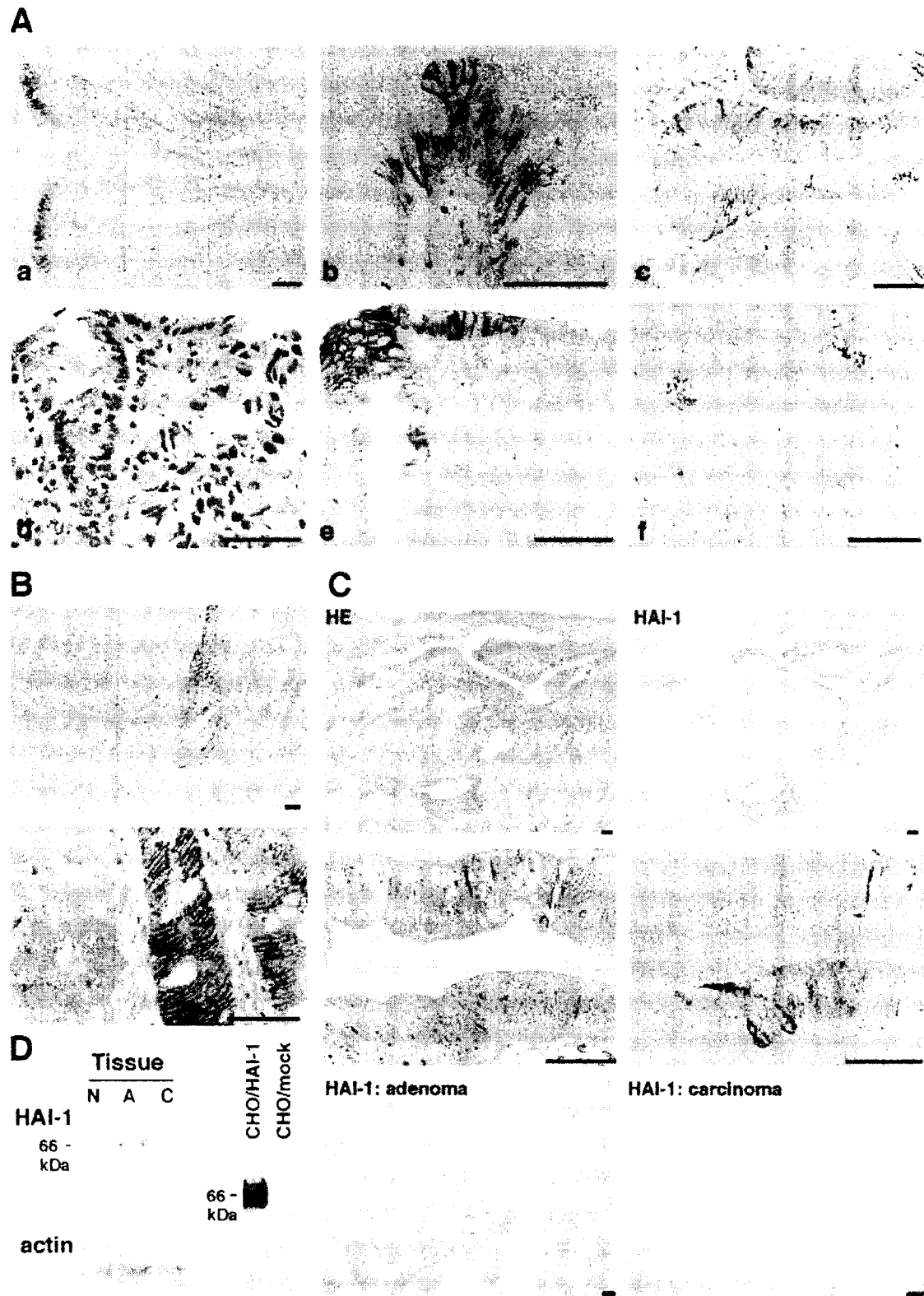


Fig. 6. Immunohistochemical detection of HAI-1. *A*, immunohistochemistry of normal colon (*a* and *b*), low-grade tubular adenoma (*c*), and carcinomas (*e* and *f*) of colon. Hematoxylin counterstain. A photo of H&E stain corresponding to *e* is shown in *d*. Bar, 100 μ m. *B*, up-regulation of HAI-1 in normal epithelium left behind in cancer tissue. In contrast, carcinoma cells are stained only faintly. Hematoxylin counterstain. Bar, 100 μ m. *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 μ m. *D*, immunoblot analysis of HAI-1 in tissue extracts of carcinoma (*C*), adenoma (*A*), and corresponding normal (*N*) mucosa using 1N7 antibody. M_r 66,000 of transmembrane form of HAI-1 was present in all specimens, and the levels of expression were decreased in carcinoma tissues. Twenty μ g 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.

