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Urinary Trypsin Inhibitor, an Alternative Therapeutic Option for Inflammatory Disorders

Ken-ichiro Inoue[1] and Hirohisa Takano[2]
[1]*Department of Public Health and Molecular Toxicology, School of Pharmacy, Kitasato University, Tokyo*
[2]*Kyoto University Graduate School of Enginnering, Department of Environmental Engineering, Kyoto*
Japan

1. Introduction

Urinary trypsin inhibitor (UTI), a serine protease inhibitor, has been widely (and sometimes experiencely) used as a supportive drug for patients with inflammatory disorders such as pancreatitis, shock, and disseminated intravascular coagulation (DIC). Also, previous in vitro studies have demonstrated that serine protease inhibitors may have anti-inflammatory properties at sites of inflammation. However, the therapeutic effects of UTI in vivo remain unclarified, since commercial UTI have been developed to act against human, with the activity and selectivity toward the relevant animal UTI being less characterized. In this review, we introduce the roles of UTI mainly in experimental endotoxin (lipopolysaccharide: LPS)-related inflammatory disorders using UTI-deficient (-/-) and corresponding wild-type (WT) mice. Our experiments employing genetic approach suggest that endogenous UTI can serve protection against the systemic inflammatory response and subsequent organ injury induced by LPS, at least partly, through the inhibition of proinflammatory cytokine and chemokine expression, which provide important in vivo evidence and understanding about a protective role of UTI in inflammatory conditions. Using genetically targeted mice selectively lacking UTI, UTI has been evidenced to provide an attractive "rescue" therapeutic option for endotoxin-related inflammatory disorders such as DIC, acute lung injury, and acute liver injury.

2. General characteristics of UTI and clinical utility

UTI, also referred to as ulinastatin, HI-30, ASPI, or bikunin, is an acidic glycoprotein with a molecular weight of 30 kDa by SDS-polyacrylamide gel electrophoresis. UTI is a multivalent Kunitz-type serine protease inhibitor found in human urine and blood [1]. It is composed of 143 amino acid residues and its sequence includes two Kunitz-type domains (Fig. 1). UTI is produced by hepatocytes as a precursor in which UTI is linked to α_1-microgloblin [2, 3]. In hepatocytes, different types of UTI-containing proteins are formed by the assembly of UTI with one or two of the three evolutionarily related heavy chains (HC) 1, HC 2, and HC 3,

through a chondroitin sulfate chain [4]; these proteins comprise inter-α-inhibitor (IαI) family members, including IαI, pre-α-inhibitor (PαI), inter-α-like inhibitor (IαLI), and free UTI. IαI, pαI, and IαLI are composed of HC1 + HC2 + UTI, HC3 + UTI, and HC2 + UTI, respectively [5, 6]. Its specific activity was 2,613 U/mg protein, one unit being the amount necessary to inhibit the activity of 2 μg trypsin (3,200 NFU/mg, Canada Packers) by 50% [7]. During inflammation, UTI is cleaved from IαI family proteins through proteolytic cleavage by neutrophil elastase in the peripheral circulation or at the inflammatory site [8-11]. Therefore, plasma UTI has been considered to be one of the acute phase reactions and indeed, the plasma UTI level and its gene expression alter in severe inflammatory conditions [9]. Further, UTI is rapidly released into urine when infection occurs and is an excellent inflammatory marker, constituting most of the urinary anti-trypsin activity [12]. Various serine proteases such as trypsin, thrombin, chymotrypsin, kallikrein, plasmin,

