Multiple Secretion of Matrix Serine Proteinases by Human Gastric Carcinoma Cell Lines

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ABSTRACT

Proteinase species secreted by 10 human gastric carcinoma cell lines were analyzed by gelatin zymography and immunoblotting. These cell lines were classified into the following three groups with respect to proteinase secretion: cell lines secreting mainly gelatinases A and/or B; those secreting multiple types of serine proteinases; and those scarcely secreting these enzymes. Two cell lines of the second group, STKM-1 and MKN28, hardly secreted metalloproteinases but secreted the following four types of serine proteinases: (a) two trypsin-like enzymes (Mr 72,000 or 64,000 gelatinase/type IV collagenase; EC 3.4.24.7), gelatinase A (Mr 72,000 or 64,000 gelatinase/type IV collagenase; EC 3.4.24.7), gelatinase B (Mr 92,000 or Mr 90,000 gelatinase/type IV collagenase; EC 3.4.24.35), andilystatin (EC 3.4.24.17), and matrilysin (matrin/pump-1; EC 3.4.24.23). We previously purified and characterized human gelatinases A and B (3), human matrilysin (4), rat stromelysin (5), and their inhibitor [tissue inhibitor of metalloproteinase 2 (TIMP-2)] (6) from a conditioned medium of tumor cells. These enzymes are secreted in latent proenzyme forms and hence must be activated for the expression of their activities. The activated enzymes potently degrade various extracellular matrix proteins such as collagens, fibronectin, laminin, and proteoglycans.

INTRODUCTION

The process of metastasis and invasion of tumor cells is thought to require the proteolytic degradation of extracellular matrix components. Recently, many studies have suggested that a family of metalloproteinases, often called "matrix metalloproteinases," plays an essential role in the matrix degradation (reviewed in Refs. 1 and 2). The matrix metalloproteinases include interstitial collagenase (EC 3.4.24.7), gelatinase A (Mr 72,000 or 64,000 gelatinase/type IV collagenase; EC 3.4.24.7), gelatinase B (Mr 92,000 or Mr 90,000 gelatinase/type IV collagenase; EC 3.4.24.35), stromelysin (EC 3.4.24.17), and matrilysin (EC 3.4.24.23). We previously purified and characterized human gelatinases A and B (3), human matrilysin (4), rat stromelysin (5), and their inhibitor [tissue inhibitor of metalloproteinase 2 (TIMP-2)] (6) from a conditioned medium of tumor cell lines. These enzymes are secreted in latent proenzyme forms and hence must be activated for the expression of their activities. The activated enzymes potently degrade various extracellular matrix proteins such as collagens, fibronectin, laminin, and proteoglycans.

In contrast to the metalloproteinases, only a few studies have been reported about the role of serine proteinases in tumor invasion/metastasis, except t-PA and u-PA, respectively. Both plasminogen activators are often secreted from tumors and malignantly transformed cells, and they can convert blood plasminogen to plasmin by limited proteolysis (7-9). It has been reported that plasmin not only directly degrades extracellular matrix proteins but also activates some metalloproteinase precursors (10-12). Recently, Koivunen et al. (13-15) purified two trypsinogen-like proteins from the serum-free conditioned medium of tumor cells and named them TAT-1 (Mr 25,000, pl 5) and TAT-2 (Mr 28,000, pl 4). TAT-1 and TAT-2 are very similar to human trypsins (1 and 2, respectively, in structure and enzymatic properties, but there are differences in their isoelectric points and substrate specificities.

Although various types of tumor cells have been studied concerning proteinase production, there have been few reports of studies on proteinase production by gastric cancer, which is the most common cancer in Costa Rica, Japan, Chile, and some other countries. In the present study, we examined proteinase secretion from 10 kinds of human gastric carcinoma cell lines using the zymographic analysis and found that two of them secreted high activities of four types of serine proteinases (two trypsin-like enzymes, a plasmin-like enzyme, a kallikrein-like enzyme, and a plasminogen activator). Some properties of a trypsin-like enzyme are also reported.