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

Lesion stained	n ^b	Score for immunostain ^a					mean score ± SE
		0	1+	2+	3+	4+	
Hyperplastic polyp	8	0	0	3	2	3	3.0 ± 0.3
Low-grade adenoma	57	0	3	21	22	11	2.7 ± 0.1
High-grade adenoma	14	0	4	5	3	1	2.1 ± 0.3
Carcinoma (primary)	63	7	29	15	9	3	1.6 ± 0.1
Metastasis (liver)	15	3	7	2	2	1	1.4 ± 0.3
Primary carcinoma							
Without liver metastasis	46	7	18	12	7	2	1.5 ± 0.2
With liver metastasis	17	0	11	3	2	1	1.6 ± 0.2

^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. Kruskal-Wallis nonparametric one-way ANOVA test.

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 acti-

vated 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 scHGF 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

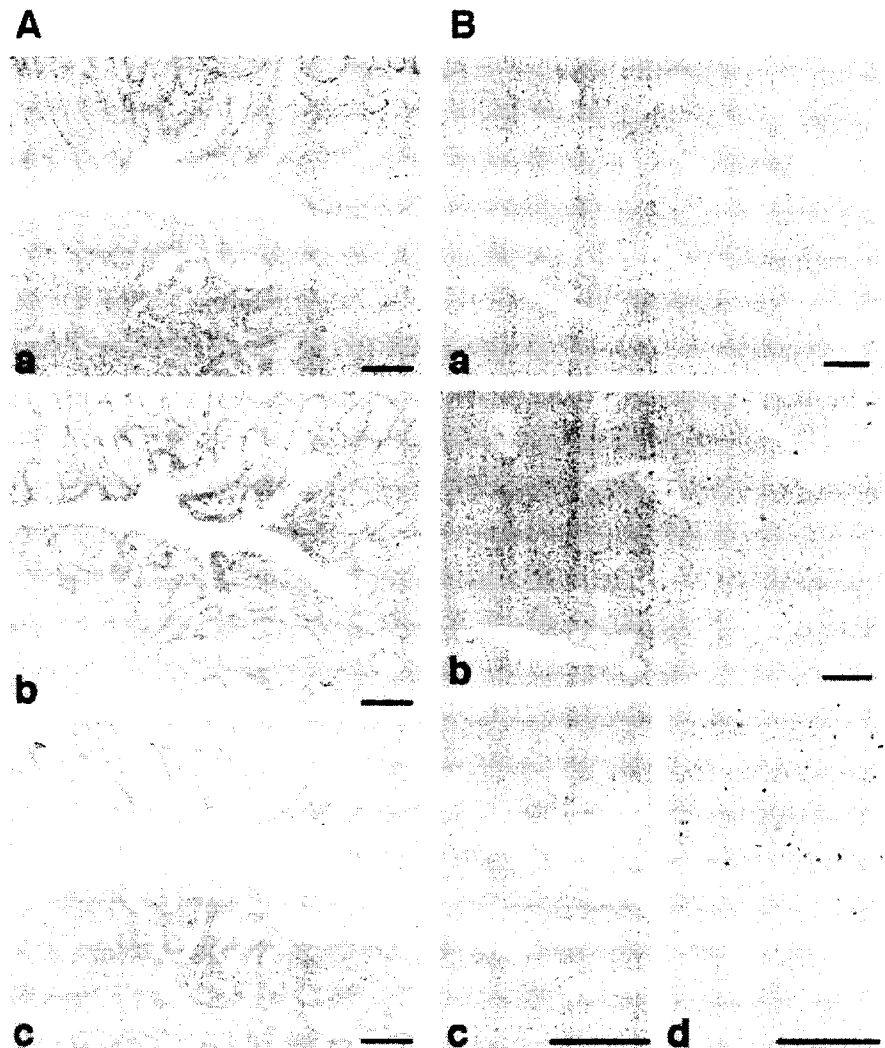


Fig. 7. Representative results of reciprocal expression pattern of HAI-1 (A, b, and B, a and c) and HGFA (A, c, and B, b and d). Both cases (A and B) are Dukes' A stage. A photo of H&E stain corresponding to A, b and c is shown in A, a. Hematoxylin counterstain. Bar, 100 μ m.

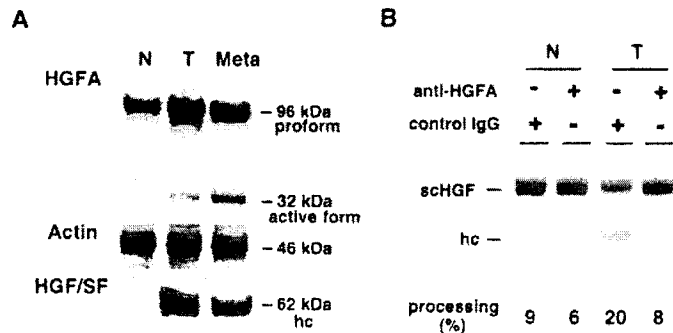


Fig. 8. A, immunoblot analysis of HGFA protein in normal mucosa (N), primary carcinoma tissue (T), and metastatic carcinoma tissue (Meta). Immunoblot using antiactin antibody was also shown as internal loading control. The same tissue extracts were processed to enrich HGF/SF using heparin Sepharose, 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 (PI-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.

