ORIGINAL ARTICLE

Development of new therapeutic agents for preterm birth by glycosaminoglycan chain remodeling of urinary trypsin inhibitor

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Aim: This study aimed to examine the effects of the glycosaminoglycan (GAG) chain in urinary trypsin inhibitor (UTI) on uterine cervical fibroblasts (UCFs) and to apply the findings to the development of more effective therapeutic drugs for the management of preterm birth.
Methods: We prepared GAG chain-remodeled UTIs by hydrolysis and/or transglycosylation by testicular hyaluronidase. These UTIs were added to UCFs obtained from gynecology operations, and the effects of UTIs on the release of IL-8, IL-6, MMP-8, and MMP-9 were examined.
Results: UTIs that were not hydrolyzed tended to reduce IL-8 release more strongly than GAG chain-hydrolyzed UTIs. IL-6 was not affected by GAG chain hydrolysis of UTIs. GAG chain-hydrolyzed UTIs tended to reduce MMP-8 and MMP-9 release more strongly than non-hydrolyzed UTIs.
Conclusions: Our findings suggest that the GAG chain of UTI might reduce hyaluronan during cervical ripening by reducing IL-8 release and has opposite effects on reducing MMP-8 and MMP-9 release related to collagen degradation. This insight may be helpful in the development of more effective therapeutic drugs for the management of preterm birth.

Introduction

Preterm birth is estimated to affect annually 12,900,000 births or 9.7% of all births in the world.[1] The preterm birth rate in some developed countries is more than 10%[1]; therefore, reducing preterm birth is a global challenge. In Japan, the preterm birth rate has recently settled at about 5.7%, and shows no sign of decreasing.[2] Prognoses for preterm infants have improved due to advances in neonatal medical care, although preterm birth remains a major cause of perinatal death.[3] The establishment of adequate care for preterm birth is an urgent issue requiring immediate attention.

The two basic mechanisms underlying cervical ripening in preterm birth are (i) degradation of collagen constituting the extracellular matrix of the cervix and (ii) the increase in glycosaminoglycan (GAG), as reflected by hyaluronan (HA) production.[4,5] Several factors, such as chorioamnionitis (CAM), induce the migration of neutrophils to the organization, and inflammatory cytokines, such as interleukin (IL)-1, are secreted. In addition, matrix metalloproteinase (MMP) and neutrophil elastase are produced, which in turn leads to the breakdown of collagen constituting the extracellular matrix of the cervix.[6,7] Inflammatory cytokines, such as IL-1, IL-8, and TNF, which are lower cytokine, induce HA production.[8]

Urinary trypsin inhibitor (also referred to as UTI,

urinastatin, and ulinastatin) suppresses proteases, such as elastase, and IL-8 and IL-6 release.[9,10] It also controls cervical inflammation and ripening,[11,12] and prevents uterine muscle contracti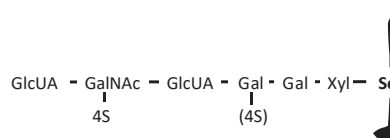on.[13] Although UTI is used as a vaginal preterm birth therapeutic drug in many clinical settings, its protective effects against preterm birth have not yet been shown.

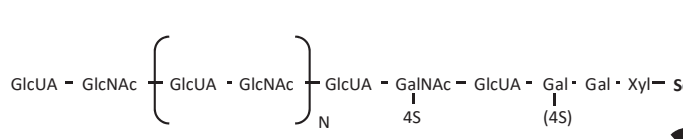UTI is a protective biological substance found in fetal
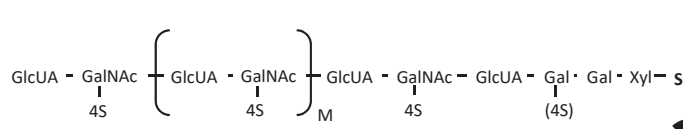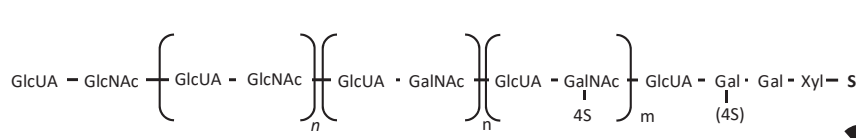
A: Native UTI
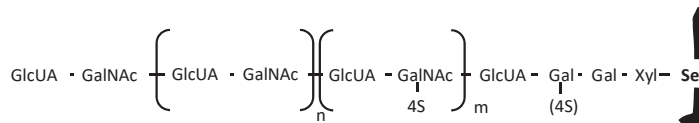
B: Linkage-UTI

C: HA-linkage-UTI

D: Ch4S-linkage-UTI

E: HA hybrid-UTI

F: Ch4S hybrid-UTI
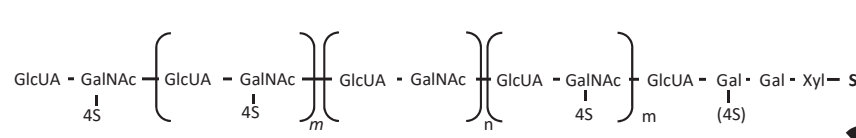
Figure 1. Molecular structure of UTIs.
(a) The core protein of native UTI has a single low-sulfated chondroitin 4-sulfate (Ch4S) chain covalently linked to the residue Ser-10 through a GlcUAβ1-3Galβ1-3Galβ1-4Xylβ1-Ser (GlcUA-Gal-Gal-Xyl-Ser) or GlcUA-Gal(4-sulfate)-Gal-Xyl-Ser linkage region. (b) GAG chain hydrolyzed UTI, i.e., linkage-UTI. (c) HA-linkage-UTI has hyaluronan (HA) ([-4GlcUAβ1-3-GlcNAcβ1-] N) linked to linkage-UTI. (d) Ch4S-linkage-UTI has Ch4S . ([-4GlcUAβ1-3-GalNAc(4-sulfate) β1-] M) linked to linkage-UTI. (e) HA hybrid-UTI has HA linked to native UTI. (f) Ch4S hybrid-UTI has Ch4S linked to native UTI. GlcUA; glucuronic acid, GlcNAc; N-acetylglucosamine, Gal; galactose, Xyl; xylose, GalNAc; N-acetylgalactosamine. M, N, n, m, n, and m; the number of repeating disaccharide units.