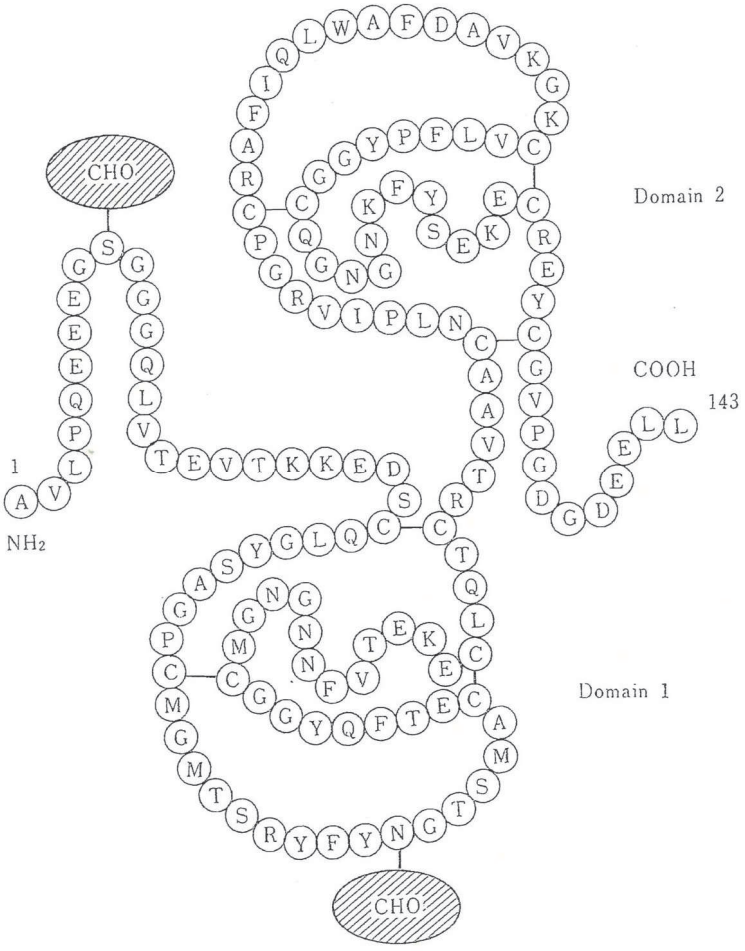

Fig. 1. Molecular structure of urinary trypsin inhibitor (UTI).

chymotrypsin, kallikrein, plasmin, elastase, cathepsin, and Factors IXa, Xa, XIa, and XIIa are inhibited by UTI [13, 14]. Furthermore, UTI can reportedly suppress urokinase-type plasminogen activator (uPA) expression through the inhibition of protein kinase C (PKC) [15, 16]. UTI appears to prevent organ injury by inhibiting the activity of these proteases [17, 18]. Based on the multivalent nature of protease inhibition, clinically, UTI is widely used, especially in Japan, to treat acute pancreatitis including post-endoscopic retrograde cholangiopancreatography pancreatitis, in which proteases are thought to play a pathophysiological role [19]; however, current understanding as for the target mechanisms/pathways remains limited.

3. Anti-inflammatory potential of UTI in *in vitro*, *in vivo*, and humans

Beyond its inhibition of inflammatory proteases mentioned above, UTI exhibits anti-inflammatory activity and suppresses the infiltration of neutrophils and release of elastase and chemical mediators from them [11, 20, 21]. Likewise, UTI reportedly inhibits the production of tumor necrosis factor (TNF)-α[22, 23] and interleukin (IL)-1 [23] in LPS-stimulated human monocytes and LPS- or neutrophil elastase-stimulated IL-8 gene expression in HL60 cells [24] or bronchial epithelial cells [25] *in vitro*. Matsuzaki et al. demonstrated that UTI inhibits LPS-induced TNF-α and subsequent IL-1β and IL-6 induction by macrophages, at least partly, through the suppression of mitogen-activated protein kinase (MAPK) signaling pathways such as ERK1/2, JNK, and p38 *in vitro* [26]. Nakatani and colleagues demonstrated that UTI inhibits neutrophil-mediated endothelial cell injury *in vitro*, suggesting that UTI can act directly/indirectly on neutrophils and suppress the production and secretion of activated elastase from them [21]. Furthermore, UTI down-regulates stimulated arachidonic acid metabolism such as thromboxane B2 production *in vitro* [27], which plays a role in the pathogenesis of sepsis [28].