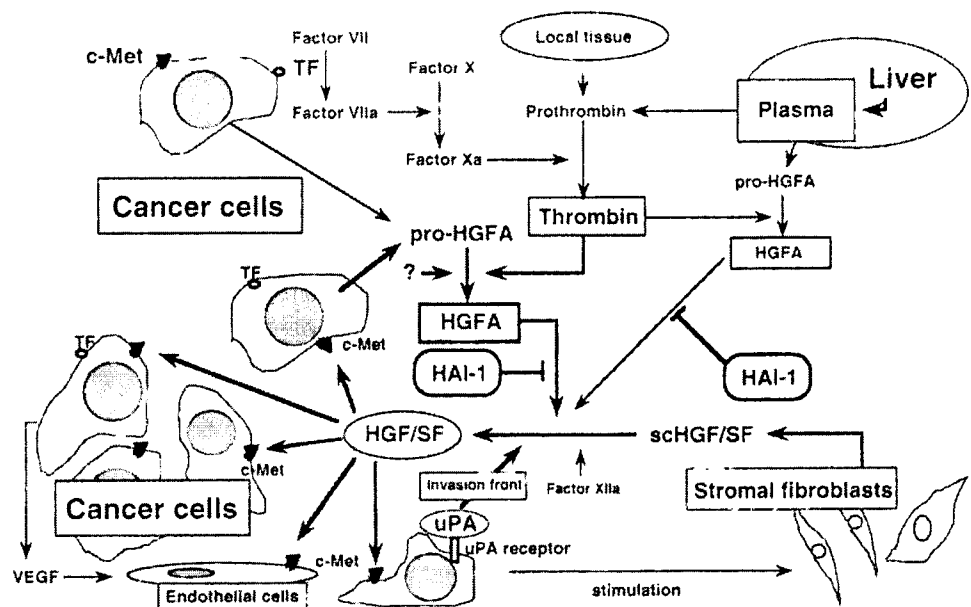
adequate method for the titration of HGFA activity are necessary, both of which are currently under development.

In contrast to HGFA, the immunoreactivity for the HAI-1 protein was highly dependent on cellular situations and correlated inversely with 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 localization 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 adenoma)-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 carcinoma 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 adjacent 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 independent 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 immunoreactivity 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 experimental 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 κB, 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. 9. Hypothetical model for intermolecular interactions in the activation of HGF/SF in colorectal carcinoma. Cancer cells stimulate the stromal fibroblasts to synthesize and secrete scHGF (12). Pro-HGFA produced by cancer cells or derived from plasma can be activated by thrombin and/or by unknown proteinase(s) in cancer tissue. The presence of functionally intact prothrombin in extrahepatic tissues and increased procoagulant activity in carcinoma cells accompanying pericellular fibrin generation have been reported (40, 41, 52). Upon activation, HGFA acquires affinity for heparin (25), ensuring the copresence of HGFA with scHGF stored in the extracellular space in cancer tissue, and efficiently activates scHGF. In addition, although the activity of uPA on the processing is weak, the activity would be stimulated by the uPA receptor, which is expressed highly in the invasion front (19, 22, 38), and local factor XIIIa may also contribute to the activation (21). The concerted actions of these proteinases on scHGF would result in significantly increased HGF/SF activity in carcinoma tissue. Activated HGF/SF acts on cancer cells and endothelial cells via Met receptor tyrosine kinase. TF, tissue factor.



proteinase designated as matriptase 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 scHGF 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.

ACKNOWLEDGMENTS

We thank Dr. H. Tsubouchi for kind suggestions and discussion, T. Miyamoto for help in processing the figures, and N. Iwakiri for preparing the tissue sections.

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Activation of hepatocyte growth factor by two homologous proteases, blood-coagulation factor XIIa and hepatocyte growth factor activator

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(Received 1 December 1994/9 February 1995) – EJB 94 1855/4

Hepatocyte growth factor (HGF) is secreted as an inactive single-chain precursor from the producing cells, and normally remains in this form associated with the extracellular matrix. In response to tissue injury, the single-chain precursor is converted to a biologically active heterodimer by a serine protease, the activity of which is induced in the injured tissue. We have previously identified HGF activator, a serum serine protease that activates single-chain HGF. The sequence of HGF activator cDNA revealed that the HGF activator is homologous to blood-coagulation factor XIIa. In this study, we found that coagulation factor XIIa has an ability to activate single-chain HGF. Factor XIIa exhibited a significant level of HGF-converting activity in the presence of dextran sulfate, although the specific activity of factor XIIa was slightly lower than that of the HGF activator. Since factor XIIa is activated during the initiation of contact activation induced by tissue injury, factor XIIa may function as an HGF-converting enzyme together with HGF activator in the injured tissue. C1-inhibitor, antithrombin III and α_2 -antiplasmin, that regulate the blood-clotting activity of factor XIIa, were also effective against the HGF-converting activity of factor XIIa. Furthermore, factor XIIa was not active in the HGF-converting activity in serum. Thus, the HGF-converting activity of factor XIIa may be regulated by these serum inhibitors.

