Inhibition of the Soluble and the Tumor Cell Receptor-bound Plasmin by Urinary Trypsin Inhibitor and Subsequent Effects on Tumor Cell Invasion and Metastasis

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INTRODUCTION

The serine-protease plasmin plays an important role in fibrinolysis, cell migration, tissue remodeling, angiogenesis, tumor invasion, and formation of metastasis (1, 2). A high affinity lysine-binding site located in the first three Kringles is important for the interaction of plasminogen with fibrin (3). In solution, native glutamic acid-plasminogen is only poorly activated to plasmin, which in plasma is very rapidly inactivated by a2AP, but in the presence of fibrin, plasmin activation is very rapid and the plasmin formed is protected from inactivation. It has also been reported that cell surface receptor-bound plasmin is protected from inhibition by a2AP and a2M (4).

The acid-labile ITI, which is a serine-protease inhibitor present in human serum and plasma, is the precursor of UTI. Hochstrasser et al. (5, 6) were the first to describe a physiological UTI (HIII-30) which had an apparent molecular mass of 34 kDa as estimated by gel chromatography. The characterization of complementary DNAs clearly indicates that ITI is a complex protein composed of several distinct polypeptide chains, two heavy chains and one light chain, probably with a NH2-terminal region of the polypeptide chain. UTI is composed of two tandem Kunitz-type domains connected with a small peptide. The amino acid sequences surrounding the reactive sites of the Kunitz-type domains in the inhibitory parts of UTI had already been reported (7—10). Although UTI inhibits trypsin, chymotrypsin, plasmin, and human leukocyte elastase, as well as cathepsin B and H, little is known about its physiological function (11). UTI has been shown to increase in several disease states including inflammation and cancer (11). This compound is at present clinically prescribed for treatment of acute pancreatitis and hemorrhagic shock in Japan.

MATERIALS AND METHODS

Cells and Culture. The ovarian cancer cell line HOC-I was established from a recurrent human ovarian endometrioid carcinoma (13), and the choriocarcinoma cell line SMT-ccl was established from a pulmonary metastatic region of human gestational choriocarcinoma (14). The murine 3LL xenograft, selected for its high lung colonization potential, was kindly provided by Chugai Pharmaceutical Co., Tokyo, Japan. The tumor was maintained by serial s.c. transplantation in C57BL/6 or C57BL/129 F1 (B6C3F1) mice. The 3LL cells were maintained as adherent monolayers (in plastic dishes) in Eagle’s minimal essential medium supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, L-glutamine, and anti-rabbit polyclonal antibody against UTI. The 3LL cells were grown to confluence in 75-cm2 tissue culture flasks (Falcon) washed three times with PBS containing 2% (v/v) BSA (Iwai, Tokyo, Japan), and then harvested after a brief treatment with 0.25% trypsin (Sigma Chemical Co., St. Louis, MO). The cell viability was determined by trypan blue dye exclusion prior to use.

Animals. Specific-pathogen-free female C57BL/6 mice, 6—8 weeks old, were purchased from Charles River Japan, Inc. (Kanagawa, Japan). The care and use of the animals were in accordance with the Institution’s guidelines.

Antibodies and Plasmin Inhibitors. The antibodies used were anti-rabbit polyclonal antibody against human plasminogen (Cosmo Bio, Kyoto, Japan) and anti-rabbit polyclonal antibody against UTI (supplied by Mochida Pharmaceutical Co., Tokyo, Japan). Aprotinin, a nonspecific inhibitor for serine proteases, was a gift from Bayer Werk, Frankfurt, Germany. All other reagents were of analytical grade. Glutamic acid-plasminogen and plasmin were purchased from KabiVitrum, Stockholm, Sweden.
DIP-plasmin was prepared by incubating plasmin twice with 10 mM diisopropylphosphorofluoridate (1 h, 23°C). Excess diisopropylphosphorofluoridate was removed by dialysis against PBS (4). α2AP was purchased (Athenes Research) or purified according to the procedure of Wiman et al. (3), and α2M was purchased (Cosmo Bio, Kyoto). A highly purified preparation of UTI (specific activity, 2,330 units/mg soluble protein, with a molecular mass of 67,000 Da) was kindly supplied by Mochida Pharmaceutical Co. The covalent structure of the polypeptide chain of the physiological inhibitor UTI (HI-30) has been analyzed and described elsewhere (5–7).