A large number of *in vivo* reports have provided evidence that UTI protects against pathological traits related to septic shock induced by gram-negative bacteria: UTI reduces LPS-elicited circulatory failure such as hypotension, lactic acidosis, and hyperglycemia [29-31] through modulating TNF-α production via the inhibition of early growth response factor (Egr)-1 in monocytes and pulmonary induction of inducible nitric oxide synthase (iNOS) [29] and reduces mortality caused by sepsis [32]. Also, UTI can alleviate coagulatory disturbance accompanied by sepsis such as an increase in the serum level of fibrinogen and fibrinogen degradation products [33]. Likewise, UTI has a protective effect against ischemia-reperfusion injury in the liver [35], kidney [36], heart [37], and lung [38] *in vivo* via the actions of its radical scavenging elements [39]. As for its mechanism, UTI reduces C-X-C chemotactic molecule production during liver ischemia/reperfusion *in vivo* [40]. In humans, prepump administration (5,000 U/kg) of UTI reportedly improves cardiopulmonary bypass-induced hemodynamic instability and pulmonary dysfunction through the attenuation of IL-6 and IL-8 production/release in humans [41]. Also, UTI can inhibit coagulatory activation accompanied by severe inflammation such as tissue factor (TF) expression on monocytes *in vitro* and *in vivo* [33] as well as coagulation and fibrinolysis during surgery in humans [42].

Koizumi et al. have shown that UTI prevents experimental crescentic glomerulonephritis in rats, at least in part, by inhibiting the intraglomerular infiltration of inflammatory cells [50]. Interestingly, Tsujimura and colleagues reported a case of infectious interstitial pneumonia

associated with mixed connective tissue disease, in whom the bolus infusion of UTI improved the pathology [52]. Also, Komori et al. illustrated that UTI improves peripheral microcirculation and relieves bronchospasm associated with systemic anaphylaxis in rabbits [53].
Moreover, UTI has been shown to down-regulate the expression of the cancer metastasis-associated molecules uPA and uPA receptor (uPAR) possibly through MAPK- dependent signaling cascades *in vitro* and *in vivo* [61, 62]. In addition, UTI has anti-inflammatory effects against several forms of malignancy *in vitro* [58, 63]. These studies suggest that UTI is a candidate anti-cancer drug, although further studies are required in the future.

4. *In vivo* mouse model supporting role of UTI in physiologic and pathologic conditions

4.1 Generation of *UTI*-gene knockout mouse

To further investigate the physiobiological functions of UTI *in vivo*, we generated UTI (-/-) mice [64]. UTI (-/-) mice were produced as follows: a targeting vector was designed to disrupt the exons encoding UTI, leaving the exons encoding α1m intact. Germline transmission was observed in 3 chimeric male mice derived from 3 independent targeted ES clones. We generated mice that were homozygous for the mutant UTI gene (UTI [-/-] mice) by intercrossing the heterozygous mice. Under specific pathogen-free conditions, UTI (-/-) mice were born and developed normally. They grew to a normal body size and showed no apparent behavioral abnormalities. A histological study of various organs revealed no apparent differences between wild-type (WT) and UTI (-/-) mice. The ages at vaginal opening during postnatal development and the estrous cycle of UTI (-/-) female mice determined by the vaginal smear method were also normal [64].
Thereafter, we conducted a series of studies on the role of UTI in the inflammation related to LPS using the UTI (-/-) mice.

4.2 Protective role of UTI in systemic inflammation

In a study [65], both UTI (-/-) and wild-type (C57/BL6: WT) mice were injected intraperitoneally (i.p.) with vehicle or LPS at a dose of 1 mg/kg body weight. Evaluation of the coagulatory and fibrinolytic parameters and white blood cell (WBC) counts at 72 hours after i.p. challenge showed that fibrinogen levels were significantly greater in LPS- than in vehicle-challenged mice with the same genotypes. In the presence of LPS, however, they were also significantly higher in UTI (-/-) than in WT mice. WBC counts significantly decreased after LPS challenge in UTI (-/-) mice. In the presence of LPS, the prothrombin time was significantly shorter in UTI (-/-) than in WT mice. Furthermore, histopathological changes in the lung, kidney, and liver of both genotypes after LPS challenge revealed severe neutrophilic inflammation in UTI (-/-) lungs challenged with LPS, whereas little neutrophilic infiltration was found in LPS-treated WT mice. The overall trend was similar regarding findings in the kidney and liver.
The protein expression levels of proinflammatory molecules such as macrophage chemoattractant protein (MCP)-1 in the lungs, MCP-1 and keratinocyte-derived chemoattractant (KC) in the kidneys, and IL-1β, macrophage inflammatory protein (MIP)-2, MCP-1, and KC in the livers, were significantly greater in UTI (-/-) than in WT mice after LPS challenge. These results indicate that UTI protects against systemic inflammation induced by the intraperitoneal administration of LPS, at least partly, through the inhibition

of proinflamatory cytokine production/release [65], suggesting that UTI may be therapeutic against sepsis in humans.

