Urinary trypsin inhibitor suppresses the production of interstitial procollagenase/proMMP-1 and prostromelysin 1/proMMP-3 in human uterine cervical fibroblasts and chorionic cells

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Abstract The mechanisms by which urinary trypsin inhibitor (UTI) prevents preterm premature rupture of fetal membrane and premature cervical ripening were investigated. We, therefore, examined the effects of UTI on the production of matrix metalloproteinases (MMPs) which closely participate in the breakdown of extracellular matrix in cultured human uterine cervical fibroblasts and human chorionic cells. UTI suppressed specifically the production of interstitial procollagenase/proMMP-1 and prostromelysin 1/proMMP-3 from both cells in a dose-dependent manner (0.32–1.28 μM). This suppression was accompanied by a decrease in steady-state levels of their mRNAs. These results indicate for the first time that UTI down-regulates the production of proMMP-1 and proMMP-3 accompanying with the decrease in the expression of their mRNAs, and therefore UTI actually participates in the maintenance of fetal membranes and/or uterine cervix by overall suppression of MMP production along with the known inhibitory actions towards serine proteinases.

Key words: Urinary trypsin inhibitor (UTI); Matrix metalloproteinase; Collagenase; Stromelysin 1; Premature rupture of fetal membrane; Human uterine cervix; Human fetal membrane

1. Introduction

The uterine cervix and fetal membrane are typical connective tissues consisting of type I and III collagens and proteoglycans, and their tensile strength and/or toughness are considered to be maintained by extracellular matrices in those tissues. Thus abnormal advance in the breakdown of extracellular matrix may lead the preterm premature rupture of fetal membrane (preterm PROM) and/or premature cervical ripening which lead to preterm labor [1–3]. The breakdown of extracellular matrix components is mainly accomplished by matrix metalloproteinases (MMPs) ([4] for review). In general, most connective tissue cells produce precursor forms of MMPs (proMMPs) together with their endogenous inhibitors of TIMPs, and then proMMPs are activated by some proteinases such as plasmin [4]. TIMPs inhibit the activities of MMPs through the formation of 1:1 stoichiometrical complex with activated MMPs [4]. Thus the balance between MMPs and TIMPs is important in maintaining or breaking down the extracellular matrix. The production of proMMPs and TIMPs is regulated by various cytokines, growth factors and hormones, and their production is finely timed in normal parturition [4–7].

Urinary trypsin inhibitor (UTI), an acid-stable glycoprotein with a molecular mass of 67 kDa which is a light chain of inter-α trypsin inhibitor in plasma, is one of the Kunitz-type proteinase inhibitors [8,9]. The glycoside chains of UTI are composed of chondroitin-sulfate, and UTI is therefore characterized as one of proteoglycans [10,11]. UTI inhibits the serine proteinases including trypsin, chymotrypsin, neutrophil elastase, cathepsin G and plasmin [8]. UTI is found in human amniotic fluid as well as human urine, and the level of UTI in amniotic fluid decreases in patients with PROM as compared with normal pregnancy [12]. Recently it was reported that UTI inhibits the production of interleukin 8 (IL-8) in macrophages [13] and the contraction of uterine muscle by modifying the intracellular Ca^{2+} concentration [14]. Furthermore, UTI is known to prevent the preterm PROM [15], preeclampsia [16], premature cervical ripening in rabbit [17] and preterm delivery in mouse [18]. These observations suggest that UTI might closely participate in the production and/or regulation of MMPs in fetal membrane and uterine cervix.

In this report, we have investigated the effect of UTI on the production of MMPs and TIMPs in human uterine cervical fibroblasts and chorionic cells, and demonstrated that UTI suppresses specifically the production of proMMP-1/interstitial procollagenase and proMMP-3/prostromelysin 1 accompanying the decrease in the expression of their mRNAs.