urine and in large quantities in amniotic fluid. It is a small proteoglycan (PG) with a low-sulfated chondroitin 4-sulfate (Ch4S) chain that binds to the tenth serine residue of the core protein as a GAG moiety via the linkage region[14-17]; the length of the GAG moiety can vary (Figure 1A).[18,19] UTI exerts its function based on the protease inhibitor activity of its core protein structure. However, the structure of the GAG moiety, which accounts for nearly 30% of the molecular weight of UTI, has not been elucidated. We previously remodeled the chondroitin sulfate chain moiety of UTI, with the core protein intact, by glycotechnology using testicular hyaluronidase.[20]

In this study, we aimed to clarify the role of the GAG chain in UTI's function. If GAG chain remodeling can impart a new function to UTI, our findings may help in the development of more effective therapeutic drugs for preterm birth. First, GAG chain-remodeled UTIs were prepared by glycotechnology using testicular hyaluronidase. Then, these GAG chain-remodeled UTIs were added to cultured human uterine cervical fibroblasts (UCFs) which were treated with lipopolysaccharide (LPS) to induce inflammation. The effects of the UTIs on the release of IL-8, IL-6, MMP-8, and MMP-9 were then examined.

Materials and methods

Materials

Dulbecco's modified Eagle medium (DMEM) was purchased from Nippon Suisan Kaisha, Ltd. (Tokyo, Japan). Fetal bovine serum (FBS), LPS (a somatic component of gram-negative rod bacteria, *Escherichia coli* 055: B5), bovine testicular hyaluronidase (BTH, type 1-S), cellulase (from *Aspergillus niger*), and Ch4S (from bovine trachea) were purchased from Sigma-Aldrich (St. Louis, MO, USA). UTI (Biotech Center, Shanghai Institute of Pharmaceutical Industry, China) was purchased and further purified by DEAE-Cellufine column chromatography.[21] Endotoxin-free HA (from *Streptococcus zooepidemicus*) was kindly donated by Shiseido Co., Ltd. (Tokyo, Japan). CNBr-activated Sepharose 4 Fast Flow resin was purchased from GE Healthcare, Japan (Tokyo, Japan). Amicon Ultra-15 was purchased from Merck Millipore Ltd. (Tullagreen, Ireland). PAGEL (5-20% Polyaminogel) was purchased from Atto Co. (Tokyo, Japan). Other reagents were obtained from commercial sources, and were of analytical grade. We used endotoxin-free reagents and distilled water in addition to UCFs.

Remodeling of GAG chain in UTI

For hydrolysis and transglycosylation by BTH, we prepared a reaction column packed with BTH-immobilized resin (internal diameter, 5.0 cm; height, 1.8 cm; bed vol., 36 ml) by using CNBr-activated Sepharose 4 Fast Flow. To obtain GAG chain-hydrolyzed UTI (i.e., linkage-UTI), native UTI was incubated in 0.1 M sodium acetate buffer (pH 4.0) containing 150 mM NaCl (optimal for hydrolysis) for 16 h at 37°C using the BTH-immobilized reaction column. Reaction products were eluted with 0.1 M sodium acetate buffer (pH 4.0) containing 0.5 M NaCl, and the fractions were collected by monitoring absorbance at 280 nm and desalted. Fractions were then concentrated by Amicon Ultra-15 (Figure 1B).

HA hybrid-linkage-UTI (HA-linkage-UTI) was generated by transglycosylating HA to linkage-UTI by BTH. Linkage-UTI (acceptor) and HA (donor) were incubated in 0.15 M Tris-HCl (pH 7.0) in the absence of NaCl (optimal for transglycosylation) for 16 h at 4°C using the BTH-immobilized reaction column. Reaction products were eluted with 0.1 M Tris-HCl buffer (pH 7.0) without NaCl, and the fractions were collected by monitoring the absorbance at 280 nm and desalted. The fractions were then concentrated by Amicon Ultra-15 (Figure 1C). Ch4S hybrid-linkage-UTI (Ch4S-linkage-UTI) was generated using the same method mentioned above, except with Ch4S as the donor (Figure 1D). HA hybrid-UTI or Ch4S hybrid-UTI, which has HA or Ch4S at the non-reducing terminus of the original low-sulfated Ch4S, respectively, was generated using native UTI as an acceptor and HA or Ch4S as a donor by the same method, without the hydrolysis step (Figures. 1E, 1F). These GAG chain-remodeled UTIs were concentrated by ethanol precipitation and prepared at a concentration of 20 or 60 μg/ml. Protein concentration was measured with Bradford's method.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with PAGEL by the method of Laemmli.[22] Gels were stained with Coomassie brilliant blue (CBB) R-250 for proteins and Alcian blue for GAGs.

Primary UCF culture

Uterine cervical tissue was obtained from non-pregnant women undergoing total hysterectomy for myoma in the uterus at Hirosaki University Hospital after obtaining written informed consent. This study was approved by the Hirosaki University Graduate School of Medicine institutional review board. Uterine cervical tissue was cut into small pieces and incubated in DMEM containing

10% FBS in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.[23] The culture medium was changed once every two days. Once UCFs reached confluence, they were used from the fourth to sixth passage for the experiments described herein.

