

The Comparison of the Protective Effects of α - and β -Antithrombin against Vascular Endothelial Cell Damage Induced by Histone in Vitro

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TH Open 2017;1:e3–e10.

Abstract

Antithrombin is a promising option for the treatment of sepsis, and vascular endothelium is an important target for this fatal condition. Here, we aimed to evaluate the protective effects of different glycoforms of antithrombin on histone-induced endothelial cell damage and explore the responsible mechanisms in an experimental model in vitro. Endothelial cells were treated in vitro using histone H4 to induce cellular damage. Various doses of either α - or β -antithrombin were used as treatment interventions, and both cell viability and the levels of lactate dehydrogenase (LDH) in the medium were assessed. Endothelial cell damage was also assessed using microscopic examination and immunofluorescent staining with anti-syndecan-4 and anti-antithrombin antibodies. As a result, both glycoforms of antithrombin significantly improved cell viability when administered at a physiological dose (150 $\mu\text{g}/\text{mL}$). Cellular injury as evaluated using the LDH level was significantly suppressed by β -antithrombin at a supranormal dose (600 $\mu\text{g}/\text{mL}$). Microscopic observation suggested that β -antithrombin suppressed the endothelial cell damage more efficiently than α -antithrombin. β -Antithrombin suppressed the intensity of syndecan-4 staining which became evident after treatment with histone H4, more prominently than α -antithrombin. The distribution of antithrombin was identical to that of syndecan-4. In conclusion, both α - and β -antithrombin can protect vascular endothelial cells from histone H4-induced damage, although the effect was stronger for β -antithrombin. The responsible mechanisms might involve the binding of antithrombin to the glycocalyx on the endothelial surface. These results provide a theoretical basis for the application of antithrombin to the prevention and treatment of sepsis-related endothelial damage.

Keywords

- ▶ antithrombin
- ▶ glycoform
- ▶ vascular endothelial cell
- ▶ glycocalyx
- ▶ syndecan-4

Introduction

Sepsis is often accompanied by vascular damage, which leads to organ dysfunction. Proinflammatory mediators including thrombin, a serine protease, play pivotal roles in sepsis. Antithrombin is a 58-kDa plasma glycoprotein that belongs

to the serine protease inhibitor family.¹ Though antithrombin is a predominant and naturally occurring inhibitor that targets thrombin and other coagulation factors,² it is also known to exert anti-inflammatory actions through both anticoagulation-dependent and anticoagulation-independent mechanisms.^{3,4}



There are two different antithrombin isoforms: α and β . These forms differ in the amount of glycosylation present on the polypeptide chain.⁵ α -Antithrombin has four N-glycans, while β -antithrombin has three. Because of the lack of N-glycan at Asn135, β -antithrombin is thought to have a higher affinity for heparin, and the absence of the N-glycan at Asn135 increases the affinity for heparin by two- to fourfold.⁶ Since the capacity of antithrombin to inhibit thrombin is known to increase dramatically in the presence of heparin, β -antithrombin is considered to be the major antithrombogenic contributor in plasma, even though it is present in much smaller amounts than other contributors. The higher affinity of β -antithrombin enables it to act as the predominant thrombin inhibitor on the vascular surface, through glycosaminoglycan binding. Glycosaminoglycans, the major component of the glycocalyx, are heparin-like molecules that contribute to the antithrombotic capacity on the endothelium.⁷ de la Morena-Barrio et al⁸ reported an elevated β -antithrombin level in patients with ischemic cerebrovascular disease, and this report supports the notion that β -antithrombin is the dominant isoform located on the endothelium.

Despite the successful treatment of an animal model of sepsis,⁹ antithrombin has failed to show any efficacy in clinical studies.¹⁰ We speculated that one of the reasons for this failure was the concomitant use of heparins.¹¹ As the binding of heparin and antithrombin abrogates its binding to glycosaminoglycan, the protective effects of the treatment may be reduced. Another possibility is the isoform of antithrombin that was used in the trial. β -isoform dominant antithrombin was more suitable. Although more than 90% of antithrombin concentrate is composed of α -antithrombin, the actual proportion of the isoforms produced in the liver is as yet unknown. Since β -antithrombin dominantly attaches to the glycosaminoglycan on the cellular surface, the amount of β -isoform that is produced cannot be easily measured. However, for the same reason, we speculated that a β -isoform-rich antithrombin formulation might act more efficiently than commercially available antithrombin concentrates. To substantiate these speculations, we attempted to elucidate the differences in the protective effects of α - and β -antithrombin, in a histone-induced model of endothelial damage *in vitro*. In this article, we addressed this issue using microscopic examination and immunofluorescent staining. We also examined the changes of syndecan-4 expression to approach the mechanism of action. In addition, cell viability and cellular injury were measured to support the phenotypic data.