MATERIALS AND METHODS

Cells and Culture Conditions. Human gastric cancer cell lines tested for the secretion of proteinases were MKN1 (adenosquamous carcinoma), MKN28 (6TG-1, adenosquamous carcinoma), MKN45 (adenocarcinoma), MKN74 (adenocarcinoma), NUGC-2 (adenocarcinoma), NUGC-3 (adenocarcinoma), AZ-521 (unspecified), KATO III (signet ring cell carcinoma), SCH (chorionic carcinoma), and STKM-1 (adenocarcinoma). Cell lines were grown to confluence in 90-mm dishes containing 10 ml of RPMI 1640 supplemented with 15% FCS (HyClone, Logan, UT) (RPMI 1640 plus 15% fetal calf serum). Cells were cultured at 37°C in a humidified atmosphere of 5% CO2 and 95% air. RPMI 1640 supplemented with 15% FCS was used as the basal medium. Cultures were maintained in the basal medium supplemented with 10% fetal calf serum (HyClone, Logan, UT) (RPMI 1640 plus 10% fetal calf serum). Plastic culture dishes were generous gifts from Sumibe Medical (Tokyo, Japan), and plastic roller bottles were purchased from Becton Dickinson (Oxnard, CA).

Preparation of Serum-free Conditioned Medium of 10 Kinds of Gastric Carcinoma Cells. The human gastric carcinoma cell lines were grown to confluence in 90-mm dishes containing 10 ml of RPMI 1640 plus 10% fetal calf serum. Cultures were rinsed three times with Ca2+- and Mg2+-free Hanks' balanced salt solution and then replaced with 10 ml of serum-free RPMI 1640. After each culture was high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TAT, tumor-associated trypsinogen; t-PA, tissue-type plasminogen activator; PBS, phosphate-buffered saline; TAME, N-tosyl-L-arginine methyl ester hydrochloride; C1s, first component of complement.
incubated for 2 days, the serum-free conditioned medium was clarified by sequential centrifugation at 1,500 rpm for 15 min and at 19,000 rpm for 30 min. The supernatant was added with ammonium sulfate to a final saturation of 80% and allowed to stand overnight at 4°C. The resultant protein precipitate was collected by centrifugation at 19,000 rpm for 30 min, dissolved in and dialyzed against 10 mM Tris-HCl (pH 7.5) containing 0.1% (w/v) Brij-35, and used as concentrated conditioned medium.

Column Chromatographies. All procedures for proteinase purification, except reverse-phase HPLC, were performed at 4°C. Conditioned medium was prepared from confluent serum-free culture of STKM-1 cells in roller bottles and concentrated as described above. The concentrated conditioned medium was dialyzed against 20 mM Tris-HCl (pH 7.5) containing 0.5 mM NaCl and 0.01% (w/v) Brij-35 and subjected to molecular sieve chromatography on a Cellulofine GCL-2000m column (2.6 x 98 cm) (Chisso, Tokyo, Japan) as reported previously (4). Rabbit polyclonal antibody against human trypsin was chemically conjugated to protein A-Sepharose by an ImmunoPure IgG Orientation kit (Pierce, Rockford, IL). The proteinase sample was applied to the antibody-conjugated column (0.8 x 4.0 cm), previously equilibrated with 20 mM Tris-HCl (pH 7.5) containing 0.01% Brij-35. After the column was washed with the buffer, the adsorbed proteinases were eluted with 10 ml of 0.1 M glycine (pH 2.5) at a flow rate of 8 ml/h. The eluted proteinase fraction was dialyzed against 0.05% (v/v) trifluoroacetic acid. The dialyzed material was subjected to reverse-phase HPLC on a SynChrom RP-4 column (0.41 x 25 cm) (SynChrom), pre-equilibrated with 0.05% trifluoroacetic acid, and eluted with a linear gradient of 0–80% acetonitrile in 30 ml of 0.05% trifluoroacetic acid at a flow rate of 0.5 ml/min.

