Human Neutrophil Elastase Degrades Inter-α-Trypsin Inhibitor to Liberate Urinary Trypsin Inhibitor Related Proteins

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Urinary tryptin inhibitor (UTI) is a physiological protease inhibitor and inter-α-tryptsin inhibitor (ITI) is regarded as a precursor of UTI. The purpose of this study is to determine the mechanism of the UTI release from UTI. To examine this, UTI was digested by human neutrophil elastase at various concentrations, and UTI-related proteins which were of the same size as UTI were obtained. The amino acid sequence of the 15 amino acid residues at the N-terminal of UTI-related proteins, corresponded to that of UTI. The amino acid sequences of the small amount of peptides detected corresponded to those of peptides from the heavy chain (H1) and the heavy chain (H2) of ITI, suggesting that most UTI-related proteins do not combine with peptides from the H1 and H2 of ITI. It was also revealed that UTI-related proteins have several physiological activities similar to those of UTI, i.e., human tryptin inhibitory activity, human neutrophil elastase inhibitory activity, inhibition of tumor necrosis factor-α (TNF-α) production from rat macrophages and of superoxide production from rabbit leukocytes.

These results demonstrated that ITI is a precursor of UTI which is digested by human neutrophil elastase to release UTI, and that its elastase inhibitory activity is derived from UTI.

Key words urinary tryptin inhibitor (UTI); inter-α-tryptisn inhibitor (ITI); elastase

UTI, an intrinsic tryptin inhibitor isolated and purified from human urine, inhibits various serine proteases such as plasmin, chymotrypsin and neutrophil elastase as well as tryptin. UTI consists of 143 amino acid residues and contains one glycaminoglycan chain at Ser10 and one N-linked carbohydrate at Asp24, so it is regarded as a kind of proteoglycan. UTI is also called bikunin because it consists of two tandemly arranged Kunitz-type protease inhibitor domains. Moreover, UTI also suppresses the production of inflammatory cytokines, including tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1), which are produced from human macrophages through lipopolysaccharide (LPS) stimulation, and it has a cytoprotective effect. In Japan, UTI is used in the treatment of acute pancreatitis and acute circulatory deficiency.

Reisinger et al. investigated the physiological mechanism of the production of UTI and reported that inter-α-tryptsin inhibitor (ITI), a serine protease inhibitor present in plasma, when treated with excess tryptsin released an acid-stable tryptsin inhibitor called HI-30 and that the N-terminal amino acid sequence of HI-30 corresponded with that of UTI. Recently, the structure of ITI has been revealed; ITI consists of two heavy chains (H1, H2) and a light (L) chain, and the amino acid sequence of the ITL chain corresponds with that of UTI except for 4 amino acid residues attached to the C-terminal of UTI. Based on these results, ITI is regarded as a precursor of UTI. However, the mechanism by which UTI is released from ITI has not been clarified as yet. It is known that the concentration of UTI in urine increases from several times to dozens of times the normal level under physiological or pathological conditions such as, e.g., pregnancy, infection, carcinoma and surgical operation. These observations suggest that UTI is produced under various stressful conditions from ITI by activated inflammatory proteases.

Thus, we hypothesized that neutrophil elastase, a typical inflammatory protease, might be related to UTI production; to test our hypothesis, we treated ITI with various concentrations of neutrophil elastase and analyzed the molecular properties and physiological activities of the released substance.

MATERIALS AND METHODS

(1) Materials

Human tryptin and human neutrophil elastase were from Calbiochem Novabiochem Co. (La Jolla, CA, U.S.A.). The purity and specific activity of human neutrophil elastase were more than 90% and 20 units/mg, respectively. Cytochrome c and concanavalin A (Con A) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Mono Q and Vyde C4 columns were from Pharmacia Biotech (Uppsala, Sweden) and The Separations Group (Hesperia, CA, U.S.A.), respectively. Peroxidase-conjugated goat anti-rabbit immunoglobulins and 3,3',5,5'-tetramethylbenzidine (TMB) precipitating reagent were from Dakopatts (Glostrup, Denmark) and Sycet Laboratory (Logan, UT, U.S.A.), respectively. Carbobenzoxy-l-alanyl-p-nitrophenyl ester and LPS were from Kokusan Chemical Works, Ltd. (Tokyo, Japan) and Difco Laboratories (Detroit, MI, U.S.A.), respectively. Rat TNF-α ELISA Kit (Factor-Test-X) and cytochalasin E were from Genzyme Co. (Cambridge, MA, U.S.A.) and Aldrich Chem Co., Inc. (Milwaukee, WI, U.S.A.), respectively.