Keywords. Hepatocyte growth factor; proteolytic activation; blood-coagulation factor XIIa; hepatocyte growth factor activator.

Hepatocyte growth factor (HGF) is a potent mitogen for hepatocytes and renal tubular epithelial cells (Gohda et al., 1988; Igawa et al., 1991) and may play an important role in regeneration following hepatic and renal injury (Tsubouchi et al., 1991; Okajima et al., 1990; Igawa et al., 1993). HGF also has several other biological effects on its various target cells. It is identical to the scatter factor which dissociates epithelial cell colonies into individual cells and stimulates the motility of epithelial cells (Weidner et al., 1991). It is also identical to tumor cytotoxic factor that inhibits the growth of some tumor cells (Shima et al., 1991). Furthermore, it functions as a mediator of epithelial tubulogenesis (Montesano et al., 1991) and of angiogenesis (Bussolino et al., 1992; Grant et al., 1993).

HGF is secreted as an inactive single-chain precursor from producing cells (Miyazawa et al., 1991; Rubin et al., 1991). The secreted HGF normally remains as the single-chain form, probably associated with the extracellular matrix in the producing tissues (Masumoto and Yamamoto, 1991; Naldini et al., 1992). In response to tissue damage such as hepatic and renal injury, the single-chain form of HGF is cleaved at Arg494 and converted to a heterodimeric molecule exclusively in the injured tissue. This

cleavage is mediated by an enzymic activity which is induced in the injured tissues (Miyazawa et al., 1994). The heterodimeric form consists of a heavy chain and a light chain, joined by a disulfide bond, and is required for the biological activities of HGF (Naka et al., 1992; Lokker et al., 1992; Gak et al., 1992; Naldini et al., 1992; Hartmann et al., 1992). Thus, the biological effects of HGF in the injured tissues are regulated through proteolytic processing by a serine protease. However, the serine protease which is involved in this regulation in the injured tissues has not yet been identified.

Tissue injury often leads to local blood coagulation (Kaplan and Silverberg, 1988). When blood coagulation is initiated, many serine proteases are generated from their zymogens by limited proteolysis. We identified HGF activator from fetal bovine serum (Shimomura et al., 1992) and from human serum (Miyazawa et al., 1993), which efficiently converts single-chain HGF to the biologically active heterodimer *in vitro*. The sequence of the HGF activator cDNA revealed that the HGF activator is homologous to blood-coagulation factor XII, and that the HGF activator precursor consists of multiple putative domains homologous to those observed in factor XII (Miyazawa et al., 1993). HGF activator circulates in the blood as an inactive zymogen. The zymogen is activated by limited proteolysis by thrombin (Shimomura et al., 1993). Naldini et al. (1992) and Mars et al. (1993) reported that urokinase and tissue-type plasminogen activator (tPA) convert single-chain HGF to the heterodimer *in vitro*. These serine proteases may function as HGF-converting enzymes in injured tissues. However, other serine proteases which are activated during blood coagulation and fibri-

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Abbreviations. HGF, hepatocyte growth factor; tPA, tissue-type plasminogen activator.

Enzymes. Plasma kallikrein (EC 3.4.21.34); plasmin (EC 3.4.21.7); thrombin (EC 3.4.21.5); coagulation factor Xa (EC 3.4.21.6); coagulation factor XIa (EC 3.4.21.27); coagulation factor IXa (EC 3.4.21.22); coagulation factor XIIa (EC 3.4.21.38); urokinase (EC 3.4.21.31).

olysis, could be responsible for the activation of HGF. In this study, we therefore examined whether these serine proteases convert single-chain HGF to the heterodimer. We found that coagulation factor XIIa has an ability to activate single-chain HGF. However, we did not detect HGF-converting activity in urokinase and tPA. Since the sequence of factor XIIa is homologous to that of the HGF activator, they may share similar biological activities. Thus, we compared the properties of factor XIIa with those of the HGF activator.

EXPERIMENTAL PROCEDURES

Materials. Reagents were obtained as follows: human plasma kallikrein (15 U/mg) and plasmin (10 U/mg) from Protogen AG; thrombin (3800 NIH U/mg), and blood-coagulation factors Xa (125 U/mg), XIa (160 U/mg), IXa (174 U/mg) and XIIa (11 U/mg) from Enzyme Research Lab. Inc.; tPA (550 000–650 000 IU/mg) from Mitsubishi Kasei Co.; dextran sulfate (average molecular mass 500 000 Da), *p*-nitrophenyl *p*'-guanidinobenzoate, urokinase (20–30 U/mg) and human α_2 -antiplasmin from Sigma; aprotinin from Pentapharm Ltd.; benzimidazole from Aldrich Chemical Inc.; 6-amidino-2-naphthyl *p*-guanidinobenzoate dimethanesulfonate (nafamostat mesilate) from Torii Co. Ltd; human antithrombin III and bovine α_1 -proteinase inhibitor from Boehringer Mannheim GmbH; human C1-inhibitor from Cordis Lab. Single-chain HGF and the HGF activator were prepared as described (Shimomura et al., 1992; Miyazawa et al., 1993).