SDS-Polyacrylamide Gel Electrophoresis and Zymographic Analysis of Proteinases. Unless otherwise noted, SDS-PAGE and gelatin zymography of proteinases were carried out on 12.5% polyacrylamide slab gels (90 mm long, 90 mm wide, 0.75 mm thick) under nonreducing conditions as described previously (3–6). For the zymography, proteinases separated on the gels containing 1 mg/ml gelatin were renatured and then incubated in 50 mM Tris-HCl (pH 7.5) with or without 10 mM CaCl2 at 37°C for 18 h. The gels were stained with Coomassie Brilliant Blue R-250. The molecular weight markers used are rabbit muscle phosphorylase b (M, 97,400), bovine serum albumin (M, 66,200), hen egg albumin (M, 42,700), bovine carbonic anhydrase (M, 29,000), soybean trypsin inhibitor (M, 21,500), and hen egg lysozyme (M, 14,000).

Zymography of plasminogen activator was performed on 1.25% (w/v) agarose plates containing 2 mg/ml fibrin and 5 μg/ml human plasminogen as described before (17). Sample proteins were separated by nonreducing SDS-PAGE, and the resultant SDS-polyacrylamide gel was washed with 2.5% (v/v) Triton X-100, layered on the fibrin-agarose plate, and incubated overnight at 37°C. The activity of plasminogen as described before (17). Sample proteins were separated by nonreducing SDS-PAGE, and the resultant SDS-polyacrylamide gel was washed with 2.5% (v/v) Triton X-100, layered on the fibrin-agarose plate, and incubated overnight at 37°C. The activity of plasminogen activator was visualized as a transparent fibrinolysis band.

Immunoblotting Analysis. Sample proteins were separated by SDS-PAGE. After the electrophoresis, the proteins separated on the gels were transferred onto nitrocellulose filters using a Bio-Rad Mini Trans-Blot apparatus (Richmond, CA), according to the method of Towbin et al. (18). The blotted filters were blocked with PBS containing 5% (w/v) skim milk at 37°C for 2 h, washed with a mixture of PBS and 0.05% Tween 20 (Tween-PBS), and then incubated overnight at room temperature with the first antibody, which had been diluted 1000-fold with PBS. After a washing with Tween-PBS, the filters were incubated with a 1000-fold diluted biotinylated anti-rabbit or anti-goat IgG antibody (Vector Laboratories), washed with Tween-PBS, and then incubated with avidin–alkaline phosphatase (Vector) at room temperature for 1 h. The filters were washed with Tween-PBS and then incubated at room temperature for about 5 min in a reaction mixture containing 5-bromo-4-chloro-3-indolylphosphate and nitro blue tetrazolium to develop colored product on the filters.

Activity Assay of Trypsin-like Enzyme. The purified proenzyme form of trypsin-like enzyme was activated by incubating with bovine enterokinase (1:1, w/w) in 28 mM succinate buffer (pH 5.6) containing 1 mM CaCl2 at room temperature for 20 min. The activated enzyme was incubated in a reaction mixture containing 10 mM TAME and 46 mM Tris-HCl (pH 8.1) at 30°C, and the increase of absorbance at 247 nm was measured with a spectrophotometer.

Determination of Protein Concentration. Protein concentration was determined by the dye method with a Bio-Rad protein assay kit, using bovine serum albumin as the standard.

RESULTS

Secretion of Proteinases from Human Gastric Carcinoma Cell Lines. Serum-free culture medium conditioned by 10 kinds of human gastric cancer cell lines was assayed for gelatinolytic activity by gelatin zymography in the presence and absence of Ca2+. In the absence of Ca2+, two cell lines, MKN28 and STKM-1, showed strong and heterogeneous gelatinolytic bands (Fig. 1A). Three bands with molecular weights of 70,000, 26,000, and 24,000 were common to both cell lines. The Mr 26,000 activity was separated into three distinct bands when a smaller amount of the sample was analyzed. Additional activities were observed at Mr, 33,000 and 28,000 in MKN28 and at Mr, 150,000 and 45,000 in STKM-1. Such gelatinolytic activities, especially those of Mr, 150,000, 45,000, 33,000, and 28,000 bands, varied considerably among different preparations of the respective conditioned media, suggesting that some of them might be proteolytic fragments of other proteinases. The Mr, 150,000 activity was detected in different preparations of the MKN28 conditioned medium. The Mr, 70,000 activity was detected at lower levels in the conditioned media of MKN1, MKN45, NUGC-3, and KATO III.

