Inhibition of Membrane-Type Serine Protease 1/Matriptase by Natural and Synthetic Protease Inhibitors

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(Received August 26, 2002)

Summary Membrane-type serine protease 1 (MT-SP1), identical to matriptase, is a recently identified type II transmembrane serine protease. MT-SP1/matriptase is of considerable interest for the development, homeostasis, and cancer invasion and metastasis of epithelial tissues. The administration of inhibitors for MT-SP1/matriptase may be effective to suppress the development of tumors where the enzyme may be involved. In the present study, we produced a secreted form of recombinant MT-SP1/matriptase (ekMT-SP1s) that can be activated by enterokinase in vitro and investigated the inhibitory ability of various protease inhibitors toward the recombinant enzyme. The enterokinase-treated ekMT-SP1s (active ekMT-SP1s) cleaved various peptidyl-4-methylcoumaryl-7-amide (MCA) substrates with arginine (or lysine) residue at position P1, and the best substrate was t-butyloxycarbonyl (Boc)-Gln-Ala-Arg-MCA. The specificity for the synthetic and natural substrates of the active ekMT-SP1s was in good agreement with that of the natural enzyme. Endogenous protease inhibitors tested, except for antithrombin III, showed no or little inhibition on the cleavage of Boc-Gln-Ala-Arg-MCA by the active ekMT-SP1s. Aprotinin showed strong inhibitory activity toward the cleavage. Food-derived inhibitors, such as soybean trypsin inhibitor, Bowman-Birk inhibitor, and lima bean trypsin inhibitor inhibited it, while chicken ovomucoid did not. Synthetic inhibitors tested inhibited it, and among them, the inhibitory effect of FOY 305 was strongest. The present findings provide important information for the suppression of cancer invasion and metastasis for which MT-SP1/matriptase is responsible.

Key Words membrane-type serine protease 1 (MT-SP1)/matriptase, protease inhibitors, suppression of tumor development

It is known that protease inhibitors suppress the development of tumors in several different tissues such as the colon, liver, lungs, esophagus, and cheek pouch (1–4). Although the mechanism of action of protease inhibitors as anticarcinogenic agents is poorly understood, it is likely that they are reversing the events in tumor development, presumably by stopping the ongoing processes. In fact, the effects of protease inhibitors that are thought to be related to anticarcinogenic activity include the ability to affect the levels of certain types of proteolytic activities (1). It has, however, remained unclear what types of proteases are responsible for tumor development that can be suppressed by the administration of protease inhibitors.

Membrane-type serine protease 1 (MT-SP1), identical to matriptase, is a recently identified type II transmembrane serine protease (Fig. 1) (5, 6). In vitro, MT-SP1/matriptase has been shown to cleave and activate single chain-urokinase type plasminogen activator (uPA) (7–9), activate protease-activated receptor-2 (PAR-2) (7), cleave the precursor form of hepatocyte growth factor (HGF) to produce its active form (8), and digest extracellular matrix proteins directly (9). The identification of these molecules as putative in vivo substrates suggests that MT-SP1/matriptase physiologically regulates the functions mediated by these molecules, such as cell adhesion and/or migration. It has also been shown that the activity of MT-SP1/matriptase is inhibited by hepatocyte growth factor activator inhibitor-1 (HAI-1) (10). HAI-1 is found predominantly in the columnar epithelium of many tissue types and is upregulated in injured or regenerative tissues (11). HAI-1 has been considered to suppress the growth and motility of carcinoma cells by inhibiting the generation of active uPA by MT-SP1/matriptase, active HGF by MT-SP1/matriptase or hepatocyte growth factor activator, or by inhibiting the activity of additional unidentified serine proteases. Recently, HAI-1 immunoreactivity in human primary colorectal carcinomas was found to be decreased significantly in cells within colon carcinomas.
relative to adjacent normal mucosa or adenomas (12). In addition, it has very recently been reported that the increased expression of MT-SP1/matriptase occurs in advanced colorectal cancers possibly because the enzyme is stabilized by glycosylation in which β1-6-N-acetylglucosaminyltransferase is involved (13). These findings indicate an imbalance in the MT-SP1/matriptase: HAI-1 ratio could be critical in the development of tumors. At least, it is believed that tumor development responsible for such an imbalance may be suppressed by the administration of inhibitors for MT-SP1/matriptase. Thus, the investigation of MT-SP1/matriptase inhibitors would be of importance.