UTI purified from healthy volunteers' plasma was kindly provided by Dr. Shigeharu Nagasawa (Hokkaido University, Sapporo, Japan). UTI was purified from healthy volunteers’ urine. Activity of UTI was assessed according to Kassell's method. One inhibitory unit was defined as the amount which inhibited the activity of 2 μg of bovine trypsin (3200 National Formulary Unit, NFU/mg, Canada Packers) by 50%. Rabbit anti-human UTI antiserum was prepared from a rabbit which had been administered high-purity human UTI as the antigen. The serum obtained was purified by chromatography using Bakerbond ABx column (J. P. Baker, Inc., Phillipsburg, NJ, U.S.A.).

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(2) ITI Digestion by Human Neutrophil Elastase
Human neutrophil elastase was incubated with ITI in various molar ratios in 50 mM Tris HCl (pH 8.0) buffer at 37°C for various periods of time. After the reaction mixture was frozen to stop the enzymatic reaction, UTI-related proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting using an anti-UTI antibody.

(3) SDS-PAGE and Western Blotting
SDS-PAGE was performed with the use of 12.5% Page® (Atto Co., Tokyo, Japan) following Laemmli's method. After electrophoresis, Western blotting was performed as described by Towbin et al., i.e., the gel was placed on a polyvinylidene difluoride (PVDF) membrane (Atto Co.), and proteins were transferred for 1 h under a current of 200 mA. Thereafter, the PVDF membrane was incubated with rabbit anti-UTI antibody at 37°C for 1 h and washed with phosphate buffered saline containing 0.1% Tween 20. Then, the membrane was incubated with peroxidase-conjugated goat anti rabbit immunoglobulins at room temperature for 1 h. The blotted proteins were detected using TMB precipitating reagent.

(4) Purification of UTI-Related Proteins
Human neutrophil elastase (25.1 μg) and ITI (185 mg) were mixed and incubated with 50 mM Tris HCl (pH 8.0) at 37°C for 24 h. Then, the same amount of elastase was added and the reaction mixture was incubated at 37°C for 48 h. The reaction mixture was loaded onto a Mono Q column equilibrated with 50 mM Tris HCl (pH 8.0) and eluted with a linear gradient of 0—0.5 M NaCl. After fractions including UTI-related proteins were detected by SDS-PAGE and Western blotting, they were pooled, diluted with 50 mM Tris HCl (pH 8.0) buffer and loaded onto a Mono Q column again. The eluted fractions containing UTI-related proteins were pooled and diluted with an equal volume of 0.04% trifluoroacetic acid. The diluted sample was loaded onto a C4 column (i.d. 10×250 mm) equilibrated with 0.04% trifluoroacetic acid and eluted with a linear gradient of acetonitrile from 0 to 70% in the presence of 0.04% trifluoroacetic acid. After the eluted fractions were subjected to SDS-PAGE and Western blotting, the fractions containing UTI-related proteins were pooled and freeze-dried.

(5) N-Terminal Amino Acid Sequencing
Amino acid sequence analysis was conducted in a 477A-120A Sequencer (Applied Biosystems Division, Perkin Elmer Co., Ltd., Foster City, CA, U.S.A.).

(6) Inhibitory Effects of Human Trypsin and Human Neutrophil Elastase by UTI and UTI-Related Proteins
The activity of UTI-related proteins was assessed according to Kasselle's method using UTI as the standard. The inhibition of human trypsin was measured as described by Morishita et al. The inhibition of human neutrophil elastase was assayed as follows: 290 μl of UTI or UTI-related proteins at various concentrations (0.01 M CaCl₂/0.15 M Tris HCl (pH 7.8)), with mixed with 10 μl of 1.47 μg/ml human neutrophil elastase (0.01 M CaCl₂/0.15 M Tris HCl (pH 7.8)), and the reaction mixture was preincubated at 37°C for 1 min. Thereafter, 2 μl of 3.89 mg/ml carbobenzoxy-L-alanyl-p-nitrophenyl ester was added, and the absorbance at 347.5 nm was measured at 37°C for 10 min.