When the reaction mixture was supplemented with 10 mM Ca2+, two gelatinolytic activities at bands with molecular weights of 90,000 and 64,000 appeared in the conditioned media of four cell lines: the Mr, 64,000 activity in SCF, the Mr, 90,000 activity in NUGC-3, and both activities in MKN1 and MKN74 (Fig. 1B). When the effects of various proteinase inhibitors on these activities were examined, 1 mM 1,10-phenanthroline, but not inhibitors for serine, cysteine, and aspartic proteinases, completely inhibited the activities of Mr, 64,000 and 90,000 bands, showing that both gelatinolytic enzymes were metalloproteinases (data not shown). The electrophoretic mobilities of the Mr, 64,000 and Mr, 90,000 enzymes in zymography were identical to those of gelatinase A (Mr, 72,000 gelatinase) and gelatinase B (Mr, 92,000 gelatinase), respectively, which we had previously isolated from the conditioned medium of human schwannoma cell line (3) (data not shown). These results indicated that the Mr, 64,000 and Mr, 90,000 gelatinolytic activities corresponded to gelatinases A and B, respectively.
Fig. 1. Zymographic analysis of gelatinolytic enzymes secreted from 10 kinds of gastric carcinoma cells. The serum-free conditioned media of the 10 carcinoma cell lines were concentrated 30-fold by ammonium sulfate precipitation, and aliquots (10 µl) of the concentrated conditioned media were subjected to zymography on gelatin-containing gels. After electrophoresis, proteins on the gels were renatured and then incubated at 37°C for 18 h in the reaction mixture without (A) or with (B) 10 mM CaCl₂. Left to right, MKN1, MKN28, MKN45, MKN74, NUGC-2, NUGC-3, AZ-521, KATO III, STKM-1, and SCH. Ordinate, molecular weight in thousands. Arrows, positions of gelatinolytic enzymes with molecular weights of 150,000, 70,000, 26,000, and 24,000 in A, and gelatinases A (M, 64,000) and B (M, 90,000) in B. Other experimental conditions are given in the text.

Partial Characterization of Ca²⁺-independent Proteinases. To classify the Ca²⁺-independent proteinases, the effects of various proteinase inhibitors on their gelatinolytic activity were tested by gelatin zymography of the STKM-1-conditioned medium. Diisopropylfluorophosphatase effectively inhibited all gelatinolytic activities including those of Mr 150,000, 70,000, 26,000, and 24,000 (Fig. 2). Leupeptin, but not chymostatin, inhibited these activities, indicating that they had trypsin-like sensitivity to the proteinase inhibitors (data not shown). These activities were not affected by 1,10-phenanthroline (a metalloproteinase inhibitor), p-aminophenyl mercuric acetate (a cysteine proteinase inhibitor), and pepstatin (an aspartic proteinase inhibitor) (data not shown). When the STKM-1-conditioned medium was subjected to zymography on a casein-containing gel, proteolytic bands were observed at the same positions as on the gelatin-containing gel (data not shown), indicating that the serine proteinases with molecular weights of 150,000, 70,000, 26,000, and 24,000 could digest both gelatin and casein.

In order to identify these serine proteinases, the concentrated conditioned medium of STKM-1 was analyzed by immunoblotting analyses with antibodies against some serine proteinases. Polyclonal antibody against human trypsin showed two immunochemically stained bands with Mr 26,000 and 24,000 under nonreducing conditions, suggesting that the Mr 26,000 and 24,000 gelatinolytic enzymes were trypsin-like enzymes (see Fig. 6). These enzymes were further characterized as described later.