In the current experiment, vascular endothelial cell damage was induced by histone H4. Histones are cationic proteins located in the nucleus of eukaryotic cells. Upon cell death during sepsis, histones leak into the cytoplasm and pass outside of the cell. Under normal situations, the circulating level of histones is usually very low or even undetectable because of rapid clearance.¹² However, once massive cell death occurs, histones will injure the host through the damage of the endothelial cell.¹³

Materials and Methods

Endothelial Cell Culture

Rat aortic endothelial cells were purchased from Cell Applications, Inc. (San Diego, California, United States), and their

uptake of Dil-Ac-LDL (CA022K; Toyobo, Osaka, Japan) was confirmed. Cells were routinely cultured in Rat Endothelial Cell Basal Medium with growth supplements (Cell Applications, Inc.). For the experiments, the cells were seeded in 96-well tissue culture plates at a concentration of 20,000 cells per well in Rat Endothelial Cell Basal Medium with growth supplements and grown to confluence.

Histone-Induced Cytotoxicity

Histone H4 was purchased from New England Biolabs (Ipswich, Massachusetts, United States). Cells were washed in two changes of phosphate-buffered saline (PBS; Gibco) before use in the experiments, and the culture medium was changed to Opti-MEM (Life Technologies, Carisbad, California, United States) without FBS at 2 hours before the addition of each reagent. Histone H4 was added to 0.2 mL of Opti-MEM, and the final concentrations were adjusted to 10, 20, 30, 40, or 50 μ g/mL. Eight hours after the treatment, the media were collected and used for the assessment of cell viability and injury.

Antithrombin Purification

The α - and β -glycoforms of antithrombin were purified from human plasma affinity chromatography on a Heparin–Sepharose column (GE Healthcare, Chicago, Illinois, United States) as described previously.⁵ Electrophoretic evaluation for purity and separation of proteins were checked by SDS-PAGE, performed in 7.5% (w/v) polyacrylamide (**► Fig. S1**, supplementary figure available in the online version only).

Treatment with α - or β -Antithrombin

The effects of α - or β -antithrombin on histone-treated endothelial cell were examined. The culture medium was changed to a serum-free medium, and then either α - or β -antithrombin was added to 0.2 mL of Opti-MEM containing histone H4. The final concentrations of both antithrombins were 75, 150, 300, or 600 μ g/mL, and the concentration of histone H4 was 35 μ g/mL. After 8 hours, the media were harvested from the cultures and stored at -70°C for the assay. Each experiment was performed three times in duplicate.

Assessment of Cell Viability and Cellular Injury

Cell viability and injury were evaluated using the Cell Counting Kit 8 (CCK-8; Dojindo, Kumamoto, Japan) and a LDH assay kit (TAKARA Bio, Shiga, Japan), respectively. The assay kits were used as per the manufacturers' instructions. Regarding CCK-8, the principal of the assay was based on the conversion of a water-soluble tetrazolium salt, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-8), to a water-soluble formazan dye upon reduction by dehydrogenases in the presence of an electron carrier.¹⁴ The media were harvested from the cultures and centrifuged to remove the cell debris, and then CCK-8 solution was added to each well, followed by incubation for 1 hour at 37°C ; the absorbance at 450 nm was then determined. For the LDH assay, the media was mixed with the assay reagent. After the incubation, the absorbance was read at 490 nm. Both experiments were done three times in duplicate.