Digestion of single-chain HGF with proteases involved in blood coagulation and fibrinolysis. Single-chain HGF (200 μ g/ml) was incubated at 37°C for 2 h with proteases in 20 mM sodium phosphate, pH 7.3, containing 100 mM NaCl and 0.01% Chaps. After incubation, 50 μ l of the reaction mixture was analyzed by SDS/PAGE (12.5% acrylamide) under reducing conditions. The gel was stained with Coomassie brilliant blue.

Effects of serine protease inhibitors on HGF-converting activity. 5 μ l HGF activator (50 ng/ml) or factor XIIa (3 μ g/ml) was mixed with 5 μ l inhibitor solutions and 38 μ l 50 mM Tris/HCl, pH 8.0, containing 150 mM NaCl and 0.01% Chaps, then incubated at 37°C for 20 min. Thereafter, 2 μ l single-chain HGF was added to the mixture at a final concentration of 200 μ g/ml and further incubated at 37°C for 2 h. The products were analyzed by SDS/PAGE (12.5% acrylamide) under reducing conditions. The gel was stained with Coomassie brilliant blue and the bands were scanned using a densitometer.

Preparation of a monoclonal antibody which specifically inhibits the HGF-converting activity of HGF activator. mAbs were obtained as described previously (Shimomura et al., 1993). One of them, P1-4 mAb, inhibited the HGF-converting activity of the HGF activator. To assess the specificity of the inhibition, HGF activator (80 ng/ml) or factor XIIa (1.5 μ g/ml) was incubated with or without P1-4 mAb (20 μ g/ml or 40 μ g/ml) at 37°C for 5 min in 20 mM sodium phosphate, pH 7.3, containing 100 mM NaCl and 0.01% Chaps. After incubation, single-chain HGF was added to the incubated mixture at a final concentration of 200 μ g/ml and further incubated at 37°C for 2 h. The incubated mixtures (50 μ l) were analyzed by SDS/PAGE (12.5% acrylamide) under reducing conditions. The gel was stained with Coomassie brilliant blue.

Inhibition of HGF-converting activity in human serum by P1-4 monoclonal antibody. Human serum was diluted to 5% (by vol.) in 10 mM sodium phosphate, pH 7.4, containing 150 mM NaCl and 0.1% Chaps, and incubated with or without P1-4 mAb (150 μ g/ml) at 37°C for 30 min (total volume, 100 μ l). Single-chain HGF was then added at a final concentra-

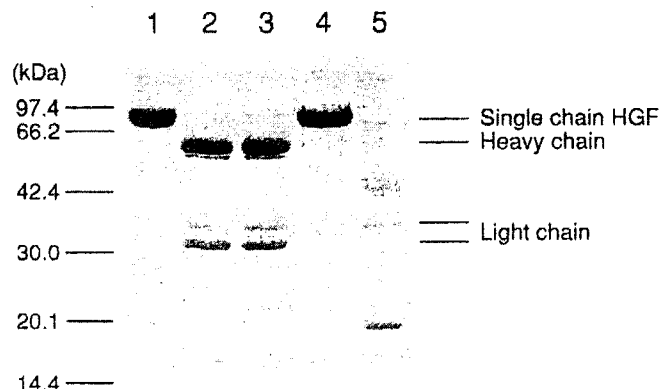


Fig. 1. Digestion of single-chain HGF with proteases. Single-chain HGF (200 μ g/ml) was incubated at 37°C for 2 h without proteases (lane 1) or with 80 ng/ml HGF activator (lane 2), 1.5 μ g/ml α -factor XIIa (lane 3), 10 μ g/ml urokinase (lane 4) or 10 μ g/ml plasmin (lane 5). After incubation, 50 μ l reaction mixture aliquots were analyzed by SDS/PAGE. Molecular-mass markers are shown in kilodaltons (kDa) on the left.

tion of 50 μ g/ml, and the mixture was further incubated at 37°C for 4 h. The reaction was terminated with 1.4 ml 50 mM Tris/HCl, pH 8.5, containing 150 mM NaCl, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 100 μ M nafamostat mesilate, 0.1% Chaps and 0.7 mg/ml rat liver soluble proteins as carriers. The mixture was loaded onto an S-Sepharose column (0.65 cm $\times$ 0.8 cm) equilibrated with 20 mM Tris/HCl, pH 8.5, containing 150 mM NaCl, 0.1% Chaps and 100 μ M nafamostat mesilate. The column was washed with 2 ml of the same buffer. HGF was eluted with 2 ml buffer containing 1 M NaCl. The eluate was concentrated by ultrafiltration (Centricon 30, Amicon) and analyzed by immunoblotting using an anti-(HGF heavy chain) monoclonal antibody as described previously (Miyazawa et al., 1994).