When human plasmin and the STKM-1-conditioned medium were run on the same gelatin-containing gel under nonreducing conditions, plasmin showed a major gelatinolytic band with the same mobility as the Mr 70,000 activity of the conditioned medium (Fig. 3). Plasmin showed two minor additional bands with molecular weights of 45,000 and 33,000. These results suggested that the Mr 70,000 proteinase secreted from STKM-1 and other carcinoma lines might be plasmin and that the minor activities of Mr 45,000, 33,000, and 28,000 bands in the conditioned media of STKM-1 and/or MKN28 were due to plasmin fragments. Immunoblotting analyses with antibodies against human plasminogen and human tissue kallikrein showed multiple immunostained bands and were unable to identify specific bands (data not shown).

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The activity of the Mr 70,000 proteinase secreted from STKM-1 cells did not significantly change during at least 2 weeks in serum-free culture with 4 medium changes (data not shown). This ruled out the possibility that the enzyme was a contaminant from fetal calf serum which had been used in the initial cell plating. The activity of plasminogen, which showed
hardly showed gelatinolytic activity on zymography, immunoblotting analyses with anti-u-PA and anti-t-PA polyclonal antibodies and zymography on a fibrin-agarose plate were carried out with the STKM-1-conditioned medium. The anti-u-PA antibody showed a major immunostained Mr, 57,000 band at the same position as control two-chain u-PA (Fig. 4, Lane 1). The fibrin zymography in the presence of plasminogen showed a major fibrinolytic band at Mr, 57,000 (Fig. 4, Lane 5). The activity was not detected when the same volume of the conditioned medium was analyzed on the fibrin-agarose plate without plasminogen. These results revealed that STKM-1 cells secreted u-PA. Any specific band was not detected by immunoblotting with the anti-t-PA antibody (Fig. 4, Lane 3). In contrast to the STKM-1-conditioned medium, the conditioned medium of MKN28 cells exhibited an immunostained band at Mr, 70,000 on immunoblotting analysis with the anti-t-PA antibody but no band resulting from the analysis with the anti-u-PA antibody (data not shown).

Purification of Trypsin-like Proteinases. STKM-1 cells were cultured in serum-free RPMI 1640 medium. The resultant conditioned medium (2.6 liters) was concentrated by ammonium sulfate precipitation and subjected to molecular sieve chromatography on a Cellulose GCL-2000m column (Fig. 5). The fractions of Mr, 26,000 and 24,000 proteins (fractions 58–70), which also contained a part of the Mr, 70,000 and 150,000 activities, were applied to an immunoaffinity column conjugated with anti-trypsin polyclonal antibody. Most of proteins passed through the column, whereas the majority of the serine proteinase activities of Mr, 24,000, 26,000, 70,000, and 150,000 bands were bound to the column and eluted with 0.1 M glycine (pH 2.5). When analyzed by SDS-PAGE under nonreducing conditions, the eluted fraction showed a major band at Mr, 24,000 and a minor band at Mr, 26,000 (Fig. 6, Lane 1). Gelatin zymography of the eluted fraction showed two strong bands with molecular weights of 26,000 and 24,000 corresponding to a gelatinolytic band with a molecular weight of 78,000 on zymography (data not shown), was hardly detected with the STKM-1-conditioned medium.

We also attempted to detect plasminogen activators in the conditioned medium of STKM-1 cells. Since u-PA and t-PA hardly showed gelatinolytic activity on zymography, immunoblotting analyses with anti-u-PA and anti-t-PA polyclonal antibodies and zymography on a fibrin-agarose plate were carried out with the STKM-1-conditioned medium. The anti-u-PA antibody showed a major immunostained Mr, 57,000 band at the same position as control two-chain u-PA (Fig. 4, Lane 1). The fibrin zymography in the presence of plasminogen showed a major fibrinolytic band at Mr, 57,000 (Fig. 4, Lane 5). The activity was not detected when the same volume of the conditioned medium was analyzed on the fibrin-agarose plate without plasminogen. These results revealed that STKM-1 cells secreted u-PA. Any specific band was not detected by immunoblotting with the anti-t-PA antibody (Fig. 4, Lane 3). In contrast to the STKM-1-conditioned medium, the conditioned medium of MKN28 cells exhibited an immunostained band at Mr, 70,000 on immunoblotting analysis with the anti-t-PA antibody but no band resulting from the analysis with the anti-u-PA antibody (data not shown).