In the present study, we investigated natural and synthetic inhibitors for MT-SP1/matriptase. Inhibition of the enzyme by protease inhibitors was determined using a secreted form of recombinant MT-SP1/matriptase. Cleavage of a peptidyl-4-methylcoumaryl-7-amide (MCA) substrate by recombinant MT-SP1/matriptase was inhibited by plant-derived natural inhibitors but not by an animal-derived inhibitor, ovomucoid, or endogenous protease inhibitors such as pancreatic secretory trypsin inhibitor. A synthetic trypsin inhibitor, FOY-305 strongly inhibited it. The administration of plant-derived inhibitors or some synthetic inhibitors may be effective for the suppression of cancer invasion and metastasis, at least in which MT-SP1/matriptase may be involved.

**MATERIALS AND METHODS**

*Materials.* COS-1 monkey kidney cells were obtained from the Health Science Research Resources Bank (Osaka, Japan). Soybean trypsin inhibitor (SBTI, type I-S), Bowman-Birk inhibitor (BBI), lima bean trypsin inhibitor (LBTI), chicken ovomucoid, α1-antitrypsin, α2-macroglobulin, and 9-aminocaproic acid were purchased from Sigma (St. Louis, MO, USA). Aprotinin and a recombinant enterokinase were purchased from Takara Shuzo Co. (Kyoto, Japan). Antithrombin III and phenylmethylsulfonyl fluoride were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). FOY-305 and human recombinant pancreatic secretory trypsin inhibitor (PSTI) were gifts from Mochida Pharmaceutical Co. (Tokyo, Japan). Various peptidyl-4-methylcoumaryl-7-amide substrates and leupeptin were purchased from Toyobo (Osaka, Japan). The synthetic oligonucleotides used were purchased from ESPEC Oligoservice Inc. (Ibaraki, Japan). Restriction endonucleases and protein size marker (broad range) were purchased from Toyobo (Osaka, Japan). KODdash® used for polymerase chain reaction (PCR) amplification, and other enzymes used for vector construction were purchased from Toyobo (Osaka, Japan). Other reagents used were of analytical grade.

*Production of recombinant MT-SP1/matriptase (ekMT-SP1s).* A secreted form of recombinant rat MT-SP1/matriptase named ekMT-SP1s, in which the activation cleavage motif, T610KQAR614, of the wild-type MT-SP1/matriptase was substituted to DDDDK, the enterokinase cleavage motif, was prepared in the present study (Fig. 1). The vector construction was performed as follows. PCR amplification was carried out using plasmid pT7-MT-SP1s (9) as the template and two sets of primers 5′-acgacaagGTGGTGTTGCGACG-3′, 5′-GAATCTGATCTGAGTC-3′ and 5′-TAGTGGACCACGACTTCA-3′, 5′-cgtcatcAAAGATGCCAGCCCAC-3′. The two PCR products were blunt-ended with T4 DNA polymerase, digested with AlwNI, and then ligated to each other to generate plasmid pT7-eMT-SP1s. A BamHI digested 2.0 kb fragment of pT7-eMT-SP1s was ligated into pSec-MT-SP1s digested by BamHI to generate pSec-eMT-SP1s. The procedure for the production and purification of ekMT-SP1s was essentially the same as done on a secreted form of recombinant MT-SP1/matriptase named MT-SP1s (9) except for the final preparation of the active form of ekMT-SP1s. The purified latent ekMT-SP1s was incubated with a recombinant enterokinase for 16h at room temperature, and then the enterokinase was removed by Enterokinase Capture Agarose® (Novagen Inc., Madison, WI, USA).

**Enzyme and inhibitor assays.** The substrate specificity of the ekMT-SP1s was determined as described previously (9). For the inhibitor assay, t-butyloxyccar-
bonyl (Boc)-Gln-Ala-Arg-MCA substrate was used. Aliquots (1 ng) of the enterokinase-treated ekMT-SP1s were preincubated with each inhibitor in a buffer (25 mM HEPES, pH 7.5, 145 mM NaCl, and 0.1% Triton X-100) for 10 min at 37°C in the final volume of 80 μL. The reaction was initiated by adding the substrate to a final concentration of 125 μM. After incubation for 20 min, the reaction was terminated by adding 350 μL of 0.1 M sodium acetate buffer, pH 4.0, containing 0.05 M monochloroacetic acid, and absorbance at 370 nm was measured.

