Inhibition of Tumor Cell Invasion through Matrigel by a Peptide Derived from the Domain II Region in Urinary Trypsin Inhibition

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Abstract

Urinary trypsin inhibitor (UTI) has a multipotent inhibitory effect on proteases such as trypsin, chymotrypsin, plasmin, human leukocyte elastase, or hyaluronidase. UTI can bind easily to its receptors on various types of tumor cells (human ovarian cancer HO-1 cells, human choriocarcinoma SMT-ccl cells, and murine Lewis lung carcinoma 3LL cells). Our results show that the UTI receptors of some tumor cells have a possible role in modulating plasmin activity on the cell surface and prevention of tumor cell invasion and metastasis (H. Kobayashi et al., J. Biol. Chem., 269: 20642–20647, 1994). UTI interacts with tumor cells as a negative modulator of the invasive cells. We investigated whether this effect may be mediated by UTI binding to the cell surface receptors. In addition, the role of peptide sequences from each UTI domain and their interaction with tumor cells were investigated. UTI derivatized with biotin or FITC was taken up by tumor cells in a dose-dependent manner. This cell association was inhibited with a monoclonal antibody D1, which specifically recognizes NH2-terminus (domain I) of UTI. The binding was inhibited by fluid phase UTI, but not HI-8, COOH-terminus (domain II) of UTI, suggesting that UTI binds to cells through a site in the UTI domain I. Furthermore, we found that UTI, HI-8 and a number of peptides containing Arg-Gly-Pro-Cys-Arg-Ala-Phe-Ile promoted the inhibition of tumor cell invasion. This site corresponds to the plasmin-inhibiting domain within HI-8. The possibility that UTI binding to tumor cells might be involved in the prevention of tumor cell invasion in vitro was excluded since HI-8, lacking domain I, promotes the inhibition of tumor cell invasion with essentially the same affinity as UTI. All these data allow us to conclude that inhibition of tumor cell invasion is mediated by domain II, which possesses anti-plasmin activity.

Introduction

UTI2 has been found to play a role in the prevention of tumor cell invasion and metastasis induced by human ovarian carcinoma HO-1 cells, choriocarcinoma SMT-ccl cells, and mouse Lewis lung carcinoma 3LL cells. These antinvasive and antimetastatic activities depend upon the anti-plasmin activity of UTI (1–4). It is generally accepted that UTI inhibits several proteases including trypsin, chymotrypsin, human leukocyte elastase, plasmin, and hyaluronidase. UTI is composed of two tandem Kunitz-type domains [NH2-terminus (domain I) and carboxyl-terminus (domain II; HI-8) of UTI] connected with a small peptide. It was found that UTI can be produced in vivo by limited proteolysis of UTI in the NH2-terminal region of the polypeptide chain (5–8). It is likely that inhibition of the cell surface receptor-bound plasmin by UTI is associated with significant reduction of tumor cell invasiveness and metastasis (1–4).

Received 12/30/94; accepted 3/17/95.

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1 To whom requests for reprints should be addressed.
2 UTI, urinary trypsin inhibitor; HI-8, carboxyl-terminus (domain II) of UTI; ITI, inter-a-trypsin inhibitor; pAb, polyclonal antibody; kDa, kilodalton(s); PAGE, polyacrylamide gel electrophoresis; FITC-UTI, FITC-conjugated UTI; FITC-HI-8, FITC-conjugated HI-8.
m NaCl. The major active fractions from a trypsin-Sepharose column were applied to a Sephadex G-100 gel chromatography. Each fraction was pooled, and NH₄Cl-terminal amino acid sequencing was carried out to obtain purified HI-8 (Fig. 4, Lane 2).

Deglycosylated UTI was obtained as follows: UTI (1 μM) was incubated with chondroitinase ABC (0.1 μM for 1 h at 37°C; Sigma). Reaction was terminated by freezing the reaction mixtures in liquid nitrogen. Chondroitinase-treated UTI was purified and isolated by reverse-phase HPLC. Each peak of purified UTI was monitored by dot blot analysis using pAb D2.