4.3 Protective role of UTI in acute lung inflammation

A previous study showed that UTI improves acute lung injury *in vivo* [66]; however, no evidence has been reported using a genetic approach. In another series of studies [67, 68], therefore, UTI (-/-) and WT mice were intratracheally treated with vehicle or LPS (125μg/kg), and sacrificed 24 hours later. In both genotypes, LPS treatment induced significant increases in the numbers of total cells and neutrophils in bronchoalveolar lavage (BAL) fluid as compared with vehicle treatment, which was significantly greater in UTI (-/-) than in WT mice. Also, UTI (-/-) mice showed a significantly greater increase in the lung water content when compared to WT mice following LPS treatment. Lung specimens stained with hematoxylin and eosin 24 hours after intratracheal instillation showed that, in the presence of LPS, WT mice showed the moderate infiltration of neutrophils, whereas in UTI (-/-) mice, LPS treatment led to the marked recruitment of neutrophils and interstitial edema. LPS treatment induced a significant elevation of the protein levels of IL-1β, MIP-1α, MCP-1, and KC in lung homogenates when compared to vehicle treatment in both genotypes; however, in the presence of LPS, the expression was higher in UTI (-/-) than in WT mice. Furthermore, immunohistochemical examination showed that, in the presence of LPS, immunoreactive 8-hydroxy-2′-deoxyguanosine was detected in the lungs of both genotypes of mice, but the staining was more prominent in UTI (-/-) than in WT mice. In addition, immunoreactive nitrotyrosine was strongly detected only in UTI (-/-) mice challenged with LPS. Quantitative gene expression analyses of lung homogenates after intratracheal challenge showed that, compared to vehicle treatment, LPS treatment resulted in a significant elevation of gene expression for iNOS in both genotypes of mice; however, in the presence of LPS, the expression was higher in UTI (-/-) than in WT mice. These results indicate that UTI also protects against acute lung inflammation induced by the intratracheal administration of LPS, at least in part, via the local suppression of proinflammatory cytokines [67] and oxidative stress [68], suggesting that UTI may be a therapeutical tool for acute lung injury in humans.

4.4 Protective role of UTI in acute liver inflammation

One study has shown that plasma UTI levels increase in patients with acute hepatitis and markedly decrease in those with fulminant hepatitis, suggesting that the plasma UTI level is closely linked to the severity of liver damage [69]. Further, the plasma UTI level is reportedly correlated with the degree of liver damage in patients with chronic liver diseases such as liver cirrhosis and hepatocellular carcinoma [70]. In a liver inflammation and coagulatory disturbance model induced by LPS (3μg/kg) and D-galactosamine (800 mg/kg: LPS/D-GalN), LPS/D-GalN treatment caused severe liver injury characterized by neutrophilic inflammation, hemorrhagic change, necrosis, and apoptosis, which was more prominent in UTI (-/-) than in WT mice [71]. In both genotypes of mice, interestingly, LPS/D-GalN challenge caused elevations of aspartate amino-transferase and alanine amino-transferase, prolongation of the prothrombin and activated partial thromboplastin time, and decreases in fibrinogen and platelet counts, as compared with vehicle challenge. These changes, however, were significantly greater in UTI (-/-) than in WT mice. Circulatory levels of TNF-α and interferon (IFN)-γ were also greater in UTI (-/-) than in WT mice after LPS/D-GalN challenge. These results suggest that UTI protects against severe liver injury and subsequent coagulatory

disturbance induced by LPS/D-GalN, which was mediated, at least partly, through the suppression of TNF-α production along with its anti-protease activity [71]. Furthermore, after LPS/D-GalN challenge, protein levels of IL-1β, TNF-α, IFN-γ, MIP-1α, and MCP-1 in the lung homogenates were elevated in both genotypes, but to a greater extent in UTI (-/-) than in WT mice. The IFN-γ level was also significantly greater in LPS/D-GalN-challenged UTI (-/-) than in other mice. These results indicate that UTI protects against the local inflammatory response accompanied by severe liver injury, which supports its anti-inflammatory properties *in vivo* [72], implicating a therapeutic potential of UTI in fulminant hepatitis in humans. In this regard, Nobuoka and colleagues have recently implicated UTI in normal liver regeneration using UTI (-/-) mice via the regulation of systemic (serum) levels of cytokines such as IL-6 and IL-10 and chemokines such as MCP-1 and MIP-1α [73].