Microscopic Observation

To evaluate the morphological changes, the endothelial cells were observed using the Eclipse Pol microscopic system (Nikon, Tokyo, Japan) 8 hours after histone H4 administration. In each well, three successive fields were selected, and each field was photographed (EOS 5D Mark III; Canon, Tokyo, Japan). Immunofluorescent staining for syndecan-4 was performed as per the manufacturer's instructions using anti-syndecan-4 antibody (ab24511; Abcam PLC, Cambridge, United Kingdom). The cells were washed three times with Hank's balanced salt solution (BSS) before the addition of fluorescein isothiocyanate (FITC)-labeled rabbit anti-polyclonal to syndecan-4 in Eagle's minimal essential medium and kept at 37°C for 2 hours. After incubation, the cells were washed five times with BSS and then used for the immunofluorescent microscopic examination. Similarly, antithrombin was stained using anti-antithrombin III antibody (ab180614; Abcam PLC, Cambridge, United Kingdom).

Statistical Analysis

A statistical analysis was performed using a one-way ANOVA with the Dunnett post hoc test and the Student *t*-test using the statistical software StatView II. Data were presented as the means \pm SDs. Differences were considered statistically significant at $p < 0.05$.

Result

► **Fig. 1** shows the levels of cell viability (CCK-8) and injury (LDH) in cultured endothelial cells treated with different histone H4 doses ranging from 0 to 50 $\mu\text{g/mL}$. Significant changes in the CCK-8 and LDH levels were not observed when

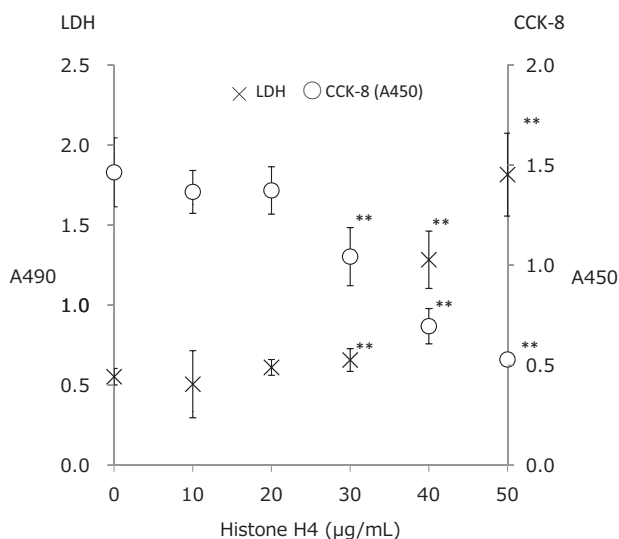


Fig. 1 Cell viability and cellular injury after treatment with histone H4. Vascular endothelial cell viability was measured based on the CCK-8 level, while cell injury was represented by the lactate dehydrogenase (LDH) level in the culture medium. Cell viability started to decrease when the histone H4 level was 30 $\mu\text{g/mL}$, and the level decreased in a dose-dependent manner. The LDH level also started to increase when the histone H4 level was 30 $\mu\text{g/mL}$, and the level increased in a dose-dependent manner. Data are expressed as the mean \pm standard error. ** $p < 0.01$, compared with a histone dose of 0 $\mu\text{g/mL}$.

the histone H4 concentration was less than 20 $\mu\text{g/mL}$. At a histone concentration of 30 $\mu\text{g/mL}$, the cell viability was reduced and the LDH level was elevated ($p = 0.01$, respectively); these changes intensified as the histone H4 dose increased.

► **Fig. 2** (left) shows the cellular viability as represented by the CCK-8 value. Data were expressed as the percentage of the control value (without antithrombin treatment). The CCK-8 value increased as the antithrombin concentration increased, and the differences when compared with the untreated control became significant when the α - and β -antithrombin doses exceeded 150 $\mu\text{g/mL}$ ($p = 0.01$, 0.05, respectively). ► **Fig. 2** (right) shows the LDH level after treatment with different doses of α - or β -antithrombin. The cellular injury represented by LDH was significantly suppressed only by β -antithrombin at supra-physiologic levels (600 $\mu\text{g/mL}$; $p = 0.01$). However, neither α -antithrombin (at any dose) nor smaller doses of β -antithrombin (75, 150, 300 $\mu\text{g/mL}$) induced a significant decrease in LDH. Regarding comparisons between α - and β -antithrombin, no significant differences were observed at any of the doses.