Anti-UTI Antibodies Binding to Immobilized UTI and HI-8. Microtiter plate wells were coated with UTI or HI-8 by incubation of 100-μl aliquots of protein solution (10 μg/ml protein in 0.1 M sodium carbonate, pH 9.5; 4°C for 16 h). After washing the wells, nonspecific binding sites were blocked with 200-μl aliquots of PBS-2% BSA, pH 7.4.

Anti-UTI antibodies (mAb D1 and pAb D2) diluted in 50 μl PBS-2% BSA was allowed to bind for 2 h at 23°C. Then the plates were washed three times with PBS, and bound antibodies were incubated with biotinylated second antibody (0.5 μg/ml for 1 h at 23°C; Dako, Copenhagen, Denmark), followed by incubation with avidin-peroxidase (0.5 μg/ml for 1 h at 23°C; Dako). After washing, wells were allowed to react with enzyme substrate (3.3'-5.5'-tetramethylbenzidine). Absorbance was read at 450 nm with an automated 96-well spectrophotometer (Bio-Rad, Richmond, CA). All samples were tested in triplicate, and the results shown are the means obtained in a typical experiment from a series.

Electrophoresis and Western Blot. SDS-PAGE (5-18% SDS-PAGE; nonreducing conditions) was carried out as described previously (9).

Immunocytochemistry. U937 cells and SMT-ccl cells were rinsed twice with serum-free culture medium. Cells were subjected to indirect immunofluorescence labeling with anti-UTI antibodies (mAb D1 and pAb D2).

Quantitative Assessment of FITC-UTI Binding to Tumor Cells by Flow Cytometry. SMT-ccl cells (10⁶ cells) were suspended in 1.0 ml of PBS-0.1% BSA containing FITC-UTI or FITC-HI-8 and rocked gently for 3 h at 37°C.

Cell-associated fluorescence (argon laser excitation at 488 nm) at real time (not washed) was immediately analyzed by an EPICS PROFILE flow cytometer. Cell attachment assay and chemotactic assay were also conducted as described previously (1, 3, 11, 12).

UTI-related Synthetic Peptides. Peptides were synthesized according to sequences of human UTI deduced from a cDNA sequence for human UTI (6, 8). The peptides used in this study (Fig. 1) were synthesized on an automated peptide synthesizer (Applied Biosystems 430A Peptide synthesizer, Tokyo, Japan) using standard Merrifield solid phase synthesis protocols and purified by reverse-phase HPLC (Senshu Pack; ODS-H-5251; 20 x 250 mm). Identities of peptides were verified by complete amino acid sequence analysis. Some of peptide solutions were neutralized by the addition of dilute NaOH and used immediately.

Cell Invasion Assay. The invasion of SMT-ccl cells were assayed in a modified Boyden chamber with an 8-μm pore size polycarbonate filter as described previously (11, 12). Cell attachment assay and chemotactic assay were also conducted as described previously (1-3, 11, 12).

Results

Characterization of Anti-UTI Antibodies. Binding of anti-UTI antibodies to immobilized solid-phase UTI and HI-8 was studied by direct ELISA (Fig. 2). The kinetics of binding of antibodies to immobilized UTI were dose dependent and saturable. Both mouse monoclonal (mAb D1) and rabbit polyclonal (pAb D2) antibodies against UTI reacted with immobilized UTI, whereas mAb D1 did not react with HI-8. Preincubation of mAb D1 (2 nm) with UTI (0-100 nm) produced a dose-dependent inhibition of antibody binding to immobilized UTI, whereas preincubation of mAb D1 with HI-8 had no significant effect (Fig. 3).

This was confirmed by SDS-PAGE followed by Western blotting using anti-UTI antibodies. Characterization of the anti-UTI antibodies used in this study was investigated (Fig. 4). pAb D2 recognizes UTI, HI-8, and chondroitinase ABC-treated UTI, and mAb D1 recognizes only UTI containing the domain I region.

To determine if the UTI receptors on tumor cells bind UTI specifically, two experiments were performed. In the first experiment, the anti-UTI antibodies were added to a cell suspension of U937 cells and analyzed by flow cytometry. UTI receptors on the cell surface of U937 cells were found to be completely saturated with endogenous UTI (4). On the other hand, exogenously applied UTI binds rapidly and with relatively high affinity to SMT-ccl cells. UTI bound to a
specific receptor that is incompletely saturated. pAb D2 reacted with receptor-bound UTI on U937 cells and SMT-cc1 cells in a dose-dependent manner, whereas mAb D1 did not react with cell-surface receptor-bound endogenous UTI (Fig. 5).