LPS and UTI treatments

UCFs (1.7×10^5 cells/well) were cultured in 24-well plates with DMEM containing 10% FBS. After UCFs had grown to confluence, they were treated as described below. UCFs were cultured in DMEM without FBS to avoid UTI action of the FBS for 16 h. Next, UTIs (native UTI, linkage-UTI, HA-linkage-UTI, Ch4S-linkage-UTI, HA hybrid-UTI, and Ch4S hybrid-UTI) were added at a concentration of either 20 or 60 μg/ml to each well. An hour later, UCFs were incubated with LPS (1 μg/ml) for 24 h,[24] followed by collection of culture medium.

Enzyme-linked immunosorbent assay

IL-8 and IL-6 concentrations in culture supernatants were assessed with enzyme-linked immunosorbent assay kits (ELISA; Invitrogen).[25,26] Absorbance at 450 nm was measured with a microplate reader (Tecan Sunrise Reader, Männedorf, Switzerland). MMP-8 and MMP-9 concentrations in culture supernatant were assayed with MMP-8 and MMP-9 activity assays (QuickZyme).[27,28] Concentrations were compared by live cell count.

Statistical analysis

Data are expressed as mean ± standard deviation of three experiments. Tukey's *post hoc* test was performed with ANOVA. Bell Curve for Excel (Tokyo, Japan) was used for data analysis. $P < 0.05$ was considered statistically significant.

Results

We prepared GAG chain-remodeled UTIs as shown in Figure 1 and confirmed the success of hydrolysis and transglycosylation by SDS-PAGE. Figure 2 (2A; CBB stain, 2B; Alcian blue stain) shows the results of SDS-PAGE of native UTIs and GAG chain-remodeled UTIs before and after BTH reactions. Native UTIs were broadly detected at approximately 32.5 kDa owing to the heterogeneity of their low-sulfated Ch4S chains (Figure 2A, lanes 1 and 2) as reported previously.[18,19] Small populations of *N*-terminal truncated UTIs without GAG chains, which are low molecular weight UTI forms, were detected at approximately 17 kDa.[20,29] After the low-sulfated Ch4S chains of native UTIs were hydrolyzed (i.e., linkage-UTIs), two bands were detected at approximately 21.5 kDa and 17 kDa (Figure 2A, lanes 3 and 4).[20] HA-linkage-UTIs and Ch4S-linkage-UTIs were similar to linkage-UTIs, but they were more broadly

distributed (Figure 2A, lanes 5, 6, 9, and 10). HA hybrid-UTIs and Ch4S hybrid-UTIs were broadly distributed up to 50 kDa (Figure 2A, lanes 7, 8, 11, and 12).[20] The success of transglycosylation was confirmed by Alcian blue staining (Figure 2B, lanes 5-12).

Figure 3 shows the effect of UTIs on IL-8 release in the culture supernatant of UCFs after LPS stimulation. After 24 h of incubation, LPS stimulated IL-8 release 4.5 times per live cell. Native UTI, Ch4S-linkage-UTI, HA hybrid-UTI, and Ch4S hybrid-UTI reduced IL-8 release to 49.5% (60 μg/ml), 48.3% (60 μg/ml), 49.0% (60 μg/ml), and 42.4% (60 μg/ml), respectively. HA hybrid-UTI and Ch4S hybrid-UTI at a dose of 20 μg/ml significantly reduced IL-8 release. In contrast, native UTI (20 μg/ml), linkage-UTI (20 and 60 μg/ml), HA-linkage-UTI (20 and 60 μg/ml), and Ch4S-linkage-UTI (20 μg/ml) did not significantly reduce IL-8 release. There were no significant differences in reduction of IL-8 release among these GAG chain-remodeled UTIs. Although not significant, UTIs prepared without hydrolysis (e.g., native UTI, HA hybrid-UTI, and Ch4S hybrid-UTI) tended to reduce IL-8 release more strongly than UTIs prepared with hydrolysis (e.g., linkage-UTI, HA-linkage-UTI, and Ch4S-linkage-UTI).

The pattern of IL-6 release was similar to but slightly different from that of IL-8. Figure 4 shows the effect of UTIs on IL-6 release in the culture supernatant of UCFs after LPS stimulation. After 24 h of incubation, LPS stimulated IL-6 release 4.4 times per live cell. Native UTI, linkage-UTI, HA-linkage-UTI, Ch4S-linkage-UTI, and HA hybrid-UTI reduced LPS-stimulated IL-6 release to 39.5% (60 μg/ml), 39.6% (60 μg/ml), 54.0% (60 μg/ml), 40.8% (60 μg/ml), and 49.7% (60 μg/ml), respectively. Ch4S hybrid-UTI reduced IL-9 release to 43.4% (60 μg/ml). All UTIs at a dose of 20 μg/ml significantly reduced IL-6 release. HA hybrid-UTI at a dose of 20 μg/ml reduced IL-6 release more strongly than HA-linkage-UTI. There were no significant differences in the reduction of IL-6 release among other GAG chain-remodeled UTIs.