(7) Inhibitory Effects of UTI and UTI-Related Proteins on TNF-α Production from Rat Macrophages
Male Wistar rats (7 weeks old) were intraperitoneally injected with 10 ml of 3% thioglycollate. After 4 days, peritoneal exudate cells were collected and cultured for 2 h at a cell density of 5 × 10⁶ cells/ml in RPMI-1640 medium supplemented with 5% fetal calf serum in 24-well plates. Non-adherent cells were then removed by aspirating the medium and washing the monolayer with culture medium. UTI or UTI-related proteins were added at various concentrations to the adherent cells (macrophages) and preincubated for 4 h in serum-free medium before adding LPS (final: 10 μg/ml) and fetal calf serum (final: 5%). After culturing the cells for 15 h, the supernatants were harvested and assayed using a rat TNF-α ELISA kit.

(8) Inhibitory Effects of UTI and UTI-Related Proteins on Superoxide Production from Rabbit Leukocytes
Rabbits were intraperitoneally injected with 200 ml of 0.2% glycogen. After 16 h, peritoneal exudates containing polymorphonuclear leukocytes were collected and treated with a hypotonic solution (0.2% NaCl) to remove erythrocytes. The cells (5 × 10⁶/ml) were preincubated for 3 min in a reaction mixture containing 66 μM cytochrome c and UTI or UTI-related proteins at 1000 U/ml at 37°C. After that, cytochalasin E (5 μg/ml) and Con A (50 μg/ml) were added to the mixture, and the reduction of cytochrome c was measured at 550 nm at 37°C by spectrophotometry.

(9) MS Analysis (MALDI-TOF MS)
UTI or UTI-related proteins (1 mg/ml) were mixed together with an equal volume of 2,5-di-hydroxybenzoic acid (DHB; 10 mg/ml of 20% ethanol). The sample/matrix solution was dropped onto a sample plate for matrix-assisted laser desorption ionization time of flight (MALDI-TOF) MS, then dried under ambient conditions. A MALDI-TOF MS spectrum was obtained on a Voyager® Elite system (PerSeptive Biosystems, Inc., Framingham, MA, U.S.A.).

(10) Other Methods
Protein concentration was determined by Lowry's method using bovine serum albumin as the standard.

RESULTS

(1) ITI Degradation by Human Neutrophil Elastase
First, we incubated ITI with human neutrophil elastase in a molar ratio 9:1 for 6 h, as described by Balduyck et al., to ensure that human neutrophil elastase would liberate a UTI-like material from ITI. As shown in Fig. 1A, SDS-PAGE of the reaction mixture demonstrated that ITI had been degraded into various products of different molecular weights. Western blotting analysis using an anti-UTI antibody (Fig. 1B) confirmed the presence of 3 specific products of 35 kDa, 20 kDa and around 94 kDa. The 35 kDa band was of the same molecular weight as UTI.

In order to find the best conditions for maximal digestion, we incubated ITI with human neutrophil elastase in various molar ratios for 72 and 75 h. It was found that a UTI-like material can be obtained within the molar ratio range of 30/1 to 1000/1 (ITI/human neutrophil elastase) (Fig. 2A, B). Then, we examined the effect of adding human neutrophil elastase twice or three times, because the enzyme might be inactivated during incubation. Based on these results, we investigated which was the lowest amount of human neutrophil elastase required to produce UTI-like material when human
**Fig. 1.** SDS-PAGE and Western Blotting of the Digestion Products of ITI by Human Neutrophil Elastase

ITI (4.4 mg) purified from human serum was incubated with 66 μg of human neutrophil elastase at a molar ratio of 9:1 in 50 mM Tris HCl (pH 8.0) for 6 h. The digestion products of ITI were analyzed by SDS-PAGE under reducing conditions, followed by silver staining (A) or Western blotting analysis using an anti-ITI antibody (B). (A) Lane 1, ITI; lane 2, the digestion products of ITI by human neutrophil elastase; (B) Lane 1, ITI; lane 2, the digestion products of ITI by human neutrophil elastase; lane 3, UTI.

**Fig. 2.** Western Blotting of the Digestion Products of ITI by Human Neutrophil Elastase

(A) (B) ITI was incubated with human neutrophil elastase at various molar ratios for 72 h. The digestion products of ITI were analyzed by SDS-PAGE under reducing conditions, followed by Western blotting analysis using anti-ITI antibody. (A) Lane 1, ITI; lane 2, ITI/elastase molar ratio = 50:1; lane 3, 100:1; lane 4, 200:1; lane 5, UTI. (B) Lanes 1 and 8, ITI/elastase molar ratio = 200:1; lane 2, 500:1; lane 3, 600:1; lane 4, 700:1; lane 5, 800:1; lane 6, 900:1; lane 7, 1000:1; lane 9, UTI; lane 10, ITI. (C) ITI was incubated with human neutrophil elastase at various molecular weights for 75 h. Elastase was added twice at 0 and 24 h (lanes 1, 2, 3 and 4) or three times at 0, 24 and 48 h (lanes 5, 6, 7 and 8). The digestion products of ITI were analyzed by SDS-PAGE under reducing conditions, followed by Western blotting analysis using an anti-ITI antibody. Lanes 1 and 5, ITI/elastase final molar ratio = 200:1; lanes 2 and 6, 300:1; lanes 3 and 7, 400:1; lanes 4 and 8, 500:1; lane 9, UTI; lane 10, ITI.