In addition, anti-UTI antibodies were added to the U937 cell suspensions. The cell-surface receptor-bound UTI was evaluated for its capacity to bind the anti-UTI antibodies (Fig. 6). Analysis of cell ELISA revealed that pAb D2 had the strongest binding affinity for cell-surface receptor-bound UTI, whereas mAb D1 had negligible binding ability. It is likely that a masking of the binding site of UTI by UTI receptors is the cause of the negligible binding of mAb D1. These results indirectly suggest that the intact domain I is required for UTI binding to UTI receptors. This was also confirmed by immunocytocchemical stainings (Fig. 5) and cell ELISA (Fig. 7).

In the second experiment, direct binding assays using flow cytometry were performed to examine the region within UTI responsible for binding to UTI receptors on tumor cells. FITC-UTI and FITC-HI-8 were tested for their binding to SMT-cc1 cells (Fig. 8A). The kinetics of binding of FITC-UTI to cells were saturable. This binding was depending on domain I region of the UTI molecule. As HI-8 is lacking to this region, it did not bind UTI receptors. FITC-UTI was tested for its binding to cells in the presence or absence of mAb D1. Preincubation of FITC-UTI with mAb D1 produced a dose-dependent inhibition of FITC-UTI binding to the cells (Fig. 8B). We confirmed that cells specifically and saturaably bound directly to UTI and not HI-8.
These results suggest that receptor binding site in UTI may be close to its domain I region.

**Protease Inhibitory Properties of UTI, HI-8, and Peptides.** We reported before that inhibition of the proteolytic enzyme plasmin by UTI specifically reduced the invasive capacity of tumor cells (1). We have also identified the active peptide within UTI responsible for plasmin inhibition. To investigate the inhibiting effects of UTI, HI-8, and peptides on plasmin, different doses (0–100 μM) were preincubated (10 min for 23°C) with 2 μM plasmin in a 96-well microtiter plate, followed by the addition of S-2251 in PBS-0.1% BSA (30 min for 23°C).

The relative enzyme activity obtained when plasmin was titrated with native UTI, HI-8, or the synthetic peptides was shown in Fig. 9. Titration of plasmin with UTI, HI-8, or peptides revealed that HI-8 inhibited plasmin with essentially the same affinity as UTI, suggesting that the affinity of UTI for plasmin is due to an interaction of domain II in UTI. It was notable that peptides 3A-1 and 3A were significantly less active or inactive, implying that these peptides are close to the amino acid sequences within reactive sites in domain II. Peptide 3 was significantly less active than peptide 3A-1. Addition of 11 residues to the carboxyl-terminus of the effective peptide 3A-1 (RGPCRAFIQLWAFDAVKGK) decreased activity. Addition of 5 residues to the amino terminus of the peptide 3A-1 and 2 residues to the carboxyl-terminus to produce peptide 3A (NLPVIRGPCRAFIQL) also decreased activity. Other peptides tested were less active or inactive.

**Effect of Peptides on Tumor Cell Invasion in Vitro.** To investigate the active site within UTI molecule in mechanisms of UTI-dependent inhibition of tumor cell invasion, HI-8 and the synthetic peptides were tested (Fig. 10). UTI, HI-8, and peptides had no effect on the viability of SMT-ccl cells in vitro. Cell growth in culture was not inhibited by these agents at concentrations as high as 1 mg/ml after 24 h, and cells were >90% viable as measured by trypan blue exclusion. In addition, peptide solutions contained no endotoxin (data not shown).

As described in previous studies, the synthetic peptide 3, RAFIQL-WAFDAVKGK, represents a potent inhibitor of tumor cell invasion (1). In this study, through a reductionist approach, we have further defined the activity within domain II region. This was carried out by systematic examination of shorter peptides derived from this active peptide and its related peptides.