2. Materials and methods

2.1. Materials

The following reagents were obtained commercially: Eagle's minimum essential medium (MEM) and Dulbecco's modified Eagle's medium/Ham's F12 mixed (1:1) medium (DMEM-Ham's F12) from Life Technologies, Inc., Grand Island, NY; fetal bovine serum (FBS) from Whittaker Bioproducts, Walkersville, MD; alkaline phosphatase-conjugated donkey anti-(sheep IgG)IgG from Sigma Chemical Co., St. Louis, MO; human glyceroaldehyde 3-phosphate dehydrogenase (GAPDH) cDNA probe from Clontech Laboratories, Inc., Palo Alto, CA. Sheep anti-(human proMMP-1)antiserum, sheep anti-(human MMP-3)antiserum, sheep anti-(human TIMP-1)antiserum, human MMP-1 cDNA (1.6 kb) and human MMP-3 cDNA (1.4 kb) were kindly provided by Prof. H. Nagase of the University of Kansas

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Abbreviations: MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinases; UTI, urinary trypsin inhibitor; PROM, premature rupture of fetal membrane; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; MEM, Eagle's minimum essential medium; IL, interleukin; SDS-PAGE, sodium dodesyl sulfate-polyacrylamide gel electrophoresis; LAH, lactalbumin hydrolysate; GAPDH, glyceroaldehyde 3-phosphate dehydrogenase; SSC, standard saline citrate

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Medical Center, Kansas City, KS. Recombinant human IL-1α (IL-1α; 2×10^7 units/mg) was a kind gift from Dainippon Pharmaceutical Co., Suita, Osaka, Japan. Urinary trypsin inhibitor (UTI; 2330 units/mg, 1 unit is defined as the amount of UTI for 50% inhibition of 2 μg of trypsin) was kindly provided by Mochida Pharmaceutical Co., Tokyo, Japan. Other reagents used were of analytical reagent grade.

2.2. Cell cultures

Human uterine cervical fibroblasts were prepared and maintained in MEM containing 10% (v/v) FBS as described previously [19]. In all experiments, cells up to the 6th passage were used.

Human trophoblast-like cells (chorionic cells) prepared from chorion leave were kindly provided by Dr. K. Ohyama, Second Department of Biochemistry, Tokyo University of Pharmacy and Life Science, and maintained in DMEM-Ham's F12 containing 20% (v/v) FBS. In all experiments, cells up to the 5th passage were used.

For most of the experiments, confluent cells in 24-multiwell plates were washed once with calcium and magnesium-free phosphate buffered saline [PBS(-)] and incubated with the serum-free respective medium/0.2% (w/v) lactalbumin hydrolysate (LAH) containing IL−1α and/or UTI for 24 h. The conditioned culture media were harvested and stored at −20°C until use. All experiments were conducted at least triplicate and typical data are presented.

2.3. Western blot analysis

Each culture medium (1 ml) from triplicate wells was mixed with 1/5 volume of 20% (w/v) trichloroacetic acid. The resultant precipitates dissolved in reducing sample buffer of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [20], were first subjected to SDS-PAGE with 10% (w/v) acrylamide gel under reducing conditions, and then proteins in the gel were electro-transferred onto a nitrocellulose membrane. The membrane was reacted with sheep anti-(human proMMP-1)antiserum, sheep anti-(human MMP-3)antiserum or sheep anti-(human TIMP-1)antiserum which was then complexed with alkaline phosphatase-conjugated donkey anti-(sheep IgG)IgG. Immunoreactive proMMP-1, proMMP-3 and TIMP-1 were visualized indirectly with 5-bromo-4-chloro-3-indolyl phosphate and Nitro Blue Tetrazolium as described previously [21].