**Fig. 3.** Purification of UTI-Related Proteins (A) SDS-PAGE, (B) Western Blotting.

(A) Lane 1, ITI; lane 2, ITI solution digested by human neutrophil elastase; lane 3, eluate of Mono Q; lane 4, eluate of C4 column; lane 5, UTI.

Table 1. N-Terminal Amino Acid Sequences of UTI-Related Proteins

<table>
<thead>
<tr>
<th>Source</th>
<th>Amino acid sequence</th>
<th>Yield (pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light chain&lt;sup&gt;a&lt;/sup&gt;</td>
<td>AVLQEESGSGGVLVVEV...</td>
<td>150</td>
</tr>
<tr>
<td>Heavy chain&lt;sup&gt;b&lt;/sup&gt;</td>
<td>SKSS...TGVDTD</td>
<td>N.C.</td>
</tr>
<tr>
<td>Heavy chain&lt;sup&gt;c&lt;/sup&gt;</td>
<td>SLPG...ISMLAQGQVLESPPPHMPVEND</td>
<td>N.C.</td>
</tr>
<tr>
<td>UTI-related proteins</td>
<td>QXEESGSGGVLVVEV</td>
<td>N.C.</td>
</tr>
<tr>
<td>QXQVLESLPXPM</td>
<td>N.C.</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> The N-terminal amino acid sequence of UTI or light chain of ITI.  
<sup>b</sup> The predicted N- and C-terminal amino acid sequences of heavy chains of ITI. N.C.: not calculated (amino acid sequence identified but not quantified). X: undetermined amino acid.

Neutrophil elastase is added to the reaction mixture intermittently. As a result, it was found that the highest yield and the lowest content of contaminating proteins can be achieved by adding human neutrophil elastase twice in a final molar ratio of 500:1 (ITI/human neutrophil elastase), and incubating for 75 h (Fig. 2C). The 35 kDa product obtained under these conditions was named UTI-related proteins.

2) **Purification of UTI-Related Proteins** ITI (185 mg) was digested to obtain UTI-related proteins, which were purified using Mono Q and Vydc C4 columns (data not shown). Through these procedures, 8.6 mg of purified UTI-related proteins was obtained.

UTI-related proteins showed a single major broad band at an apparent molecular weight of 35 kDa in SDS-PAGE (Fig. 3A). Western blotting analysis revealed no contaminating proteins except for a faint band of 20 kDa (Fig. 3B).

Specific activities of UTI and UTI-related proteins were 2700 and 3080 U/mg, respectively. The specific activity of UTI-related proteins was equivalent to that of UTI.

3) **Amino Acid Sequencing and Mass Analysis** The amino acid sequencing of purified UTI-related proteins was carried out through 15 cycles and the results showed that the main amino acid sequence, AVLP..., corresponded with the N-terminal sequence of UTI (Table 1). Additionally, some minor sequences were detected in purified UTI-related proteins. For example, QXE... corresponded with a sequence from 5th amino acid of the N-terminal of UTI, TGVDTD corresponded with a sequence of 5 amino acids of
the C-terminal of the H1 chain, ISMLA..., MLAQG... and QGXQV... corresponded with sequences from the 24th, 22th and 19th amino acids of the C-terminal of the H2 chain, respectively.

It was judged that the major molecule of UTI-related proteins was the one with the same primary structure as UTI, because each molecule containing H chain-derived sequences was less than 10% of the major molecule.

In MALDI-TOF MS analysis, the average molecular mass of UTI and UTI-related proteins was 24.3 kDa and 24.5 kDa, respectively (Fig. 4). As for their mass spectrum, UTI-related proteins had a broad spectrum compared with that of UTI.

4 Enzyme Inhibitory Spectrum Regarding the inhibitory effect of UTI and UTI-related proteins on human trypsin (Fig. 5), the IC$_{50}$ of UTI was 0.71 U/ml and that of UTI-related proteins was 0.60 U/ml. Their inhibitory activities were equivalent.