**Binding of Biotinylated Plasminogen and DIP-Plasmin.** Glutamic acid-plasminogen and DIP-plasmin were biotinylated according to the method of Guesdon et al. (15), using N-hydroxysuccinimidyl/biotinamidocaproate (Sigma).

HOC-1 and SMT-ccl cells were grown to confluence in 96-well microtiter plates (Costar, Cambridge, MA), washed twice with cold PBS containing 2% BSA, and then were treated with biotinylated plasminogen or biotinylated DIP-plasmin (10 μM, 4 h, 4°C) (4). A 10 μM concentration of plasminogen is enough to saturate cell surface plasmin(ogen) binding sites (data not shown; see Ref. 4). After the cells were washed twice with PBS-0.2% BSA, specifically bound biotinylated plasminogen (or DIP-plasmin) was detected with avidin-peroxidase (0.4 μg/ml, 1 h, 23°C; Dako, Copenhagen, Denmark). The reactions with 3,3′,5,5′-tetramethylbenzidine (AMRESCO, Inc.), were terminated, and each well was measured at 450 nm (A450) in an EIA reader (Model 2550; Bio-Rad, Richmond, CA) (16, 17).

In addition, the specific binding of biotinylated plasminogen (or DIP-plasmin) to cells pretreated with 6AHA was determined to detect the amount of total plasmin(ogen)-binding sites on their surface (18). Endogenous plasminogen was displaced by incubating the cells with 0.1 M 6AHA (30 min, 23°C) followed by thorough washing with PBS-0.1% BSA. The cells were then incubated with biotinylated plasminogen or DIP-plasmin (10 μM, 4 h, 4°C). After they were washed twice with PBS-0.1% BSA, the total binding sites were determined as described above. A time course study (0–5 h, 4°C) was carried out to assess biotinylated plasminogen binding to the cells.

Tumor cells grown to confluence in microtiter plates were incubated with 5 μM biotinylated plasminogen in the presence of increasing concentrations of either unlabeled plasminogen (0–10 μM) or DIP-plasmin (0–10 μM) at 4°C for 4 h, and binding was measured as described above. All samples were assayed at least in triplicate.

**Enzyme-linked Immunosorbent Assay.** HOC-1 and SMT-ccl cells were grown to confluence in 96-well microtiter plates, washed with PBS-2% BSA, and then allowed to react with antibody against plasminogen (100 nM, 16 h, 4°C). Non-specific binding was ascertained in reactions using rabbit immunoglobulin as the control. Antibody specifically bound to plasminogen was detected with biotin-conjugated second antibody (1.5 μg/ml, Dako; 1 h, 23°C), and then allowed to react with antibody against plasminogen (100 ng/ml, 16 h, 23°C). The reactions with 3,3′,5,5′-tetramethylbenzidine (AMRESCO, Inc.), were terminated, and each well was measured at 450 nm (A450) in an EIA reader (Model 2550; Bio-Rad, Richmond, CA) (16, 17).

**Experimental Lung Metastasis in C57BL/6 Mice.** 3LL cells (5 x 10⁶ cells) admixed with various concentrations of plasmin inhibitors (0–50 μM, 200 μM/mouse) were injected via the lateral tail vein of unanesthetized C57BL/6 mice (25). Mice were given i.p. injections of each plasmin inhibitor (0–50 μM, 200 μM/mouse/day) for 7 days immediately after tumor cell inoculation. Twenty-one days after the inoculation of tumor cells, mice were sacrificed and the lungs were removed, washed in water, and fixed in Bouin’s solution. The lung tumor colonies were counted under a dissecting microscope. The data were analyzed for significance by Student’s t test or nonparametric test.