2.4. RNA extraction and Northern blot analysis

Confluent human uterine cervical cells or human chorionic cells in 100 mm diameter dishes were treated with 15 ml of each medium/0.2% (w/v) LAH containing IL-1α and/or UTI. After 24 h of treatment, total cytoplasmic RNA was extracted with Isogen (Nippon Gene Co., Toyama, Japan) according to the manufacturer's instructions. Then extracted RNA (10 μg) was denatured with formamide and formaldehyde, electrophoresed to 1% (w/v) agarose containing 1×3-(*N*-Morpholino)propanesulfonic acid (MOPS) and 2.2 M formaldehyde, and transferred onto a nylon membrane (Gene Screen; DuPont, Boston, MA) in 10×standard saline citrate (SSC). The membrane was hybridized with ^{32}P-labeled random primed human proMMP-1, proMMP-3 or GAPDH cDNA in 50% (v/v) formamide/5×SSC/0.1% (w/v) SDS/1×Denhardt's solution/50 mM Na_3PO_4/200 μg/ml of heat-denatured salmon sperm DNA at 42°C for 18 h. After hybridization, the membrane was washed in 1×SSC/0.1% (w/v) SDS and washed in 0.1×SSC/0.1% (w/v) SDS at room temperature for 30 min, respectively, and then exposed to Konica X-ray film (Konica Co., Shinjuku, Tokyo, Japan) at −80°C.

3. Results

3.1. Suppression of the IL-1α-induced production of proMMP-1 and proMMP-3 by UTI

When confluent human uterine cervical fibroblasts were treated for 24 h with IL-1α (1 ng/ml) which stimulates most connective tissue cells to produce proMMP-1 and proMMP-3 [4,5] and participates in PROM [22,23] and uterine cervical ripening [24], the production of both proMMP-1 and proMMP-3 was augmented: about a 3-fold increase in immunoreactive respective enzyme was observed in the IL-1α-treated cells as compared to the control cells (Fig. 1). UTI effectively suppressed both basal and IL-1α-augmented production of proMMP-1 and proMMP-3 in a dose-dependent manner (0.32–1.28 μM), and 1.28 μM UTI suppressed about 40% of the IL-1α-mediated production of both proMMP-1 and proMMP-3. In addition, UTI more effectively suppressed the spontaneous production of these proMMPs whereas UTI only slightly suppressed the production of TIMP-1. These concentrations of UTI affected neither the thymidine incorporation into DNA (data not shown) nor the number of cells: when confluent cells were treated with UTI (0.32 μM) and/or IL-1α (1 ng/ml) for 24 h, the DNA content in control, UTI-, IL-1α- and UTI plus IL-1α-treated groups were 2.92±0.25, 2.91±0.14, 3.02±0.24 and 2.97±0.27 (μg DNA/well; the mean±S.D. for three wells), respectively, indicating that UTI specifically suppressed the production of proMMPs without affecting the cell growth and/or cell functions.

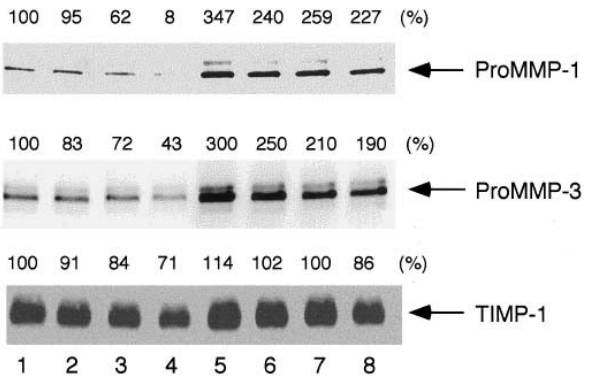

Fig. 1. Effect of UTI on the production of proMMP-1, proMMP-3 and TIMP-1 in human uterine cervical fibroblasts. Confluent human uterine cervical fibroblasts at the 6th passage were treated with IL-1α (1 ng/ml) and/or UTI (0.32 μM, 0.64 μM and 1.28 μM, respectively) for 24 h. An aliquot (1 ml) of harvested culture media from triplicate wells was subjected to Western blot analysis to determine the proMMP-1, proMMP-3 and TIMP-1 as described in the text. The relative amounts of proMMP-1, proMMP-3 and TIMP-1 were quantified by densitometric scanning, taking the untreated cells as 100%. Three independent experiments using cells of different origins at different passages were highly reproducible, and typical data are shown. Lane 1, untreated cells; lanes 2–4, cells treated with UTI (0.32 μM, 0.64 μM and 1.28 μM, respectively); lane 5, cells treated with IL-1α (1 ng/ml) and lanes 6–8, cells co-treated with IL-1α (1 ng/ml) and UTI (0.32 μM, 0.64 μM and 1.28 μM, respectively).