Moreover, their inhibitory effects on human neutrophil elastase were also comparable (Fig. 6). The IC$_{50}$ was 5.6 U/ml ($n=2$) for both UTI and UTI-related proteins.

5 Inhibitory Effects of UTI and UTI-Related Proteins on TNF-α Production from Rat Macrophages Inhibition of TNF-α production from rat macrophages was assayed as described in Materials and Methods. Inhibition of TNF-α production represents the relative amount of TNF-α against 100% control, which was assayed without UTI or UTI-related proteins.

As shown in Fig. 7, both UTI and UTI-related proteins suppressed TNF-α production from rat macrophages under LPS stimulation by approximately 50% at the dose of 1000 U/ml.

As for their effects on superoxide production from rabbit leukocytes, UTI and UTI-related proteins suppressed superoxide production by 67.5% and 48.7%, respectively, at the dose of 1000 U/ml. Their inhibitory activities were equivalent.
DISCUSSION

Although Hochstrasser et al.\(^{21}\) reported that HI-30, a protein equivalent to UTI, can be obtained by digesting ITI with trypsin, and Baldyuck et al.\(^{20,22}\) as well as Pratt et al.\(^{23}\) reported that UTI-like materials can be obtained by digesting ITI with neutrophil elastase and cathepsin G, no agreement has been reached as to whether ITI is indeed a precursor of UTI. This is probably because in study the amount of enzyme used to degrade ITI was too large to think that the same condition was attainable in vivo. In addition, the primary structure and properties of the UTI-like materials produced were not described in detail.

In this study we found that UTI-related proteins can be obtained when reacting human neutrophil elastase with ITI at any ratio between 1:9 and 1:1000 (elastase : ITI). Our results contradict the reports by Baldyuck et al.\(^{20,22}\) and Pratt et al.\(^{23}\) and suggest that UTI can be produced from ITI in the presence of low concentrations of human neutrophil elastase, thus ITI would be a substrate and not an inhibitor of human neutrophil elastase. It is considered that the inhibitory effect of ITI on human neutrophil elastase could be caused by UTI released from ITI.

We were able to obtain 8.6 mg of purified UTI-related proteins from 185 mg of ITI. The theoretical recovery was approximately 41% in all procedures; therefore, it is understood that UTI-related proteins are the major products, not the subproducts, of the digestion of ITI by elastase.

Amino acid sequencing of purified UTI-related proteins showed that the sequence of the 15 amino acid residues at the N-terminal completely corresponded with that of UTI. Although some minor sequences derived from the H1 and the H2 chains of ITI were detected in the major sequence, it was suggested that the major component of UTI-related proteins has the same amino acid sequence as UTI.

As a result of mass analysis, the approximate average molecular mass of both UTI and UTI-related proteins was 24 kDa. It was suggested that the molecular mass of the major component of UTI-related proteins is equivalent to that of UTI. We thought that the peak of the mass spectrum of UTI-related proteins was a little broader than that of UTI because of the presence of a mixed molecular species containing UTI and proteins attached to the peptides from the H chains of ITI.

Moreover, the physiological activities of UTI-related proteins corresponded with those of UTI in this study. They not only inhibited human trypsin and human neutrophil elastase, but also inhibited both the production of cytokine from rat macrophages and that of superoxide from rabbit leukocytes. Therefore, these findings showed that ITI releases a protein which is extremely similar to UTI in primary structure and function in the presence of human neutrophil elastase, and it can thus be considered a physiological precursor of UTI.

Nowadays, it has been demonstrated that leukocyte is activated by various stimuli such as LPS and cytokines, and then the inflammatory response is triggered by the released inflammatory cytokines and is enhanced by inflammatory proteases such as neutrophil elastase and cathepsin G. Although there are many elastase inhibitors in the blood, such as α1 proteinase inhibitor\(^{29}\) and α2 macroglobulin,\(^{25}\) the blood concentration of neutrophil elastase increases in the early stages, and then these inhibitors are consumed during an inflammatory process, causing the inflammation to progress. Our results raise the possibility that UTI is produced in an inflammatory process and suppresses the progression of the inflammatory reaction by acting as a protease inhibitor; furthermore, it also suppresses the production of inflammatory cytokines from leukocytes and that of superoxide, which both contribute to the progression of inflammation.\(^{16,27}\) Recently, Kobayashi et al. reported that UTI is released from ITI by elastase-like enzymes on the surface of cancer cells.\(^{28}\) Our results were supported by their results. In conclusion, our results showed that ITI is degraded to UTI by neutrophil elastase and that the released UTI has a variety of physiological activities which are associated with the in vivo mechanism of defense.

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