## RESULTS

**Immunological and Enzymological Determination of Plasminogen on the Surface of Tumor Cells.** Membrane-localized plasminogen was detected by cell enzyme-linked immunosorbent assay using a plasminogen-specific antibody (Table 1). The concentration of plasminogen-related antigen associated with SMT-ccl cells was approximately 3 times higher than that found in HOC-1 cells, although the antibody against plasminogen used in this study was unable to distinguish plasmin from plasminogen. The cells saturated with plasminogen showed highly specific binding. Immunoreactivity decreased enough to saturate cell surface plasminogen binding sites (data not shown; see Ref. 4). After the cells were washed twice with PBS-0.1% BSA, they were treated with biotinylated plasminogen or biotinylated DIP-plasmin (10 μM, 4 h, 4°C). A 10 μM concentration of plasminogen is enough to saturate cell surface plasminogen binding sites (data not shown; see Ref. 4).

**Results**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>HOC-1</th>
<th>SMT-ccl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.08</td>
<td>0.24</td>
</tr>
<tr>
<td>After saturation</td>
<td>0.06</td>
<td>0.06</td>
</tr>
<tr>
<td>6AHA treatment</td>
<td>&lt;0.01</td>
<td>0.02</td>
</tr>
<tr>
<td>6AHA treatment + saturation</td>
<td>0.07</td>
<td>0.15</td>
</tr>
</tbody>
</table>

a. Tumor cells were washed with cold PBS-2% BSA.

b. Mean ± SD (absorbance unit) of absorbance at 450 nm of assays using 1 x 10⁶ cells and carried out in triplicate.

c. The cells were treated with 0.1 M 6AHA to displace endogenous plasminogen (30 min, 23°C).

d. Again, the cells were treated with PBS-0.1% BSA containing 10 μM plasminogen (4 h, 4°C).

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**Table 1 Immunological determination of plasminogen on the surface of tumor cells by cell enzyme-linked immunosorbent assay**

Cell enzyme-linked immunosorbent assays using polyclonal antibody against plasminogen detect surface membrane-localized plasminogen.
drastically following a 6AHA treatment. The cells treated with 6AHA acquire the property of binding plasminogen.

We next performed binding studies on these cells using biotinylated plasminogen or biotinylated DIP-plasmin. Binding of biotinylated plasminogen to the cells occurred in a dose-dependent and saturable manner. Specific binding reached saturation at approximately 5 μM (Fig. 1). Total plasmin(ogen) binding was measured following treatment with 6AHA to remove endogenous plasmin(ogen) from cellular binding sites. Specific binding increased after treatment with 6AHA. Biotinylated plasminogen was able to bind to the cell-associated binding proteins uncovered by treatment with 6AHA. Similar results were obtained with respect to binding of biotinylated DIP-plasmin to the cells (data not shown).

In addition, experiments were performed to determine whether either plasminogen or DIP-plasmin could compete with biotinylated plasminogen for binding to these tumor cells. Biotinylated plasminogen (5 μM) was inhibited in a dose-dependent manner by unlabeled plasminogen or DIP-plasmin, and these competitors had similar abilities to inhibit biotinylated plasminogen binding to these cells. The concentration of unlabeled plasminogen or DIP-plasmin producing 50% inhibition of biotinylated plasminogen binding to the cells were similar (approximately 3 μM; data not shown). These results indicate that these cells express functional plasmin(ogen) binding proteins that are partially occupied by ligands.

For a quantitative assay of plasmin activity on the surface of tumor cells, we applied S-2251, a chromogenic synthetic substrate, to the cells adhered to the microtiter plates (Fig. 2). HOC-I cells alone were capable of degrading S-2251 to some extent. Note that SMT-ccl cells showed higher levels of plasmin activity than HOC-I cells. In addition, the murine tumor cell line 3LL was also able to degrade S-2251.