Figure 5 shows the effect of UTIs on the release of MMP-8 in the culture supernatant of UCFs after LPS stimulation. After 24 h of incubation, LPS significantly stimulated MMP-8 release 1.6 times per live cell. Native UTI, linkage-UTI, HA-linkage-UTI, Ch4S-linkage-UTI, HA hybrid-UTI, and Ch4S hybrid-UTI reduced LPS-stimulated MMP-8 release to 53.4% (60 μg/ml), 24.4% (60 μg/ml), 32.5% (60 μg/ml), 21.2% (60 μg/ml), 51.9% (60 μg/ml), and 52.2% (60 μg/ml), respectively. Linkage-UTI, HA-linkage-UTI, and HA hybrid-UTI at a dose of 20 μg/ml significantly reduced MMP-8 release. In contrast, native UTI, Ch4S-linkage-UTI, and Ch4S hybrid-UTI at a dose of 20 μg/ml did not significantly reduce MMP-8 release. There were no significant
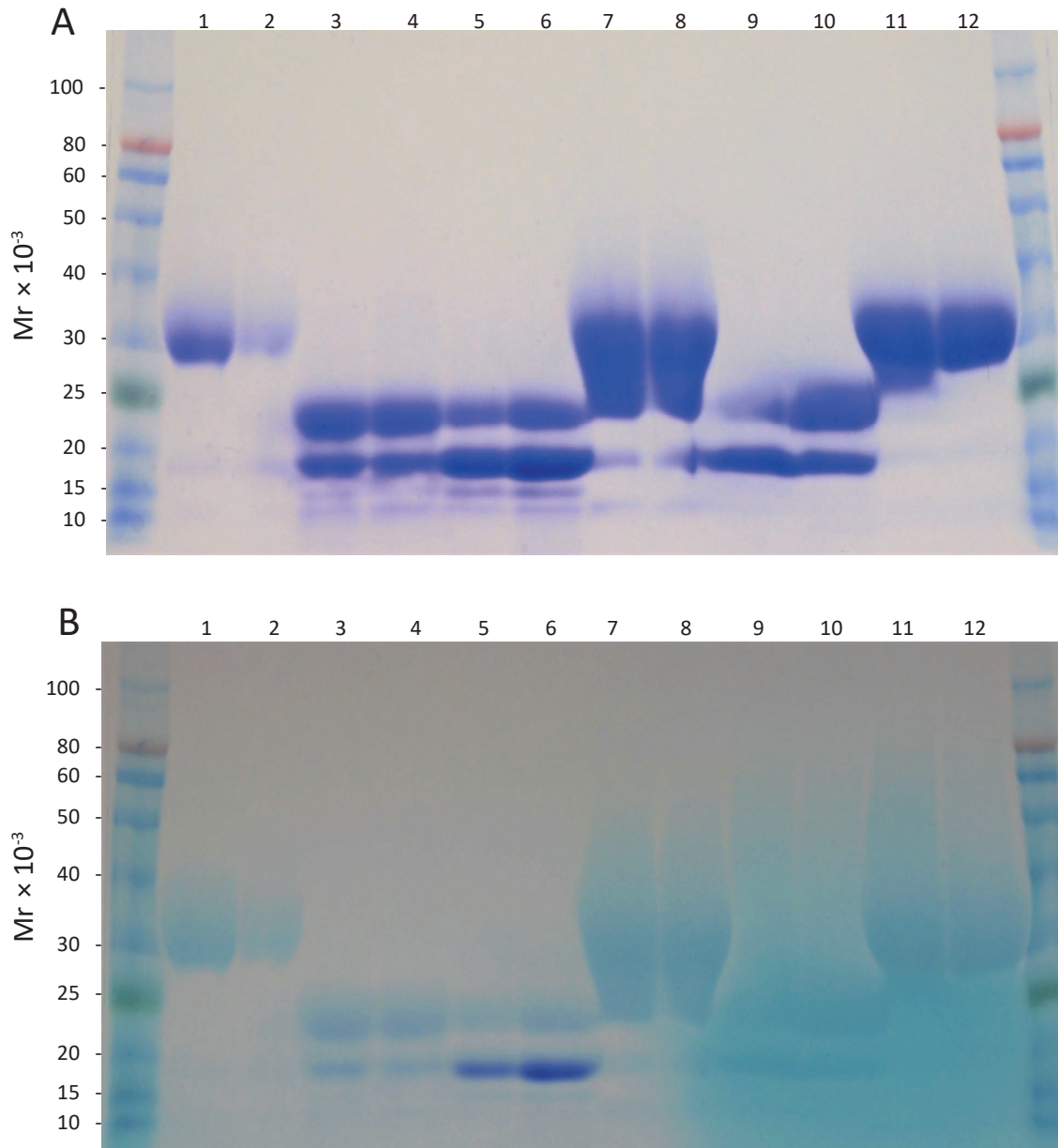
Figure 2. SDS-PAGE of UTIs.
UTIs (native UTI, linkage-UTI, HA-linkage-UTI, Ch4S-linkage-UTI, HA hybrid-UTI, and Ch4S hybrid-UTI) were separated on PAGEL (5−20% Polyaminogel) and stained with (a) Coomassie brilliant blue R250 or (b) Alcian blue. Lanes 1, 2: native UTI; lanes 3, 4: linkage-UTI; lanes 5, 6: HA-linkage-UTI; lanes 7, 8: Ch4S-linkage-UTI; lanes 9, 10: HA hybrid-UTI; lanes 11, 12: Ch4S hybrid-UTI.

differences in reduction of LPS-stimulated MMP-8 release among all GAG chain-remodeled UTIs. Although not significant, UTIs prepared with hydrolysis tended to reduce MMP-8 release more strongly than UTIs prepared without hydrolysis.