► **Fig. 3** (upper left panel) shows phase-contrast images of normal vascular endothelium. In the control, histone H4 at a concentration of 35 $\mu\text{g/mL}$ had caused significant shrinkage and separation of the cells at 8 hours (upper right panel). These changes were already apparent at 8 hours; thus, the above histone H4 dose and observation point were selected for the experiments. As shown in the lower right panel, 300 $\mu\text{g/mL}$ of β -antithrombin suppressed the morphological changes induced by histone H4. In contrast, the protective effect seemed to be less significant after treatment with 300 $\mu\text{g/mL}$ of α -antithrombin (lower left panel).

► **Fig. 4** shows representative images of endothelial cells treated with 35 $\mu\text{g/mL}$ of histone H4 and 300 $\mu\text{g/mL}$ of either α - or β -antithrombin 8 hours after treatment. The upper panels show phase-contrast images of the endothelium. The middle panels show immunofluorescent images for syndecan-4. Normal confluent endothelial cells did not exhibit syndecan-4 on their surfaces (► **Fig. S2**, supplementary figure available in the online version only); however, after treatment with histone H4, syndecan-4 was visualized mainly in the periphery of the damaged cell. The endothelial cells treated with α -antithrombin showed a higher intensity of syndecan-4 than those treated with β -antithrombin. The lower panels are the endothelial cells stained using anti-antithrombin antibody. The distribution of antithrombin was the same as that for syndecan-4. In contrast, the intensity of antithrombin seemed to be similar between the isoforms.

Discussion

The purpose of this study was to examine the effects of different isoforms of antithrombin on protecting vascular endothelium from damage in situations like sepsis experimentally. Since the affinity to heparan sulfate on vascular endothelium was different between the isoforms, the roles of α - and β -antithrombin may differ. Though commercially available antithrombin concentrates are composed mainly