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Substrate specificity of the autolytically activated $M_r$ 24,000 enzyme was examined against two cell-adhesive glycoproteins and four types of pepsin-treated collagens (Fig. 8). The enzyme potently degraded fibronectin and laminin, while it partially degraded the four collagens. Type IV and V collagens were more susceptible to the enzyme than type I and III collagens.

**Immunohistochemical Identification of Tissue Kallikrein-like Enzyme and Plasmin-like Enzyme.** As shown in Fig. 6, Lane 2, the proteinase fraction eluted from the anti-trypsin antibody

the protein bands (Fig. 6, Lane 2). Together with the SDS-PAGE result, this analysis indicated that the $M_r$ 26,000 enzyme had a higher specific activity than the $M_r$ 24,000 enzyme. The relative increase of the $M_r$ 24,000 activity compared to the $M_r$ 26,000 activity seemed due to the partial leakage of the latter activity from the affinity column. Both proteins were strongly stained by immunoblotting with anti-trypsin antibody (Fig. 6, Lane 3).

The gelatin zymography of the proteinase fraction showed additional gelatinolytic bands such as two bands at $M_r$ 66,000 and 70,000 and a broad band on the top of the gel ($M_r > 150,000$) (Fig. 6, Lane 2), although their corresponding proteins were not detected by protein staining (Fig. 6, Lane 1). These gelatinolytic activities were further characterized by immunoblotting analysis as described later (see Fig. 9).

The proteinase fraction from the immunoaffinity column was further subjected to reverse-phase HPLC on a C4 column. This chromatography separated the $M_r$ 24,000 protein from the $M_r$ 26,000 protein and other proteinases. The purified protein hardly hydrolyzed TAME, the synthetic substrate of trypsin. When treated with enterokinase, the natural activator of trypsinogen, it effectively hydrolyzed TAME; the approximate specific activities (units/mg protein) before and after the treatment were 12 and 110, respectively. This indicated that the $M_r$ 24,000 protein was a proenzyme (zymogen). The enterokinase-activated enzyme showed the same mobility as trypsin on SDS-PAGE; its estimated molecular weight was 23,000 under non-reducing conditions ($M_r$ 27,000 under reducing conditions) (Fig. 7). The $M_r$ 24,000 proenzyme was autolytically activated when incubated in a pH 7.5 solution at 37°C for more than 1 h. These results showed that the $M_r$ 24,000 protein was identical or very similar to trypsinogen. Analysis by isoelectric focusing on a thin layer polyacrylamide gel showed that the pl of the $M_r$ 24,000 protein was 6.6 (data not shown).
column contained gelatinolytic activities with $M_r$ 66,000, 70,000, and >150,000 in addition to the trypsinogen-like proteins with $M_r$ 24,000 and 26,000. It appeared that during the affinity chromatography the $M_r$ 150,000 enzyme present in the pooled fraction from the molecular sieve chromatography was aggregated to show a broad band on the top of the SDS-gel, while the plasmin-like enzyme of $M_r$ 70,000 partly converted to a lower molecular form of $M_r$ 66,000 by limited proteolysis. To identify these gelatinolytic enzymes, their reactivity with polyclonal antibodies against tissue- and plasma-types kallikreins, plasminogen, elastase, and C1s was examined by immunoblotting analysis.

The anti-plasminogen antibody exhibited strong doublet bands at $M_r$ 60,000 and 56,000 and some minor bands under reducing conditions (Fig. 9, Lane 1). This profile is very similar to that of u-PA-activated human plasmin (Fig. 9, Lane 2). These immunostained bands appeared to correspond to the large subunits of Glu- and Lys-plasmins ($M_r$ 60,000 and 56,000), their proteolytic fragments ($M_r$ 45,000 and 43,000), their small subunit ($M_r$ 23,000, and intact plasminogen ($M_r$ 83,000).