RESULTS

Production and characterization of recombinant MT-SP1/matriptase, ekMT-SP1s

In the present study, we produced a secreted form of recombinant MT-SP1/matriptase named ekMT-SP1s that can be activated by enterokinase in vitro. As shown in Fig. 1, 15 nucleotides corresponding to amino acid residues T610KQAR614 (N-terminal side at the activation cleavage site) of wild-type MT-SP1/matriptase were substituted to those corresponding to DDDDK, the enterokinase recognition sequence, to create pSec-ekMT-SP1s. Transiently transfected COS-1 cells with pSec-ekMT-SP1s secreted ekMT-SP1s to a final concentration of about 10 μg/mL in conditioned media. The purified ekMT-SP1s gave signals of about 95 and 90 kDa on sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing and non-reducing conditions, respectively (Fig. 2). After enterokinase treatment, the ekMT-SP1s gave signals of about 15, 24, and 30 kDa under reducing conditions and about 16, 24, and 50 kDa under non-reducing conditions (Fig. 2), showing that this form of recombinant was cleaved to a disulide-linked form. We previously produced a secreted form of recombinant MT-SP1/matriptase named MT-SP1s in which the activation cleavage site remains natural (9). The purified latent MT-SP1s was cleaved at the site by trypsin to be activated (9). The sizes of the latent ekMT-SP1s and fragment pattern of enterokinase-treated ekMT-SP1s (active ekMT-SP1s) are compatible with that of the latent MT-SP1s and those of trypsin-treated MT-SP1s (9), respectively.

Table 1. Comparison of substrate specificity for wild-type MT-SP1/matriptase, MT-SP1s, and ekMT-SP1s.

<table>
<thead>
<tr>
<th>MT-SP1/matriptase purified from human milka</th>
<th>MT-SP1sb</th>
<th>ekMT-SP1sc</th>
</tr>
</thead>
<tbody>
<tr>
<td>sc-uPA activation</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Trypsinogen activation</td>
<td>ND</td>
<td>No</td>
</tr>
<tr>
<td>Chymotrypsinogen activation</td>
<td>ND</td>
<td>No</td>
</tr>
<tr>
<td>Fibronectin digestion</td>
<td>ND</td>
<td>Yes</td>
</tr>
<tr>
<td>Laminin digestion</td>
<td>ND</td>
<td>Yes</td>
</tr>
</tbody>
</table>

a Data were obtained from Refs. 6 and 8.
b Data were obtained from Ref. 9.
c ND, not determined.

Boc:: t-butyloxycarbonyl. MCA: 4-methylcoumaryl-7-amide. sc-uPA: single-chain urokinase-type plasminogen activator.
ekMT-SP1s is in agreement with that for the active MT-SP1s (9) (Table 1).

**Inhibition of MT-SP1/matriptase by protease inhibitors**

The inhibition of MT-SP1/matriptase by protease inhibitors was examined using the active ekMT-SP1s and Boc-Gln-Ala-Arg-MCA. We first tested the inhibitory activity of typical endogenous protease inhibitors (Table 2). α1-Antitrypsin at the normal plasma level of 1 mg/mL (15) showed 11% inhibition toward the substrate cleavage of the active ekMT-SP1s. α2-Macroglobulin at the concentration of 0.01 mg/mL showed no inhibitory action. Antithrombin III at the normal plasma level of 0.3 mg/mL (15) showed 40% inhibition. PSTI at the normal plasma level of 1 ng/mL (16) showed no inhibition. PSTI at a super-physiological dose (0.1 mg/mL) also showed no inhibitory activity. Aprotinin (basic pancreatic trypsin inhibitor), a serine protease inhibitor produced in bovine pancreas or lung, strongly inhibited it (IC₅₀ = 10 nM). As shown in Fig. 3, all of the plant-derived protease inhibitors tested inhibited the active ekMT-SP1s in the following order: LBTI > BBI > SBTI. An animal-derived protease inhibitor, chicken ovomucoid, showed no inhibitory activity. Synthetic protease inhibitors tested showed inhibitory activity in the following order: FOY-305 > leupeptin > 9-aminoacridine > phenylmethylsulfonyl fluoride (PMSF) (Fig. 4).