The pattern of MMP-9 was similar to that of MMP-8, but different from IL-8 or IL-6. Figure 6 shows the effect of UTIs on the release of MMP-9 in the culture supernatant

of UCFs after LPS stimulation. After 24 h of incubation, LPS significantly stimulated MMP-9 release 1.9 times per live cell. Native UTI, linkage-UTI, HA-linkage-UTI, Ch4S-linkage-UTI, HA hybrid-UTI, and Ch4S hybrid-UTI reduced LPS-stimulated MMP-9 release to 51.2% (60 μg/ml), 31.9% (60 μg/ml), 28.4% (60 μg/ml), 18.3% (60 μg/ml), 45.4% (60 μg/ml), and 53.8% (60 μg/ml), respectively. All UTIs at 20 μg/ml significantly reduced
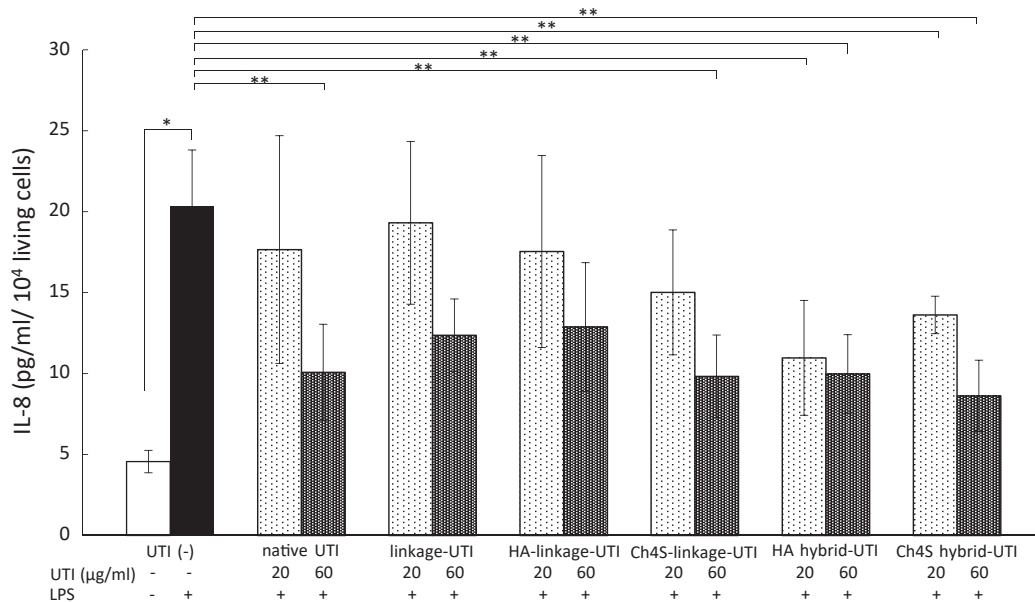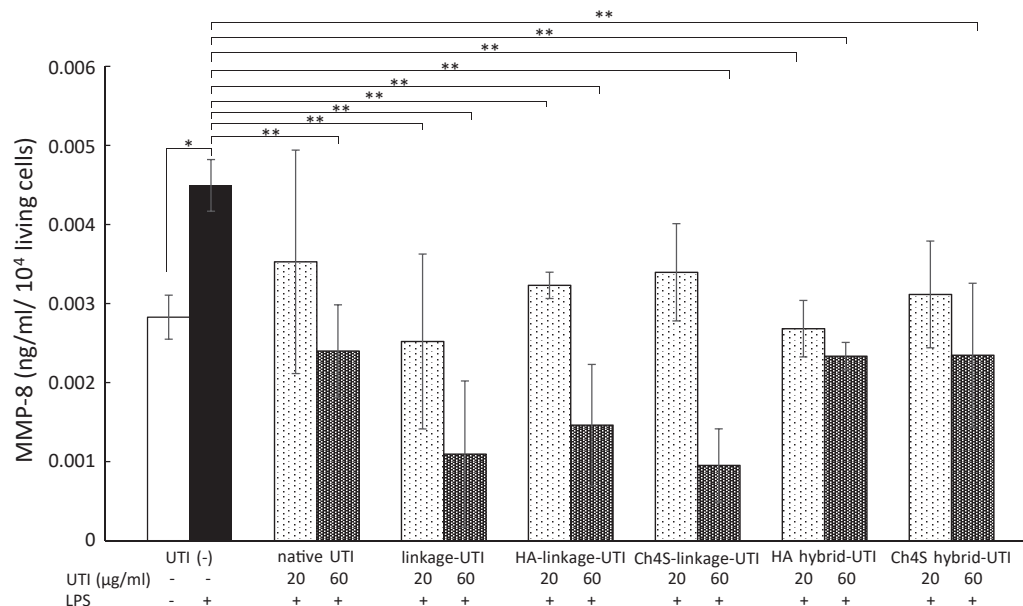
Figure 3. **Effect of UTIs on IL-8.**
Lipopolysaccharide (LPS) significantly stimulated IL-8 release after 24 h of incubation. Native UTI (60 μg/ml), Ch4S-linkage-UTI (60 μg/ml), HA hybrid-UTI (20 and 60 μg/ml), and Ch4S hybrid-UTI (20 and 60 μg/ml) significantly reduced LPS-stimulated IL-8 release. There were no significant differences in the reduction of IL-8 release among native UTI, linkage-UTI, HA-linkage-UTI, Ch4S-linkage-UTI, HA hybrid-UTI, and Ch4S hybrid-UTI at both 20 and 60 μg/ml. *Significant when comparing LPS ($-$) to LPS ($+$) ($P < 0.05$). **Significant when comparing LPS ($+$) (UTI ($-$)) to native UTI, linkage-UTI, HA-linkage-UTI, Ch4S-linkage-UTI, HA hybrid-UTI, and Ch4S hybrid-UTI ($P < 0.05$).
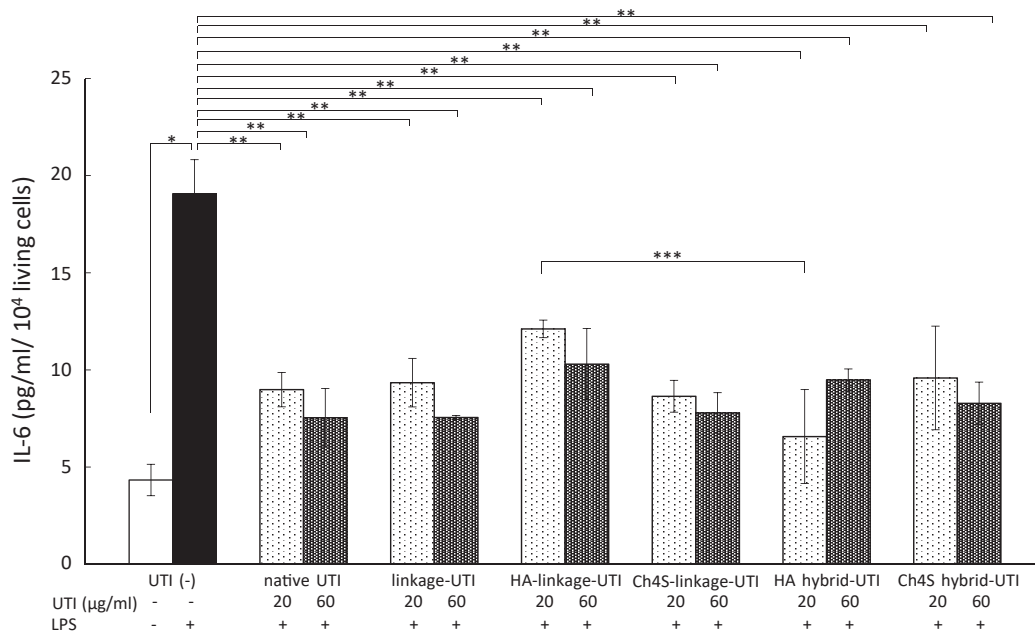
Figure 4. **Effect of UTIs on IL-6.**
LPS significantly stimulated IL-6 release after 24 h of incubation. All UTIs at both 20 and 60 μg/ml significantly reduced LPS-stimulated IL-6 release. All HA hybrid-UTIs at 20 μg/ml significantly reduced IL-6 release more strongly than HA-linkage-UTI. *Significant when comparing LPS ($-$) to LPS ($+$) ($P < 0.05$). **Significant when comparing LPS ($+$) (UTI ($-$)) to native UTI, linkage-UTI, HA-linkage-UTI, Ch4S-linkage-UTI, HA hybrid-UTI, and Ch4S hybrid-UTI ($P < 0.05$). ***Significant when comparing HA hybrid-UTI at 20 μg/ml to HA-linkage-UTI at 20 μg/ml ($P < 0.05$).