Several of the UTI peptides were tested for inhibition of SMT-ccl cell invasion in a Boyden chamber assay (1, 11, 12). UTI and HI-8 strongly inhibited tumor cell invasion with essentially the same activity. Peptides 3A and 3B, but not peptides 3C and 3D, displayed activity. In order to further locate the active sequences within peptides 3A and 3B, four shorter overlapping peptides spanning peptide 3A-1 were tested. Also, to evaluate the contribution of adjacent sequences to activity, peptide 3A, which contained the NH2-terminal sequence of peptide 3A-1, was examined in inhibition studies. The longer peptide 3A that contained the active sequences of peptide 3A-1 showed less inhibiting effect on tumor cell invasion. The peptide 3A containing the sequences that are distant from the active sequence may poorly interfere with enzyme-inhibitor interactions. These results indicate the importance of the sequence common to both active peptides 3A and 3A-1, the octapeptide RGPCRAFI. Peptide 3A-5 (PCRAFI) was not as active as peptide 3A-1, suggesting that peptide 3A-5 does not contain all of the active residues.

Peptide 3A-1 inhibited directed invasion to a lesser degree than HI-8. Inhibition of tumor cell invasion was dose dependent in the range of 1–100 μM peptide. The effective peptide corresponds to the plasmin-inhibiting domain of UTI. To be sure that the active peptides from peptide 3A-1 contain the sequences that are relevant for inhibition of tumor cell invasion, the shorter active peptides were tested as soluble inhibitors. The shorter peptide 3A-3 (RAFI), but not peptides 3A-2 (RGPC) and 3A-6 (RAF), only partially inhibits cell invasion. A combination of peptides 3A-2 and 3A-3 did not have a synergistic effect. Peptides (1, 2, and 2-1) in the domain I region were inactive. Thus far, we have identified the octapeptide RGPCRAFI as important sequences for inhibition of tumor cell invasion.

The cell chemotactic response was also tested to determine whether the inhibiting effect of peptides on cell invasion of Matrigel was due to an inhibition of chemotaxis. The cells tested showed a good chemotactic migration in the presence of UTI or peptides. The lack of
of negative effects on chemotaxis is consistent with an absence of toxicity of peptides. In addition, we examined the effects of UTI or peptides used in this study on cell attachment. No inhibition of attachment to Matrigel was seen with UTI or any of the peptides (data not shown).

Discussion

We previously showed that UTI inhibited production of experimental and spontaneous lung metastasis in an in vivo mouse model (3). When i.v. or s.c. injected with 3LL mouse tumor cells in syngeneic mice, UTI decreased the formation of lung tumor colonies. UTI binds rapidly and with relatively high affinity to tumor cells. UTI that binds to UTI receptors may be internalized and exhibited the intracellular degradation (4). UTI receptors may play a role in modulating plasmin activity to the environment close to the cell surface. In these early studies, we postulated that exogenously added UTI inhibited tumor cell invasion and metastasis formation through the binding of UTI to its receptors on tumor cells. We have confirmed that the domain I region of UTI plays a central role in the binding of UTI to cells, while the domain II of UTI acts as an inhibitor for tumor cell invasion.

To confirm that the domain I subunit functioned as a UTI-binding domain, a mAb D1 was raised in the mouse. The mouse antibody was purified and tested for reactivity against UTI by ELISA and Western blot analysis. It was also evaluated for activity in protease inhibition and tumor cell invasion assays. Interaction of the domain I region with UTI receptors is specific by several criteria: (a) high affinity binding to UTI receptors on tumor cells was obtained only with UTI, whereas HI-8 which lack domain I failed to compete with UTI binding to its receptors; (b) preincubation of FITC-UTI with HI-8 did not produce inhibition of UTI binding to the cells; and (c) UTI preincubated with mAb D1 failed to bind to its receptors on tumor cells. Amino acid sequences recognized by mAb D1 are required for direct binding of UTI to its receptors. The domains or amino acid sequences responsible for UTI binding to its receptors are currently being examined.

Based on peptide activity, antinvasive activity of UTI was assigned to the carboxyl-terminal domain II subunit. We postulated that the sequences contained in the domain II subunit, which does not depend on the ligand-receptor interactions, may be playing a fundamental role in inhibition of tumor cell invasion. However, the possibility that UTI binding to tumor cells might be involved in the prevention of tumor cell invasion was excluded since HI-8, lacking domain I, promotes the inhibition of tumor cell invasion with essentially the same affinity as UTI. UTI binding to cells is not a prerequisite for the inhibition of tumor cell invasion. Our recent interpretation is that peptides, structurally identical to the plasmin-inhibiting domain of UTI, inhibit tumor cell-associated plasmin activity and subsequently prevent tumor cell invasion. Peptides, based on the structure of the domain II subunit, may find a wide application in prevention of tumor spread.