**DISCUSSION**

In the present study, we investigated the effects of MT-SP1/matriptase inhibitors using a secreted form of the recombinant enzyme. We previously produced and characterized a secreted form of recombinant MT-SP1/matriptase named MT-SP1s (9). The MT-SP1s that was purified as single-chain proteins showed no proteolytic activity, but incubation of the latent MT-SP1s with pancreatic trypsin resulted in activation accompanied by limited cleavage at the activation cleavage site between Arg₆¹₄ and Val₆¹₅ (Fig. 1). The active MT-SP1s as well as the natural enzyme purified from human milk (8) cleaved various MCA substrates with Arg (or Lys) residue at position P1, indicating that MT-SP1/matriptase is an enzyme that has trypsin-like specificity. The most preferable substrate was Boc-Gln-Ala-Arg-MCA. However, the productivity of MT-SP1s was very low; the final concentration was approx. 0.2 μg/mL in a conditioned medium of the transiently transfected COS-1 cells. In addition, the activation step by trypsin in vitro was not easy to be controlled. In the present study, therefore, we produced ekMT-SP1s that can be activated by enterokinase, a highly specific serine protease, and found that (i) the productivity of ekMT-SP1s in COS-1 cells was more than 50 times higher than that of MT-SP1s, and (ii) the activation step of the latent ekMT-SP1s.
SP1s by enterokinase was easily controlled and reproducible. As shown in Table 1, the active ekMT-SP1s exhibits enzymatic properties similar to that of natural enzyme purified from human milk (6, 8). For this reason, we currently used ekMT-SP1s for investigation of the inhibitors.

MT-SP1/matriptase was initially identified as an 80 kDa matrix-degrading protease from conditioned medium of human breast cancer cells (T-47D) (17), and was later purified from human breast milk as a complex with a Kunitz-type serine protease inhibitor, HAI-1 (10). Recently, HAI-1 immunoreactivity in human primary colorectal carcinomas was found to be decreased significantly in cells within colon carcinomas relative to adjacent normal mucosa or adenomas (11). In contrast, it has very recently been reported that the increased expression of MT-SP1/matriptase occurs in advanced colorectal cancers possibly because of its glycosylation (13). Thus, it has been suggested that the MT-SP1/matriptase : HAI-1 ratio could be important in the development of advanced tumors (18). In addition, any of the endogenous inhibitors currently tested showed no or little inhibitory activity toward MT-SP1/matriptase. In such a case, the exogenous administration of inhibitors may be a choice for the suppression of cancer invasion and metastasis.

It has been reported that BBI to min mice drastically reduces tumor development in both the small intestine and colon of the animal (4). There is a possibility that the protease inhibitor may exert these effects through its inhibitory action on MT-SP1/matriptase. In fact, the inhibitory effect of the BBI was not only previously shown using the purified enzyme (19), but also shown currently. SBTI was previously shown to inhibit the proteolytic activity of membrane-bound arginine-specific serine proteinase, porcine orthologue of MT-SP1/matriptase (14). The inhibitory action of SBTI was confirmed by the current study (Fig. 3). LBTI showed inhibitory action on the active ekMT-SP1s, and the inhibition was strongest among the plant-derived inhibitors tested (Fig. 3). SBTI and LBTI, if they reach the colon without significant loss of activity, may be orally administered for the suppression of colorectal cancer invasion and metastasis.

It has been reported that aprotinin (basic pancreatic trypsin inhibitor) shows an inhibitory action on the membrane-bound arginine-specific serine proteinase (14, 20). The inhibitory activity was also shown using active ekMT-SP1s. In the present study, we found a strong inhibitory action of FOY-305 toward active ekMT-SP1s. These inhibitory capacities for the inhibitors were stronger than those of the plant-derived inhibitors tested. Synthetic inhibitors are more stable in the gastrointestinal tract and easily available. Moreover, it has been reported that camostate (FOY-305)-containing diets have little or no effect on the homeostasis of the small intestine of normal mice (21); thus, such a compound is highly applicable for the suppression of cancer growth and invasion without significant damage of other normal tissues.

In summary, we currently investigated the MT-SP1/matriptase inhibitors. None of endogenous inhibitors tested had strong inhibitory effects on the currently prepared recombinant MT-SP1/matriptase. Plant-derived and synthetic protease inhibitors inhibited proteolytic activity, indicating that the administration of some inhibitors may be effective for the prevention of tumor growth and invasion in which MT-SP1/matriptase participates.

Acknowledgments
We wish to thank Mochida Pharmaceutical Co. for
kindly supplying a recombinant human PSTI and FOY-305. This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan, the Uehara Memorial Foundation, and the Asahi Glass Foundation.

REFERENCES