The antibody against tissue-type kallikrein showed a strongly stained, broad band on the top of the immunoblot under non-reducing conditions (Fig. 9, Lane 3). When the sample was reduced by 2-mercaptoethanol, the immunoblot showed a sharp, single band with $M_r$ 50,000 (Fig. 9, Lane 4), which was consistent with the reported molecular weight value of tissue-type kallikrein (19). These results indicated that the $M_r$ 150,000 gelatinolytic activity found in the STKM-1 conditioned medium was identical or closely related to tissue-type kallikrein and that the $M_r$ 150,000 and higher molecular weight forms might be produced by self-aggregation or binding with a carrier protein.

The antibodies against human elastase, human plasma kallikrein, and human C1s did not show immunostained band with the serine proteinase fraction used in the above experiments (data not shown). The two trypsinogen-like proteins, the plasmin-like protein and the tissue-kallikrein-like protein, were also detected immunocohemically in the proteinase fraction obtained from the conditioned medium of MKN28 cells by the antitrypsin antibody column chromatography (data not shown).

**DISCUSSION**

In the present study we revealed proteinase species secreted by 10 human gastric carcinoma cell lines using gelatin zymography and immunoblotting analysis. These cell lines were divided into the following three groups with respect to proteinase secretion: (a) cell lines secreting gelatinases A and/or B as major components; (b) those secreting multiple serine proteinases; and (c) those scarcely secreting these enzymes. The two cell lines STKM-1 and MKN28, which belonged to the second group, secreted at least four types of serine proteinases: (a) two trypsin-like enzymes ($M_r$ 26,000 and 24,000 in proenzyme forms); (b) a tissue kallikrein-like enzyme; (c) a plasmin-like enzyme; and (d) a plasminogen activator. Such cancer cell lines have not been reported before.

Trypsinogen is normally synthesized in the pancreas, and its activated form trypsin nonspecifically digests various proteins. Koivunen et al. (15) have immunologically detected TAT-1 and/or TAT-2 in the conditioned media of three fibrosarcoma cell lines, a colon carcinoma cell line, and an erythroleukemia cell line. The trypsin-like proteins with molecular weights of 24,000 and 26,000 secreted by STKM-1 cells are likely to correspond to TAT-1 and TAT-2, respectively. Although TAT-1 and TAT-2 have been believed to be distinct from trypsinogen I and trypsinogen 2, respectively, the possibility seems to remain that they are the modified forms of trypsinogen 1 and 2. Further structural analysis is required to prove this possibility. The present study, together with the studies of Koivunen et al., demonstrates that trypsin-like enzymes are ectopically synthesized and secreted by various kinds of tumor cells.

Kallikreins are classified into plasma kallikrein and tissue kallikrein (or glandular kallikrein), the latter of which is rich in exocrine glands such as the salivary gland and pancreas. Tissue kallikrein is a member of a closely related gene family. This family consists of tissue kallikrein and at least three structurally related proteins in humans. Besides the exocrine tissues, tissue kallikrein or its related proteins or their mRNA have been found in the kidney, arterial walls, prostate, brain, colon, spleen, heart, plasma, and urine (reviewed in Ref. 20). However, production and secretion of kallikrein by cancer cells have rarely been reported except for pancreatic carcinoma cells (21). Kallikrein produces the vasodilator kinin from kininogen by limited proteolysis. Kinin is involved in various pathophysiological processes such as hypertension, inflammation, and allergy. Although kallikrein is known to hydrolyze other biologically important peptides such as pro-insulin, atrial natriuretic factor, and vasoactive intestinal peptides (20), its direct effect on extracellular matrix proteins has not been reported.
We have obtained the data that porcine urinary tissue kallikrein (Sigma) potently hydrolyzes laminin and fibronectin and to lesser extents gelatin and type I, III, and IV collagens (data not shown). In the present study, the gelatinolytic activities with $M_r 150,000$ or higher present in the STKM-1 conditioned medium and its chromatographic fractions were attributed to tissue kallikrein or its related proteinase. Such high molecular weight forms of tissue kallikrein have not been reported before (19, 20). However, human urinary kallikrein ($M_r 42,000$), which is smaller than pancreatic tissue kallikrein ($M_r 55,000$) because of the difference in the sugar moiety (19), is known to form a SDS-stable complex at $M_r 92,000$ with a specific binding protein (serpin) at $M_r 60,000$ (22, 23). It seems likely that the tumor-derived tissue kallikrein forms a larger complex with an unidentified binding protein. In addition, there is another possibility that the $M_r 150,000$ form and other high molecular weight forms of tissue kallikrein are oligomeric or polymeric forms.