**Inhibition of the Soluble and the Tumor Cell Receptor-bound Plasmin by Plasmin Inhibitors.** The inhibition of soluble plasmin by plasmin inhibitors was investigated using chromogenic assays. The relative enzyme activities obtained when plasmin (20 nM) was titrated with UTI (0.4 μM), α2AP (0.4 μM), or α2M (0.2 μM) are shown in Fig. 3. As expected, more than 90% of the plasmin activity in solution was inhibited by α2AP within 10 min at 23°C. Titrations of plasmin with UTI or α2M revealed that each of them inhibits plasmin at a lower rate. Thus, α2AP proved to be more efficient than UTI and α2M when plasmin is in solution.

The reaction of SMT-ccl cell-associated plasmin with α2AP and α2M at 23°C was extremely slow compared with the solution phase reaction, demonstrating that receptor-bound plasmin is highly protected from inhibition by α2AP and α2M. In contrast, UTI has the ability to inhibit the tumor cell-associated plasmin. Titrations of the soluble and receptor-bound plasmin with UTI revealed that 5 μM UTI inhibits cell-associated plasmin with essentially the same affinity as the solution phase plasmin in our assay system (Fig. 3). Similar results were obtained with HOC-I and 3LL cell surface receptor-bound plasmin (data not shown). These results indicate that UTI inhibits the cell-associated human plasmin and mouse plasmin-like activity.

The caseinolytic activity of SMT-ccl cells in the presence or absence of plasmin inhibitors is shown in Fig. 4. This was significantly suppressed in the presence of UTI, which confirmed the results of the amidolytic assays described above.

**Effect of Plasmin Inhibitors on Tumor Cell Invasion.** uPA and plasmin appear to be essential to the invasion process of HOC-I and SMT-ccl cells in vitro (17). Inhibition of HOC-I cell invasion in vitro was observed after treatment with specific inhibitors and antibodies for uPA and plasmin (17). These cells express both cell-associated plasmin(ogen) and functional uPA receptors that are partially occupied by ligands. SMT-ccl cells, which express 3-fold levels of cell-associated plasmin activity (by amidolytic assay; see Fig. 2), showed approximately 2-fold increase in their invasive potential (Table 2). Experiments with UTI in the in vitro invasion assay showed a statistically significant and dose-dependent inhibition of SMT-ccl cells. α2AP and

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Fig. 1. Specific binding of biotinylated plasminogen to HOC-I and SMT-ccl cells. Binding of biotinylated plasminogen to tumor cells. HOC-I and SMT-ccl cells were grown to semiconfluence in 96-well microtiter plates. After two washings with PBS-2% BSA, the cells were treated with increasing concentration of biotinylated plasminogen (0–10 μM; 4 h, 4°C). The same procedure was used in control experiments, in which biotinylated plasminogen was incubated with the cells in the presence of an excess of plasminogen (500 μM). Specific plasminogen binding reached saturation at approximately 5 μM and it increased following treatment with 6AHA. Assays were carried out in triplicate. Bar, SD. Cells were treated with PBS to detect free plasminogen binding sites (Δ), HOC-I (●), SMT-ccl (○) or with 6AHA to determine total plasminogen binding sites (△, HOC-I (●), SMT-ccl (○)).

Fig. 2. Quantitative detection of plasmin activity in tumor cells. The cell-bound plasmin activity was investigated using the chromogenic synthetic substrate S-2251. Assays were routinely carried out in triplicate (60 min; 23°C). HOC-I cells (○), SMT-ccl cells (●), and 3LL cells (□). Bar, SD.