By screening a series of overlapping subpeptides from domain II for their ability to inhibit tumor cell invasion in vitro, we have pinpointed one sequence, RGPCRAFI, which reflects the activity of UTI to some extent (13–15). The two assays (inhibition of plasmin activity and prevention of tumor cell invasion) identified the same subpeptide as containing active sequences. The active site appears to be partially cryptic in UTI, because the octapeptide RGPCRAFI inhibits plasmin activity and tumor cell invasion better than the longer peptides containing the same sequence. The lower activity of peptide 3A relative to RGPCRAFI suggests that the flanking residues do not directly interact with plasmin. Further studies will be required, however, to examine the effects of neighboring sequences on the conformation of the active sequence in this family of peptides. We have to determine the accessibility and conformation of this sequence in native UTI. This class of peptides may have multiple applications in the regulation of proteolytic events involved in tumor invasion and metastasis.

More recently, native UTI and its fragments (the domain I of UTI and HI-8) were examined to determine whether they inhibit production of spontaneous lung metastasis by murine Lewis lung carcinoma (3LL) cells. In an in vivo assay (that measures metastasis from a primary tumor), multiple s.c. injection of the UTI and HI-8 for 7 days after s.c. tumor cell inoculation significantly inhibited metastatic lung tumor colonization in a dose-dependent manner. The domain I of UTI, however, had no effect. These results indicate that occupation of UTI receptors on 3LL cells by the domain I of UTI did not reduce tumor cell invasion and the formation of metastasis. The HI-8 studies assign invasion/metastasis inhibition activity to the COOH-domain of UTI.

Although the in vivo physiological role of the UTI receptors at present is not clear, it has been postulated to function as a regulator of UTI. UTI that binds to its receptors may be internalized and exhibited intracellular degradation. UTI receptors may mediate the degradation of UTI, suggesting that receptor binding may not correlate with invasion/plasmin inhibition.

Although the biological significance of the RGPCRAFI-like sequences in these proteins is not clear at present, the high degree of conservation in the superfAMILY of Kunitz-type protease inhibitors suggests a functional role of the sequence RGPCRAFI. A search of the computer data base using the search sequence RGPCRAFI yielded several other proteins with similar sequences. One of the most intriguing protease inhibitors is ITI, a Kunitz-type protease inhibitor found in mammalian plasma (16).

Although elucidation of the genuine roles of ITI family members is still eagerly awaited, it has been reported that hyaluronic acid synthesized by cultured fibroblasts firmly bound 85-kDa proteins, which corresponds to the two heavy chains of ITI (17, 18). In addition, Chen et al. (19) have reported that ITI acts as a cumulus extracellular matrix stabilizing factor.

In addition to ITI, the peptide RGPCRAFI has interesting similarity with other proteins, including β-amyloid protein precursor and tissue factor pathway inhibitor (13). Quantitative and qualitative abnormalities of Kunitz-type inhibitors have been found in the brain of Alzheimer’s patients. The suspected role of protease inhibitors as neurite growth promoters remains to be proved (13). Also, UTI-like immunoreactive substance was found in the murine brain (20). UTI is produced by neurons in response to brain injury and fear-stress stimuli (20).

Until now, we do not know if ITI family members act as protease inhibitors in vivo. ITI may act as a carrier of hyaluronic acid or a binding protein between hyaluronic acid and other matrix proteins. It is possible that UTI, which was produced by limited proteolysis of ITI, may function as a modulator to regulate tumor cell invasion. Further studies on the relevant subunit of molecules within the ITI family will be required to clarify the genuine roles of ITI and UTI.

Acknowledgments

We thank Drs. N. Moniwa and H. Shinozawa (Department of Obstetrics and Gynecology, Hamamatsu University School of Medicine) for helpful discussion.

References

PEPTIDES DERIVED FROM URINARY TRYPsin INHIBITOR


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