STKM-1 and MKN28 cells also secreted a high gelatinolytic activity with $M_r 70,000$ into culture media. Immunoblotting analysis suggested that the enzyme might be plasmin, although the possibility cannot be ruled out that it is a different trypsin-type proteinase. We could not detect plasminogen in their conditioned media by gelatin zymography, although a plasminogen-like protein was slightly observed by immunoblotting analysis. It seems likely that plasminogen had been mostly converted to plasmin by an endogenous plasminogen activator, the latter of which was also secreted from these cells. Plasmin has been reported to degrade extracellular glycoproteins and basement membrane type IV collagen (10). There are many studies suggesting an important role of the PA/plasmin system in tumor invasion and metastasis (10, 12, 24). To our knowledge, however, the synthesis and secretion of plasminogen by nonhepatic tumors have not been reported before. It should be noted that a similar plasmin-like activity was detected in 5 of the 10 gastric carcinoma cell lines examined. Sakiyama et al. (25, 26) have isolated an extracellular matrix-degrading $M_r 88,000$ serine proteinase from the conditioned medium of malignant hamster fibroblasts and identified it as Cls. The $M_r 70,000$ proteinase secreted by STKM-1 cells was clearly distinguished from human Cls by electrophoretic mobility on zymography, and the former hardly reacted with monoclonal antibodies against human Cls on immunoblotting analysis (data not shown).

The three enzymes trypsin, tissue-type kallikrein, and plasmin can proteolytically activate each other’s proenzymes, and the activated enzymes directly degrade extracellular matrix proteins. Moreover, they can activate the proenzymes of some matrix metalloproteinases, which also hydrolyze the matrix proteins (5, 11, 12). The two carcinoma cell lines STKM-1 and MKN28 secreting these enzymes or their related enzymes are expected to exhibit a high matrix-degrading activity in vivo. Indeed, we have preliminarily observed that STKM-1 cells exhibit invasive growth in the peritoneal cavity of SCID mice and in vitro invasion through the reconstituted basement membrane (Matrigel) in Boyden chambers. Under culture conditions, both cell lines showed very poor cell-to-cell and cell-to-substratum interactions compared to other cell lines, except KATO III. KATO III cells grew in suspension in spite of the poor secretion of proteinases. Further studies are needed to clarify the relationship between the matrix-invasive potential of the gastric carcinoma cell lines and the proteinase species secreted by them.

In preliminary studies we have detected the $M_r 24,000$ and $26,000$ trypsinogen-like activities and the $M_r 70,000$ plasminogen-like activity in the conditioned media of many human cancer cell lines other than gastric carcinomas but to lesser extents than STKM-1 and MKN28. We have not been able to show the secretion of the tissue kallikrein-like enzyme in cancer cell lines other than the two gastric carcinoma cell lines on gelatin zymography. Immunochemical analysis with anti-kallikrein antibodies having a high titer may reveal its wider distribution.

As summary, the present study, together with the preliminary results, suggests the possibilities that in some kinds of tumor cells these serine proteinases, which may be called “matrix serine proteinases,” play a major role in the matrix degradation and subsequent tumor cell invasion, and that in some other tumor cells they act as the activator of the latent proenzymes of the matrix metalloproteinases.

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Multiple Secretion of Matrix Serine Proteinases by Human Gastric Carcinoma Cell Lines

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