5. Concluding remarks

As described above, UTI protects against endotoxin-related inflammatory diseases' pathology and subsequent organ damage induced by LPS in mice, at least partly, via the regulation of neutrophil-derived proteases such as elastase, proinflammatory cytokines and chemokines such as IL-1β, MIP-1α, MCP-1, and KC and oxidative stress (Fig. 2). Our

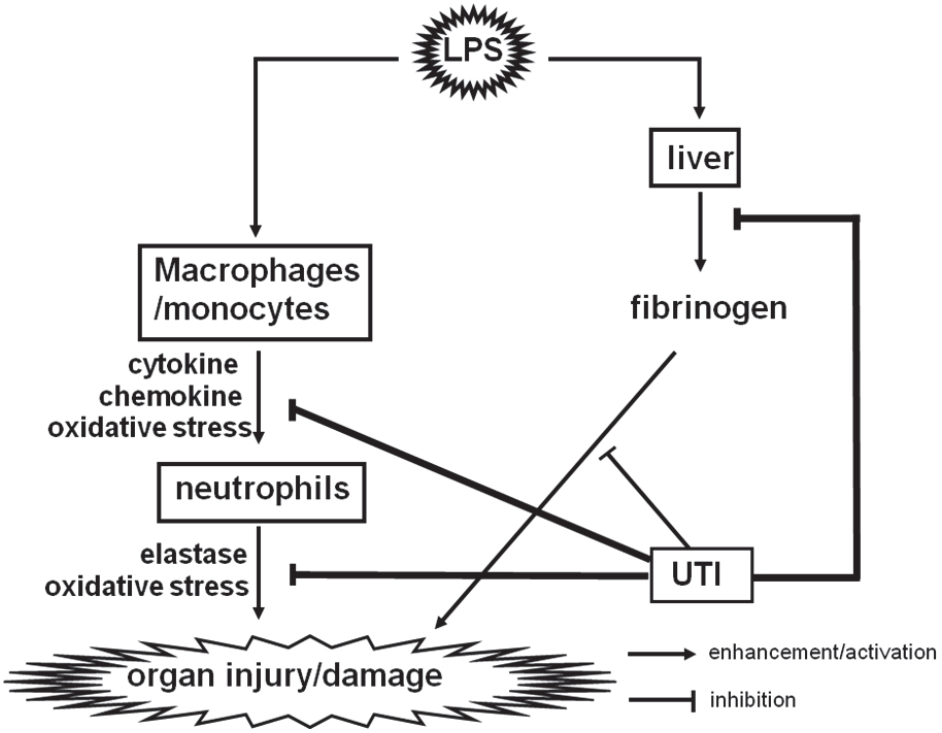

Fig. 2. Schematic representation of the protective role of UTI against endotoxin-related inflammation in mice. Our data suggest that UTI protects against: 1) endothelial activation/damage, 2) proinflammatory cytokine and chemokine production/release, 3) fibrinogen synthesis, 4) neutrophil recruitment into organs, and/or 5) organ injury.

consecutive *in vivo* results provide direct and novel molecular evidence for the "rescue" therapeutic potential of UTI against endotoxin-related inflammatory diseases such as DIC, acute lung injury, and acute liver injury.

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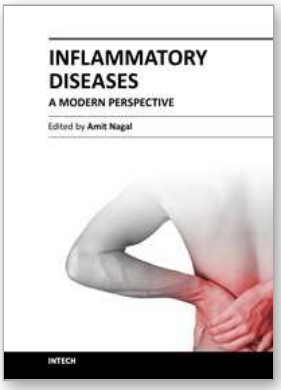

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"Inflammatory Diseases - A Modern Perspective" represents an extended and thoroughly revised collection of papers on inflammation. This book explores a wide range of topics relevant to inflammation and inflammatory diseases while its main objective is to help in understanding the molecular mechanism and a concrete review of inflammation. One of the interesting things about this book is its diversity in topics which include pharmacology, medicine, rational drug design, microbiology and biochemistry. Each topic focuses on inflammation and its related disease thus giving a unique platform which integrates all the useful information regarding inflammation.

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University Campus STeP Ri
Slavka Krautzeka 83/A
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InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821