Fig. 3. Titration of the soluble and the SMT-ccl cell surface receptor-bound plasmin with plasmin inhibitors. The hydrolysis of synthetic substrate S-2251 catalyzed by the soluble or the receptor-bound plasmin were measured at 405 nm. The soluble or the receptor-bound plasmin was preincubated with the indicated concentrations of UTI (△), α2AP (●), and α2M (○) for various time intervals (0–60 min) at 23°C. The value at 405 nm was considered as 100%, when the soluble or the receptor-bound plasmin was incubated with S-2251 for 60 min at 23°C in the absence of inhibitors. The reaction of 20 nm soluble phase plasmin (△) with 0.4 μM of each plasmin inhibitor at 23°C was also determined for comparison. After incubation for 60 min, the reaction was stopped and the amidolytic activity was determined. The concentrations of each plasmin inhibitor used were 0.2 (○), 1.0 (△), and 5.0 (□) μM. Similar results were obtained with HOC-I and 3LL cell-associated plasmin.

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The visible tumors have pulmonary metastasis by systemic injection of 3LL cells. UTI, a2AP presence of each of the plasmin inhibitors (data not shown). Plasmin inhibitors was due to an inhibition of this response. HOC-I, tested to determine if the inhibition of cell invasion induced by plasmin inhibitors had no effects. These results indicate that only after treatment with plasmin inhibitors (data not shown).

SMT-ccl, and 3LL cells showed a good chemotactic migration in the tumor cell invasion. The chemotactic response of the cells was also a2AP, not a2AP or a2M, plays an important role in the inhibition of invasion of HOC-I cells and 3LL cells, indicating that a2AP and a2M similar results were obtained with UTI, a2AP and a2M on in vitro effects of these inhibitors on cell attachment were also examined.

Control experiments were carried out in the absence of inhibitors. Either UTI, a2AP, or a2M was added to the upper chamber 60 min before the cells were plated on the Matrigel. Each experiment was performed at least in triplicate. Mean ± SD of 3 experiments.

In the present study, we compared the reaction of tumor cell-associated proteolytic activity (uPA, plasmin, cathepsin B, cathepsin D, and collagenase) (1, 2, 26), which leads to degradation of the tumor stroma and the basement membranes. In general, tumor cells express cell surface-associated uPA and plasmin activities and the inhibition of these activities by specific antibodies and proteinase inhibitors leads to a decrease in the invasive potential of tumor cells (27–33). However, it is difficult to use antibodies for cancer therapy in humans.

It has been indicated that receptor-bound plasmin is protected from inhibition by a2AP and a2M (4). However, when the concentration of a2AP approximates or exceeds that present in plasma (1.0 μM), receptor-bound plasmin is inactivated by direct reaction with a2AP at the cell surface as well as after dissociation. These data demonstrate that the plasmin receptor provides a mechanism for the expression of enzyme activity even in the presence of consistently high concentrations of a2AP and a2M.

In the present study, we compared the reaction of tumor cell-associated plasmin with highly purified plasmin inhibitors (UTI, a2AP, and a2M) with that of solution phase plasmin. Besides, we used the above mentioned plasmin inhibitors to investigate the role of cell-associated plasmin activity in tumor cell invasion and metastasis. We used two human tumor cell lines, ovarian cancer HOC-I and choriocarcinoma SMT-ccl cells, and one murine tumor cell line, Lewis lung carcinoma cells. a2AP and a2M rapidly inactivated soluble plasmin but not receptor-bound plasmin. In contrast, UTI inhibited both types of plasmin. As expected, UTI but not a2AP or a2M inhibited tumor cell invasion of basement membranes in vitro and was associated with a decreased number of lung tumor colonies in vivo. This inhibition was dose dependent and nontoxic, and correlated well with the ability of UTI to inhibit cell-associated plasmin activity. Furthermore, we concluded that the mode of action of inhibitors is not associated with an inhibition of tumor cell chemotaxis or cell attachment.