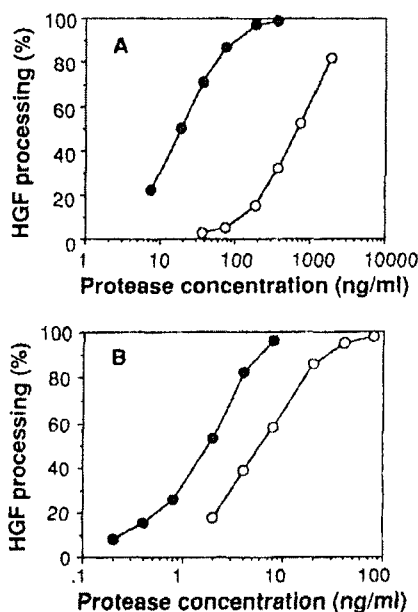
RESULTS

Conversion of single-chain HGF by coagulation factor XIIa. Single-chain HGF was incubated with various serine proteases involved in blood coagulation or fibrinolysis and the products were analyzed by SDS/PAGE (Fig. 1). Coagulation α -factor XIIa efficiently converted single-chain HGF to the heterodimer (Fig. 1, lane 3). This did not occur upon incubation with urokinase (Fig. 1, lane 4), tPA, plasma kallikrein, thrombin and factors IXa and XIa (data not shown), even when single-chain HGF (200 μ g/ml) was incubated with high concentrations of enzymes (10 μ g/ml). Factor Xa converted single-chain HGF to the heterodimer. However, high concentration (approximately 30 μ g/ml) of the enzyme was required for complete conversion (data not shown). Plasmin digested single-chain HGF, but generated smaller peptide fragments (Fig. 1, lane 5). To confirm that the α -factor XIIa digestion produced the active form of HGF, the N-terminal amino acid sequence of the band corresponding to the light chain generated by α -factor XIIa digestion was determined by the procedure described previously (Shimomura et al., 1992). The result showed that the sequence was identical to that of the light chain of the active heterodimeric form of HGF (data not shown), indicating that α -factor XIIa converted single-chain HGF to the active form.

To eliminate the possibility that the HGF-converting activity of α -factor XIIa is due to the presence of a small amount of

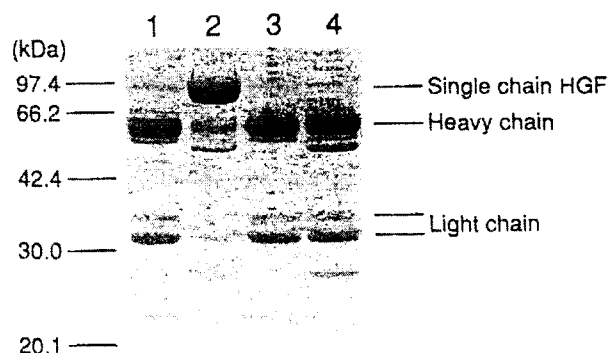
Table 1. The effects of serine protease inhibitors on the HGF-converting activity of α -factor XIIa and the HGF activator.

Protease inhibitor	Concentration	Inhibition	
		α -factor XIIa	HGF activator
		%	
Nafamostat mesilate	40 nM	1	33
	400 nM	10	100
<i>p</i> -Nitrophenyl <i>p</i> '-guanidinobenzoate	800 nM	19	31
	4 μ M	33	100
Aprotinin	25 KIU/ml	0	37
	100 KIU/ml	4	75
	500 KIU/ml	13	100
Benzamidine	1 mM	9	1
	10 mM	46	5

**Fig. 2.** Dose-dependence of the HGF-converting activity of the HGF activator and α -factor XIIa. Single-chain HGF (200 μ g/ml) was incubated at 37°C for 2 h with α -factor XIIa (A) or the HGF activator (B) in the presence (●) or absence (○) of dextran sulfate.

HGF activator in the α -factor XIIa preparation, the effects of serine protease inhibitors on the HGF-converting activity of α -factor XIIa and the HGF activator were examined (Table 1). Nafamostat mesilate, *p*-nitrophenyl *p*'-guanidinobenzoate and aprotinin effectively inhibited the HGF-converting activity of HGF activator, but were less effective on that of α -factor XIIa. In contrast, benzamidine was more effective on the activity of α -factor XIIa than that of the HGF activator. These results indicate that the HGF-converting activity is an intrinsic property of α -factor XIIa.

Comparison of HGF-converting activity of α -factor XIIa and the HGF activator. To compare the specific HGF-converting activities of α -factor XIIa and the HGF activator, the dose dependence of the activity was examined. Single-chain HGF was incubated with various concentrations of α -factor XIIa or the HGF activator purified from human serum and the conversion ratios were quantified (Fig. 2). Since the factor-XIIa-dependent activations of prekallikrein and factor XI are accelerated by

**Fig. 3.** Inhibition of HGF-converting activity by P1-4 monoclonal antibody. HGF activator (80 ng/ml) was incubated at 37°C for 5 min without (lane 1) or with (lane 2) P1-4 mAb (20 μ g/ml). α -factor XIIa (1.5 μ g/ml) was incubated at 37°C for 5 min without (lane 3) or with (lane 4) P1-4 mAb (40 μ g/ml). After incubation, single-chain HGF (200 μ g/ml) was added to the mixture and further incubated for 2 h. Thereafter, 50- μ l aliquots of the reaction mixtures were analyzed by SDS/PAGE. Molecular-mass markers are shown in kilodaltons (kDa) on the left.