Figure 5. **Effect of UTIs on MMP-8.**
Lipopolysaccharide (LPS) significantly stimulated MMP-8 release after 24 h of incubation. Native UTI (60 μg/ml), linkage-UTI (20 and 60 μg/ml), HA-linkage-UTI (20 and 60 μg/ml), Ch4S-linkage-UTI (60 μg/ml), HA hybrid-UTI (20 and 60 μg/ml), and Ch4S hybrid-UTI (60 μg/ml) significantly reduced LPS-stimulated MMP-8 release. There were no significant differences in MMP-8 release among all UTIs at both 20 and 60 μg/ml. *Significant when comparing LPS (−) to LPS (+) ($P < 0.05$). **Significant when comparing LPS (+) (UTI (−)) to native UTI, linkage-UTI, HA-linkage-UTI, Ch4S-linkage-UTI, HA hybrid-UTI, and Ch4S hybrid-UTI ($P < 0.05$).
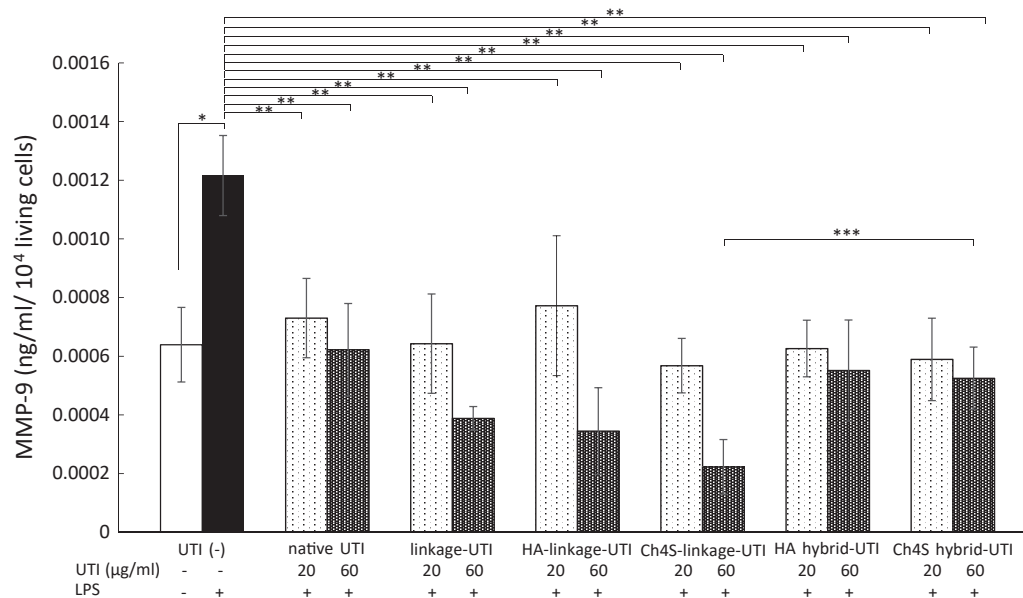

Figure 6. **Effect of UTIs on MMP-9.**
Lipopolysaccharide (LPS) significantly stimulated MMP-9 release after 24 h of incubation. All UTIs at both 20 and 60 μg/ml significantly reduced LPS-stimulated MMP-9 release. Ch4S-linkage-UTI at 60 μg/ml reduced LPS-stimulated MMP-9 release more strongly than Ch4S hybrid-UTI. *Significant when comparing LPS (−) to LPS (+) ($P < 0.05$). **Significant when comparing LPS (+) (UTI (−)) to native UTI, linkage-UTI, HA-linkage-UTI, Ch4S-linkage-UTI, HA hybrid-UTI, and Ch4S hybrid-UTI ($P < 0.05$). ***Significant when comparing Ch4S-linkage-UTI at 60 μg/ml to Ch4S hybrid-UTI at 60 μg/ml ($P < 0.05$).