The effect of UTI on the production of proMMPs and TIMP-1 was also examined in human chorionic cells. As shown in Fig. 2, UTI suppressed the IL-1α-augmented production of proMMP-1 and both basal and IL-1α-mediated production of proMMP-3 to a similar extent. Interestingly, more effective suppression (50%<) of their production was observed even when cells were treated with 0.32 μM UTI. The production of TIMP-1 was also interfered by UTI to a lesser extent than that of proMMPs. We also confirmed that UTI did not reduce the total protein synthesis in human chorionic cells; when the confluent cells were treated with UTI (0.64 μM) for 24 h and then co-incubated with [^{3}H]amino acid mixture for the last 8 h, both control and UTI-treated cells uptook 2.33±0.22 and 2.35±0.12 ($\times10^{-3}$ dpm/well; the mean±S.D. for three wells) of ^{3}H into protein fraction, respectively. It therefore appears that UTI is an effective and selective suppressor of the production of proMMP-1, proMMP-3 and TIMP-1 not only in human uterine cervical fibroblasts, but also in human chorionic cells.

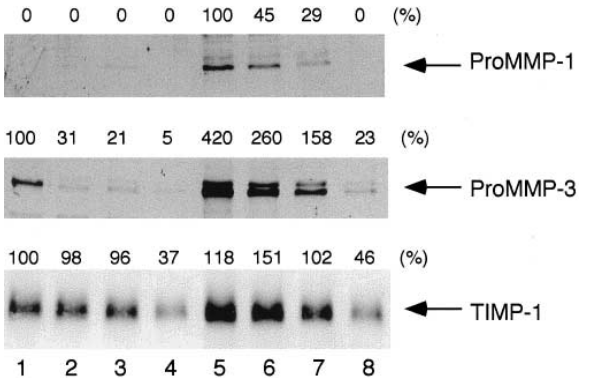

Fig. 2. Effect of UTI on the production of proMMP-1, proMMP-3 and TIMP-1 in human chorionic cells. Confluent human chorionic cells at the 4th passage were treated with IL-1α (1 ng/ml) and/or UTI (0.32 μM, 0.64 μM and 1.28 μM, respectively) for 24 h. An aliquot (1 ml) of harvested culture media from triplicate wells was subjected to Western blot analysis to detect the proMMP-1, proMMP-3 and TIMP-1 as described in the text. The relative amounts of proMMP-3 and TIMP-1 were quantified as described in Fig. 1. In the case of proMMP-1, the level of IL-1α (lane 5) was taken as 100% because chorionic cells produced basically an insignificant amount of the enzyme. Three independent experiments using cells of different origins at different passages were highly reproducible, and typical data are shown. Lane 1, untreated cells; lanes 2–4, cells treated with UTI (0.32 μM, 0.64 μM and 1.28 μM, respectively); lane 5, cells treated with IL-1α (1 ng/ml) and lanes 6–8, cells co-treated with IL-1α (1 ng/ml) and UTI (0.32 μM, 0.64 μM and 1.28 μM, respectively).

3.2. *UTI interferes the IL-1α-induced accumulation of steady-state levels of MMP-1 and MMP-3 mRNAs*

Next, we examined whether the decrease in the proMMPs level caused by UTI resulted from the changes in their mRNAs. The changes in the steady-state levels of MMP-1 and MMP-3 mRNAs were monitored by Northern blot analysis. Both non-treated uterine cervical fibroblasts and chorionic cells contained slight MMP-1 and MMP-3 transcripts, and IL-1α increased their transcripts obviously as shown in Fig. 3. UTI decreased the steady-state levels of MMP-1 and MMP-3 mRNAs in both cell species, whereas their suppression by UTI was less than that of protein levels. These results suggest that UTI controls the production of proMMP-1 and proMMP-3 in human uterine cervical cells and chorionic cells at both the transcriptional and post-transcriptional levels.