negatively charged surfaces (Tans and Rosing, 1987), the effects of dextran sulfate on the HGF-converting activity were also examined. Dextran sulfate stimulated the activity of both α -factor XIIa and the HGF activator, and the stimulation was more prominent for α -factor XIIa. The HGF-converting activities of α -factor XIIa and the HGF activator were affected by the NaCl and dextran sulfate concentrations. Optimal NaCl and dextran sulfate concentrations were around 200 mM and 10 μ g/ml, respectively, for α -factor XIIa, and 120 mM and 50 μ g/ml, respectively, for the HGF activator. Thus, incubation proceeded under these optimal NaCl and dextran sulfate conditions. The concentrations for half-maximal activity of α -factor XIIa were 20 ng/ml in the presence of dextran sulfate and 700 ng/ml in the absence of dextran sulfate. These values were 2 ng/ml and 6 ng/ml, respectively, for the HGF activator. The molecular masses of α -factor XIIa and the HGF activator purified from human serum are 80 kDa and 34 kDa, respectively. Taking this into consideration, the specific activity of α -factor XIIa was approximately fourfold lower in the presence of dextran sulfate and approximately 50-fold lower in the absence of dextran sulfate than that of the HGF activator.

Limited proteolysis of the zymogen factor XII produces two active enzyme forms, α -factor and β -factor XIIa (Dunn et al., 1982). α -factor XIIa is comprised of two chains of 52 kDa and 28 kDa, whereas β -factor XIIa consists of two chains of 2 kDa and 28 kDa. β -factor XIIa exerted a similar HGF-converting activity to α -factor XIIa in the absence of dextran sulfate. However, the activity of β -factor XIIa was not stimulated in the presence of dextran sulfate (data not shown).

HGF-converting activity in human serum. Serum appears to be the major source of an HGF-converting enzyme (Naka et al., 1992; Naldini et al., 1992). HGF activator and factor XIIa are present in serum. Thus, we examined which enzyme is attributed to HGF-converting activity in human serum, using an antibody that specifically inhibits the HGF-converting activity of the HGF activator. We previously prepared two monoclonal antibodies against HGF activator (A6 and 7E10) for a sandwich-method enzyme-linked immunosorbent assay (Shimomura et al., 1993). However, they did not efficiently inhibit the HGF-converting activity of the HGF activator (data not shown). We, therefore, prepared other monoclonal antibodies and tested their ability to inhibit the HGF-converting activity (Fig. 3). Among six mo-

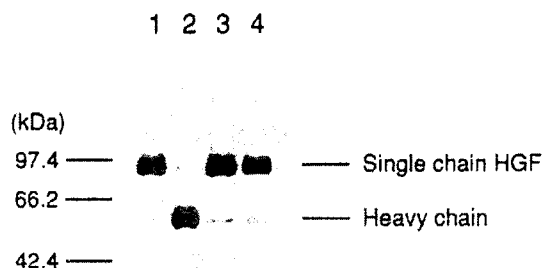


Fig. 4. Inhibition of HGF-converting activity in human serum by P1-4 mAb. Human serum was incubated without (lanes 1 and 2) or with P1-4 mAb (lanes 3 and 4) for 37°C for 30 min. After incubation, single-chain HGF was added to the mixture and incubated at 37°C for 0 h (lanes 1 and 3) or for 4 h (lanes 2 and 4). HGF was partially purified on an S-Sepharose column and immunoblotted by an anti-(HGF heavy chain) monoclonal antibody. Molecular-mass markers are shown in kilodaltons (kDa) on the left.

Table 2. The effects of serum serine protease inhibitors on the HGF-converting activity of α -factor XIIa and the HGF activator.

Protease inhibitor	Concentration	Inhibition	
		α -factor XIIa	HGF activator
		%	
Human antithrombin III (+ Heparin 5 U/ml)	3 IU/ml	93	0
Human C1-inhibitor	10 μ g/ml	100	0
Human α_2 -antiplasmin	40 μ g/ml	87	0
Bovine α_1 -proteinase inhibitor	100 μ g/ml	0	0

noclonal antibodies. P1-4 mAb most efficiently inhibited the HGF-converting activity of the HGF activator (Fig. 3, lane 2), but did not inhibit that of factor XIIa (lane 4). Thus, the P1-4 mAb was used to analyze the activity in human serum (Fig. 4). Human serum contained a high level of HGF-converting activity (Fig. 4, lane 2). This activity was almost completely abolished in the presence of P1-4 mAb (Fig. 4, lane 4). Thus, the HGF activator is the major HGF-converting enzyme present in human serum.