α2M (up to 25 μM), however, showed no significant inhibitory effects. Similar results were obtained with UTI, a2AP, and a2M on in vitro invasion of HOC-I cells and 3LL cells, indicating that α2AP and α2M have no significant inhibitory effects. These results indicate that only UTI, not α2AP or α2M, plays an important role in the inhibition of tumor cell invasion. The chemotactic response of the cells was also tested to determine if the inhibition of cell invasion induced by plasmin inhibitors was due to an inhibition of this response. HOC-I, SMT-ccl, and 3LL cells showed a good chemotactic migration in the presence of each of the plasmin inhibitors (data not shown). Plasmin inhibitors had no effects on tumor cell chemotaxis. Moreover, the effects of these inhibitors on cell attachment were also examined. Under optimal conditions, more than 80% of these cells remained attached to the well after washing. No inhibition of attachment of HOC-I, SMT-ccl, and 3LL cells to Matrigel or fibronectin was seen after treatment with plasmin inhibitors (data not shown).

**Effects of Plasmin Inhibitors on the Experimental Metastasis.**

We examined the effects of plasmin inhibitors on the experimental pulmonary metastasis by systemic injection of 3LL cells. UTI, α2AP, or α2M was admixed with tumor cells (30 min, 23°C) and the mixtures were injected i.v. into C57BL/6 mice. The visible tumors have appeared at day 7 after tumor cell inoculation. These inhibitors did not affect the local tumor growth. The results of a representative experiment are shown in Table 3. α2AP and α2M did not lead to decreased numbers of lung tumor colonies even at the highest concentrations used. In contrast, the average number of pulmonary nodules formed was significantly lower in mice treated with UTI. UTI showed a statistically significant and dose-dependent inhibition of tumor cell metastasis.

**DISCUSSION**

The increased invasive potential of tumor cells is correlated with tumor cell-associated proteolytic activity (uPA, plasmin, cathepsin B, cathepsin D, and collagenase) (1, 2, 26), which leads to degradation of the tumor stroma and the basement membranes. In general, tumor cells express cell surface-associated uPA and plasmin activities and the inhibition of these activities by specific antibodies and proteinase inhibitors leads to a decrease in the invasive potential of tumor cells (27–33). However, it is difficult to use antibodies for cancer therapy in humans.

The increased invasive potential of tumor cells is correlated with tumor cell-associated proteolytic activity (uPA, plasmin, cathepsin B, cathepsin D, and collagenase) (1, 2, 26), which leads to degradation of the tumor stroma and the basement membranes. In general, tumor cells express cell surface-associated uPA and plasmin activities and the inhibition of these activities by specific antibodies and proteinase inhibitors leads to a decrease in the invasive potential of tumor cells (27–33). However, it is difficult to use antibodies for cancer therapy in humans.

**Table 2 Effects of plasmin inhibitors on tumor cell invasion in vitro**

HOC-I and SMT-ccl cells were tested in an *in vitro* invasion assay. The number of cells that had attached to the lower surface of the filter at 12 h was used to calculate cell invasion. Control experiments were carried out in the absence of inhibitors. Either UTI, α2AP, or α2M was added to the upper chamber 60 min before the cells were plated on the Matrigel. Each experiment was performed at least in triplicate. Mean ± SD of 3 experiments.

<table>
<thead>
<tr>
<th>Plasmin inhibitor</th>
<th>Concentration (μM)</th>
<th>No. of invading cells/field</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HOC-I</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UTI</td>
<td>0.05</td>
<td>22.5 ± 20.8</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>10.5 ± 8.5*</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>3.9 ± 5.5b</td>
</tr>
<tr>
<td>α2 AP</td>
<td>0.25</td>
<td>29.0 ± 14.6</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>32.5 ± 19.0</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>23.1 ± 22.1</td>
</tr>
<tr>
<td>α2 M</td>
<td>0.25</td>
<td>25.8 ± 24.3</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>21.6 ± 19.6</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>24.8 ± 11.9</td>
</tr>
</tbody>
</table>

*P < 0.05 compared to control.

**Table 3 Inhibition by plasmin inhibitors of pulmonary metastasis of Lewis lung carcinoma cells in the in vivo C57BL/6 mouse model**

For evaluation, the number of tumor nodules in the lung was counted.