MMP-9 release. Ch4S-linkage-UTI at 60 μg/ml reduced LPS-stimulated MMP-9 release more strongly than Ch4S hybrid-UTI. There were no significant differences among other GAG chain-remodeled UTIs. Although not significant, except between Ch4S-linkage-UTI and Ch4S hybrid-UTI, UTIs prepared with hydrolysis tended to reduce MMP-9 release more strongly than UTIs prepared without hydrolysis.

Discussion

In this study, we assessed the role of the GAG chain in UTI using human UCFs stimulated with LPS and found that the GAG chain may reduce HA production during cervical ripening. LPS has been reported to induce IL-8 and IL-6 release in UCFs, indicating that these cells may represent a good model for studying inflammation.[24] Our results are consistent in that LPS induced the release of both IL-8 and IL-6. We also demonstrated that LPS promotes the release of MMP-8 and MMP-9 from human UCFs.

UTIs prepared without hydrolysis tended to reduce LPS-stimulated IL-8 increase more strongly than UTIs prepared with hydrolysis. Although not significant, our findings suggest that UTIs that have the original low-sulfated chondroitin 4-sulfate chain may have strong IL-8 suppressing effects. HA hybrid-UTI and Ch4S hybrid-UTI significantly reduced IL-8 release at 20 μg/ml, suggesting that longer GAG chains might be effective even at low doses.

Similarly, all GAG chain-remodeled UTIs significantly reduced LPS-stimulated IL-6 increase. A significant difference was observed only between HA-linkage-UTI and HA hybrid-UTI, and the results did not differ by hydrolysis status or type of GAG chain. This suggests that the GAG chain of UTI may not have a large impact on reducing IL-6 release.

In the preterm birth process, inflammatory cytokines such as IL-8 and IL-6 promote HA production in the extracellular matrix of the cervix. Since HA has high water retentivity, the organizations become soft and cervical ripening progresses by increasing HA production.[8] UTI suppresses IL-8 and IL-6 release,[9,10] and it was suggested that the suppressant effects were similar.

UTIs prepared by hydrolysis tended to reduce the LPS-stimulated increase in MMP-8 and MMP-9 more strongly than UTIs prepared without hydrolysis, suggesting that UTIs that lack the original low-sulfated chondroitin 4-sulfate chain may have strong MMP-8 and MMP-9 suppressing effects.

The GAG chain of UTI might have suppressing effects on IL-8, but opposite effects on MMP-8 and MMP-9. According to one study, UTI has only a mild effect on reducing active MMPs directly.[30] Our present findings suggest that the GAG chain of UTI might weaken the reduction of MMP release. During the process of cervical ripening in preterm birth, MMP-8 and MMP-9 are involved in the degradation of collagen, while IL-8 and IL-6 are involved in increasing HA production. This indicates that the GAG chain might reduce HA production during cervical ripening and have opposite effects on the degradation of collagen which constitutes the extracellular matrix of the cervix.

Transglycosylation of Ch4S in UTIs tended to reduce IL-8, IL-6, MMP-8, and MMP-9 release more strongly than other members of our UTI series. This might be due to the anti-inflammatory effects of chondroitin sulfate.[31]

To our knowledge, this study is the first to demonstrate the role of the GAG chain in the function of UTIs on UCFs. These findings suggest the possibility of improving the function of UTIs by remodeling the GAG chain. Although further studies will be needed, our findings suggest the possibility of new treatments for preterm birth without side effects, and could help address some of the major issues in perinatal medical care, such as reducing preterm births and improving the prognosis of preterm infants.

Acknowledgments

Conflicts of interest

References

1. Beck S, Wojdyla D, Say L, et al. The worldwide incidence of preterm birth: a systematic review of maternal mortality and morbidity. Bull World Health Organ. 2010; 88: 31–38.
2. Sakata S, Konishi S, Ng CFS, Watanabe C. Preterm birth rates in Japan from 1979 to 2014: Analysis of national vital statistics. J Obstet Gynaecol Res. 2018; 44: 390–396.
3. Lawn JE, Gravett MG, Nunes TM, Rubens CE, Stanton C. Global report on preterm birth and stillbirth (1 of 7): definitions, description of the burden and opportunities to improve data. BMC Pregnancy Childbirth. 2010; 10 Suppl 1: S1.
4. Danforth DN, Veis A, Breen M, Weinstein HG, Buckingham JC, Manalo P. The effect of pregnancy and labor on the human cervix: changes in collagen, glycoproteins, and glycosaminoglycans. Am J Obstet Gynecol. 1974; 120: 641–651.
5. Straach KJ, Shelton JM, Richardson JA, Hascall VC, Mahendroo MS. Regulation of hyaluronan expression during cervical ripening. Glycobiology. 2005; 15: 55–65.
6. Sennstrom MB, Brauner A, Bystrom B, Malmstrom A, Ekman G. Matrix metalloproteinase-8 correlates with the cervical ripening process in humans. Acta Obstet Gynecol Scand. 2003; 82: 904–911.
7. Watari M, Watari H, DiSanto ME, Chacko S, Shi GP, Strauss JF, 3rd. Pro-inflammatory cytokines induce expression of matrix-

metabolizing enzymes in human cervical smooth muscle cells. Am J Pathol. 1999; 154: 1755–1762.