4. Discussion

Although UTI is well known as an inhibitor of serine proteinases, recent reports that UTI suppresses the production of IL-8 in macrophages [13] and the lipopolysaccharide-induced Ca^{2+}-influx into cells [25] are of great interest. These observations suggest that UTI exerts various functions on the cells. In this report, we demonstrated that UTI effectively suppresses the production of proMMP-1 and proMMP-3 accompanying the decrease in their mRNA expressions in human chorionic cells and in human uterine cervical fibroblasts. These suppressive effects were more remarkable in the former than in the latter. The reason why different actions of UTI were observed in these two cell species is still unclear. The presence of two types of UTI receptor has been demonstrated on the cell surface of some tumor cells and leukocytes [26]. UTI receptors of human uterine cervical fibroblasts and chorionic cells have not been characterized, but the different effects of UTI on these two cell types might be dependent upon the different number of UTI receptors expressed. In our experiments, the production of TIMP-1 was also suppressed by UTI in these cell species, but to a lesser extent than that of proMMP-1 and proMMP-3, indicating that UTI is very likely to totally prevent the destruction of extracellular matrix.

UTI also suppressed the accumulation of steady-state levels of MMP-1 and MMP-3 mRNAs with a lesser extent than their protein levels, suggesting that UTI biphasically regulates their production at the transcriptional and post-transcriptional levels, and that UTI modulates for the most part at the latter level. Further studies are needed to clarify this complex mechanism.

A higher concentration (about 0.64 μM) of UTI was found in the neonatal urine than in adult urine, and UTI contained in amniotic fluid is considered to be derived from fetal urine [15]. It is thought that UTI in the amniotic fluid interferes with the proteolytic activity and closely participates in the maintenance of functions of fetal membrane during pregnancy [15]. Pharmacological dose of UTI also prevents premature cervical ripening by inhibiting the recruitment of neutrophils and the activity of elastase [17]. Our observation that UTI interferes the production of proMMP-1 and proMMP-3 contributes to understanding about the mechanism of protective effect of UTI against preterm cervical ripening and preterm PROM. In addition, it is very likely that UTI inhibits the activation of these proMMPs because their activation is probably accomplished by trypsin and plasmin [4] which are effectively inhibited by UTI. By contrast, we have confirmed that UTI does not directly inhibit MMPs activities (data not shown).

In conclusion, we have demonstrated for the first time that in human uterine cervical fibroblasts and chorionic cells UTI down-regulates the production of proMMP-1 and proMMP-3 accompanying with the decrease in expression of their mRNAs, and therefore UTI is very likely to participate in

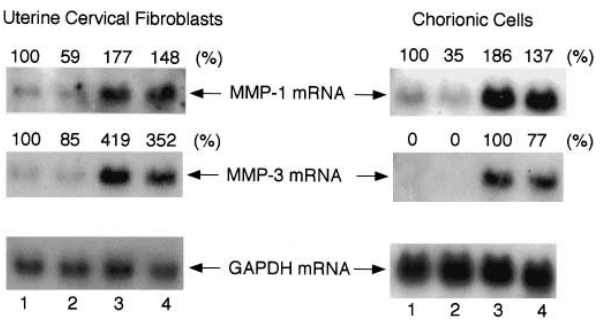

Fig. 3. Effect of UTI on the steady-state mRNA levels of MMP-1 and MMP-3. Confluent human uterine cervical fibroblasts at the 3rd passage and human chorionic cells at the 5th passage were treated with IL-1α (1 ng/ml) and/or UTI (1.28 μM) for 24 h. Total RNA (10 μg) was subjected to Northern blot analysis as described in the text. The relative amounts of respective mRNA were quantified by densitometric scanning, and normalized with that of GAPDH mRNA taking the untreated cells as 100%. Three independent experiments using cells of different origins at different passages were highly reproducible, and typical data are shown. Lane 1, untreated cells; lane 2, cells treated with UTI (1.28 μM); lane 3, cells treated with IL-1α (1 ng/ml) and lane 4, cells co-treated with IL-1α (1 ng/ml) and UTI (1.28 μM).

the maintenance of uterine cervix and fetal membranes by overall prevention of matrix breakdown.

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