<table>
<thead>
<tr>
<th>Plasmin inhibitor</th>
<th>n</th>
<th>Concentration (μM)</th>
<th>No. of tumor nodules in the lung</th>
<th>Range</th>
<th>Rate of inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9</td>
<td></td>
<td>64.8 ± 52.7</td>
<td>21–200</td>
<td></td>
</tr>
<tr>
<td>UTI</td>
<td>9</td>
<td>0.5</td>
<td>53.7 ± 30.0</td>
<td>12–125</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>5.0</td>
<td>36.9 ± 24.4*</td>
<td>0–69</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>50</td>
<td>11.0 ± 13.9b</td>
<td>0–30</td>
<td>83</td>
</tr>
<tr>
<td>α2 AP</td>
<td>7</td>
<td>0.5</td>
<td>51.4 ± 35.7</td>
<td>25–159</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>5.0</td>
<td>54.0 ± 49.5</td>
<td>20–200</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>50</td>
<td>45.5 ± 48.2</td>
<td>18–200</td>
<td>11</td>
</tr>
<tr>
<td>α2 M</td>
<td>8</td>
<td>0.5</td>
<td>58.1 ± 34.1</td>
<td>16–180</td>
<td>0</td>
</tr>
</tbody>
</table>

*P < 0.05 compared to control.

**Fig. 4. Caseinolytic activity of SMT-ccl cells in the presence or absence of plasmin inhibitors. SMT-ccl cells were briefly digested with trypsin. After a washing, the cells were incubated with 0.1 μM plasmin (2 h, 4°C) and washed again. Then, the cell suspension (2 × 10⁶ cells) was placed on top of the agarose-plasminogen gel in a standing tube in the presence or absence of plasmin inhibitors (UTI (0.4 μM), α2AP (0.4 μM), and α2M (0.4 μM)). The lysis volumes were compared after a 4-h incubation period at 23°C. Bars, SD; *P < 0.05 compared to control (cells alone); **P < 0.001 compared to control.**

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attachment to fibronectin, a component in basement membranes. The amino acid sequence of UTI, which is derived from limited proteolysis of UTI, includes two Kunitz-type domains connected with a small peptide (5–11). The amino acid sequences surrounding the reactive sites of the Kunitz-type domains in the inhibitory parts of UTI are Gly—Pro—Cys—Met—Gly—Met—Thr for elastase inhibition and Gly—Pro—Cys—Arg—Ala—Phe—Ile for trypsin/plasmin inhibition, respectively (5–11). Furthermore, UTI and its-related fragment, which contains a plasmin-inhibiting domain (composed of 15 amino acid sequences including Gly—Pro—Cys—Arg—Ala—Phe—Ile), significantly inhibit tumor cell invasion in a dose-dependent manner in an in vitro reconstituted basement membrane assay.  

The cell surface-bound proteinases, uPA and plasmin, would create more favorable conditions for localized proteolysis (34–36). This enhancing effect of uPA/plasmin may be due to proteolysis in critical sites of contact between tumor cells and substratum, or to increased interaction between the enzyme and the substrate as a result of higher local enzyme concentration, or because receptor-bound plasmin is protected from inhibition by serum inhibitors. The role that plasmin plays in tumor invasion has been clearly demonstrated by previous studies (30, 35). Pretreatment of murine B16 melanoma cells with plasmin resulted in a 200–300% increase in the number of pulmonary metastasis at plasmin concentrations above 1 ng/ml (30). Mignatti et al. (35) reported that the uPA/plasmin system plays an important role in the handing of cell invasion exerted by UTI is due to inhibition of the enzyme activity adsorbed on the membrane. If the serum is not eliminated roles for plasmin in the initial steps (intravasation) of the metastatic process or in both the early and late steps of the migration process. The exact reasons for the efficient inhibition of cancer cell invasion and metastasis by the UTI molecule remain to be explored.

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17. Terranova, V. P., Hujanen, E. S., and Martin, G. R. Basement membrane and the metastatic process or in both the early and late steps of the migration process. The exact reasons for the efficient inhibition of cancer cell invasion and metastasis by the UTI molecule remain to be explored.

4 Unpublished data.


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