8. Ogawa M, Hirano H, Tsubaki H, Kodama H, Tanaka T. The role of cytokines in cervical ripening: correlations between the concentrations of cytokines and hyaluronic acid in cervical mucus and the induction of hyaluronic acid production by inflammatory cytokines by human cervical fibroblasts. Am J Obstet Gynecol. 1998; 179: 105–110.

9. Maehara K, Kanayama N, Halim A, et al. Down-regulation of interleukin-8 gene expression in HL60 cell line by human Kunitz-type trypsin inhibitor. Biochem Biophys Res Commun. 1995; 206: 927–934.

10. Zhao X, Sun X, Gao F, Luo J, Sun Z. Effects of ulinastatin and docataxel on breast tumor growth and expression of IL-6, IL-8, and TNF-alpha. J Exp Clin Cancer Res. 2011; 30: 22.

11. Kanayama N, el Maradny E, Halim A, Maehara K, Kajiwara Y, Terao T. Urinary trypsin inhibitor suppresses premature cervical ripening. Eur J Obstet Gynecol Reprod Biol. 1995; 60: 181–186.

12. Kanayama N, Chinarong L, Naruse H, et al. The effect of granulocyte elastase inhibitor (urinastatin) vaginal suppository on patients with imminent premature delivery. Nihon Sanka Fujinka Gakkai zasshi. 1992; 44: 477–482.

13. Kanayama N, el Maradny E, Halim A, et al. Urinary trypsin inhibitor prevents uterine muscle contraction by inhibition of Ca++ influx. Am J Obstet Gynecol. 1995; 173: 192–199.

14. Balduyck M, Mizon C, Loutfi H, Richet C, Roussel P, Mizon J. The major human urinary trypsin inhibitor is a proteoglycan. Eur J Biochem. 1986; 158: 417–422.

15. Yamada S, Oyama M, Yuki Y, Kato K, Sugahara K. The uniform galactose 4-sulfate structure in the carbohydrate-protein linkage region of human urinary trypsin inhibitor. Eur J Biochem. 1995; 233: 687–693.

16. Enghild JJ, Thogersen IB, Cheng F, Fransson LA, Roepstorff P, Rahbek-Nielsen H. Organization of the inter-alpha-inhibitor heavy chains on the chondroitin sulfate originating from Ser(10) of bikunin: posttranslational modification of IalphaI-derived bikunin. Biochemistry. 1999; 38: 11804–11813.

17. Ly M, Leach FE, 3rd, Laremore TN, Toida T, Amster IJ, Linhardt RJ. The proteoglycan bikunin has a defined sequence. Nat Chem Biol. 2011; 7: 827–833.

18. Yuki Y, Nomura K, Kirihara M, et al. Charge isomers of urinary bikunin (trypsin inhibitor). Biochimica et biophysica acta. 1993; 1203: 298–303.

19. Kakizaki I, Takahashi R, Ibori N, et al. Diversity in the degree of sulfation and chain length of the glycosaminoglycan moiety of urinary trypsin inhibitor isomers. Biochim Biophys Acta. 2007; 1770: 171–177.

20. Kakizaki I, Takahashi R, Yanagisawa M, Yoshida F, Takagaki K. Enzymatic synthesis of hyaluronan hybrid urinary trypsin inhibitor. Carbohydr Res. 2015; 413: 129–134.

21. Proksch GJ, Routh JI. The purification of the trypsin inhibitor from human pregnancy urine. J Lab Clin Med. 1972; 79: 491–499.

22. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970; 227: 680–685.

23. Tanaka K, Nakamura T, Takagaki K, Funahashi M, Saito Y, Endo M. Regulation of hyaluronate metabolism by progesterone in cultured fibroblasts from the human uterine cervix. FEBS Lett. 1997; 402: 223–226.

24. Fukuyama A, Tanaka K, Kakizaki I, et al. Anti-inflammatory effect of proteoglycan and progesterone on human uterine cervical fibroblasts. Life Sci. 2012; 90: 484–488.

25. Baggiolini M, Walz A, Kunkel SL. Neutrophil-activating peptide-1/interleukin 8, a novel cytokine that activates neutrophils. J Clin Invest. 1989; 84: 1045–1049.

26. Houssiau FA, Devogelaer JP, Van Damme J, de Deuxchaisnes CN, Van Snick J. Interleukin-6 in synovial fluid and serum of patients with rheumatoid arthritis and other inflammatory arthritides. Arthritis Rheum. 1988; 31: 784–788.

27. Tester AM, Cox JH, Connor AR, et al. LPS responsiveness and neutrophil chemotaxis in vivo require PMN MMP-8 activity. PloS one. 2007; 2: e312.

28. Tanaka A, Yamane Y, Matsuda H. Mast cell MMP-9 production enhanced by bacterial lipopolysaccharide. J Vet Med Sci. 2001; 63: 811–813.

29. Pugia MJ, Valdes R, Jr., Jortani SA. Bikunin (urinary trypsin inhibitor): structure, biological relevance, and measurement. ADV CLIN CHEM. 2007; 44: 223–245.

30. Hashimoto K, Nagao Y, Kato K, Mori Y, Ito A. Human urinary trypsin inhibitor inhibits the activation of pro-matrix metalloproteinases and proteoglycans release in rabbit articular cartilage. Life Sci. 1998; 63: 205–213.

31. Chan PS, Caron JP, Rosa GJ, Orth MW. Glucosamine and chondroitin sulfate regulate gene expression and synthesis of nitric oxide and prostaglandin E(2) in articular cartilage explants. OSTEOARTHR CARTILAGE. 2005; 13: 387–394.