Effects of serum serine protease inhibitors on the HGF-converting activity of factor XIIa and the HGF activator. There are a number of serine protease inhibitors in serum which regulate the activities of serine proteases involved in blood coagulation and fibrinolysis. Among them, antithrombin III, C1-inhibitor and α_2 -antiplasmin inhibit the blood-clotting activity of factor XIIa (Tans and Rosing, 1987). We, therefore, examined the effects of these inhibitors on the HGF-converting activity of α -factor XIIa (Table 2). All these inhibitors efficiently inhibited the HGF-converting activity of α -factor XIIa and none of them prevented the activity of the HGF activator. Bovine α_1 -proteinase inhibitor did not inhibit the activity of either α -factor XIIa or the HGF activator.

DISCUSSION

We have previously identified the HGF activator, which efficiently activates single-chain HGF from human serum *in vitro*.

Its sequence is homologous to that of factor XII (Miyazawa et al., 1993). In this study, we found that factor XIIa has an ability to convert single-chain HGF to the active two-chain form. Factor XIIa exhibited a significant level of HGF-converting activity in the presence of dextran sulfate, although the specific activity of factor XIIa for the activity was slightly lower than that of the HGF activator. Factor XIIa plays a central role in the contact activation system (Tans and Rosing, 1987). Factor XII circulates in the blood as the inactive precursor (zymogen) of a serine protease. The initiation of contact activation reactions leads to the conversion of the zymogen factor XII into an active proteolytic enzyme (factor XIIa). The activated factor XIIa is then responsible for the initiation of the blood-coagulation cascade. The HGF activator also circulates in the blood as an inactive zymogen. Since the zymogen of the HGF activator is converted into the active form by thrombin (Shimomura et al., 1993), this conversion appears to be linked to blood coagulation. HGF is proteolytically activated by a serine protease, the activity of which is induced in the injured tissue (Miyazawa et al., 1994). Since tissue injury leads to the initiation of contact activation followed by blood coagulation, the zymogens of factor XIIa and the HGF activator are converted into active enzymes following tissue injury. The activated factor XIIa may function as an HGF-converting enzyme together with the activated HGF activator in the injured tissue.

Several serum protein inhibitors of serine proteases appear to be involved in the regulation of contact activation. C1-inhibitor, antithrombin III and α_2 -antiplasmin can inhibit the blood-clotting activity of factor XIIa (Tans and Rosing, 1987). These inhibitors were also effective against the HGF-converting activity of factor XIIa. Since the HGF-converting activity detected in serum was almost completely prevented by an antibody against the HGF activator, this activity is mainly attributed to the HGF activator. Thus, the HGF-converting activity of factor XIIa in serum appears to be inactivated by these serum protein inhibitors. The HGF-converting activity of the HGF activator was not prevented by the serum protein inhibitors, and the HGF activator was active in human serum. Thus, the HGF-converting activity of the HGF activator does not appear to be regulated by serum protein inhibitors.

Naldini et al. (1992) and Mars et al. (1993) reported that urokinase and tPA converted single-chain HGF to the two-chain form *in vitro*. However, high doses of enzymes were required to achieve complete conversion (approximately 10–100 ng urokinase or tPA to 1 ng single-chain HGF). We did not detect any HGF-converting activity in urokinase and tPA under our experimental conditions, in which 10 μ g/ml urokinase or tPA was used for 200 μ g/ml single-chain HGF. Similar results were obtained by Mizuno et al. (1992), showing that urokinase or tPA (1 μ g/ml) did not convert single-chain HGF (10 μ g/ml) to the two-chain form. In contrast, much lower doses of the HGF activator and factor XIIa were sufficient for complete conversion (approximately 10 ng/ml HGF activator or 200 ng/ml α -factor XIIa to 200 μ g/ml single-chain HGF). Thus, the specific activities of urokinase and tPA for the HGF-converting activity are more than 1000-fold lower than those of the HGF activator and factor XIIa. Urokinase and tPA may not be the enzymes responsible for activation of single-chain HGF in injured tissues. However, the enzymic reaction may be stimulated by a cofactor(s), such as dextran sulfate for the HGF activator and factor XIIa. In fact, soluble urokinase is less active in plasminogen activation than receptor-bound urokinase (Ellis et al., 1991). Thus, urokinase and tPA could efficiently function as HGF-converting enzymes *in vivo*.

The genes for HGF and its receptor are expressed in many tissues in adults (Okajima et al., 1990; Tashiro et al., 1990) and

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during the embryonic periods (Sonnenberg et al., 1993). HGF is thought to mediate a signal exchange between mesenchyme and epithelia during mouse development (Sonnenberg et al., 1993). Thus, a proteolytic activation system appears to be required during the development. Such a system exists in pattern formation of the *Drosophila* embryo (Hecht and Anderson, 1992). Extracellular serine proteases are involved in the generation of a localized extracellular ligand for a membrane receptor, which is required for the organization of the dorsal-ventral pattern of the *Drosophila* embryo. We identified HGF activator and factor XIIa, which may be involved in the generation of biologically active growth factor in injured tissues. Further study is required to determine whether or not these serine proteases also function as HGF-converting enzymes during mammalian development.

This work was supported in part by research grants from the Ministry of Education, Science and Culture of Japan and the Japan Private School Promotion Foundation.

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