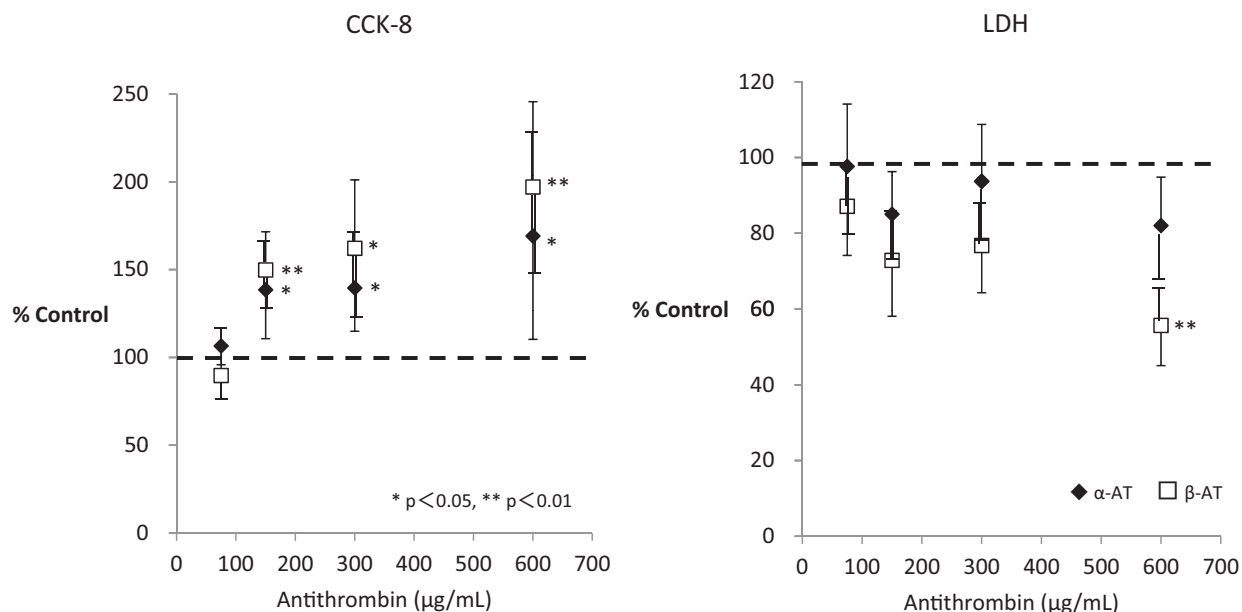


Fig. 2 Changes in cell viability and cellular injury after treatment with α - or β -antithrombin. Cell viability increased after treatment with antithrombin in a dose-dependent manner, and a significant increase was recognized when the α - or β -antithrombin dose was 150 $\mu\text{g/mL}$ or more. The increase in the lactate dehydrogenase (LDH) level was suppressed at a β -antithrombin dose of 600 $\mu\text{g/mL}$. In contrast, the suppression was not significant after treatment with α -antithrombin. Data are expressed as the mean \pm standard deviation. AT, antithrombin, * $p < 0.05$, ** $p < 0.01$, compared with control.

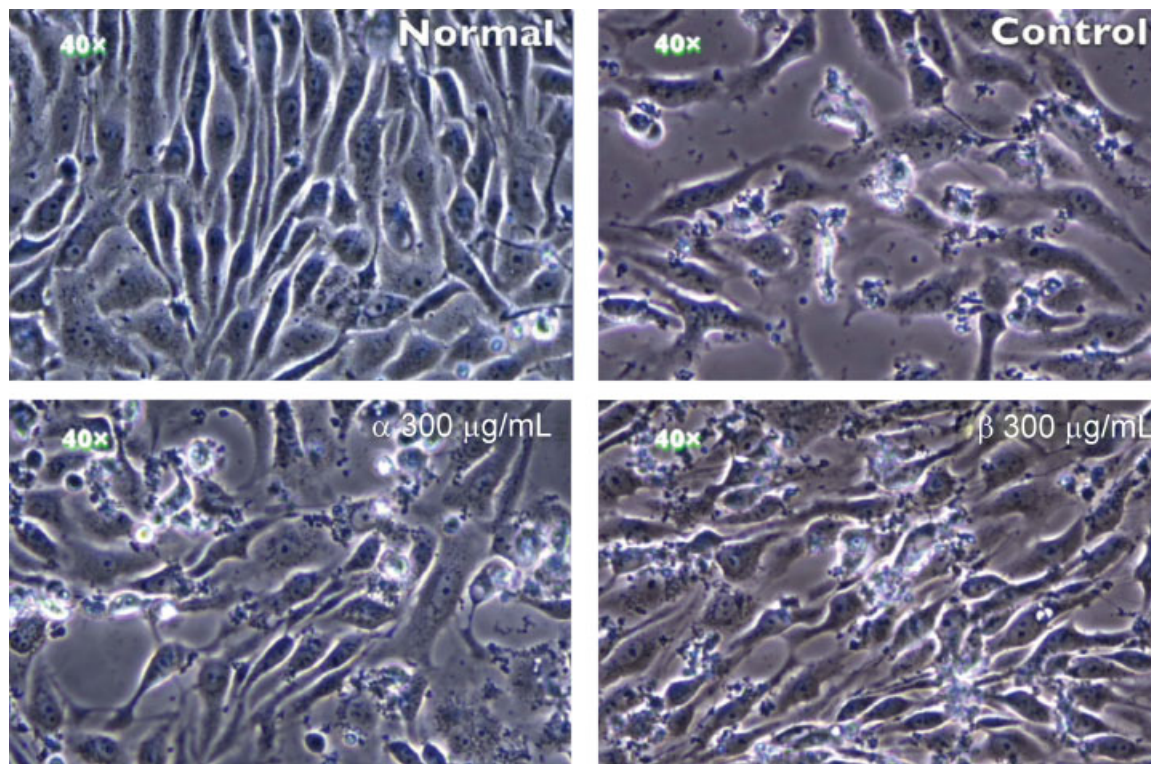


Fig. 3 Morphological changes of endothelial cells after treatment with histone H4. After exposure to various concentrations of histone H4, the morphological changes of endothelial cells were observed. Increasing concentrations of histone H4 resulted in typical signs of cellular damage, including pleomorphism, rupture of the nuclear or plasma membrane, a shrunken cytosol, disruption of the intercellular junction, and the presence of cellular debris. The above changes were attenuated by 300 $\mu\text{g/mL}$ of α - or β -antithrombin, and the effect of β -antithrombin was more prominent than that of α -antithrombin.

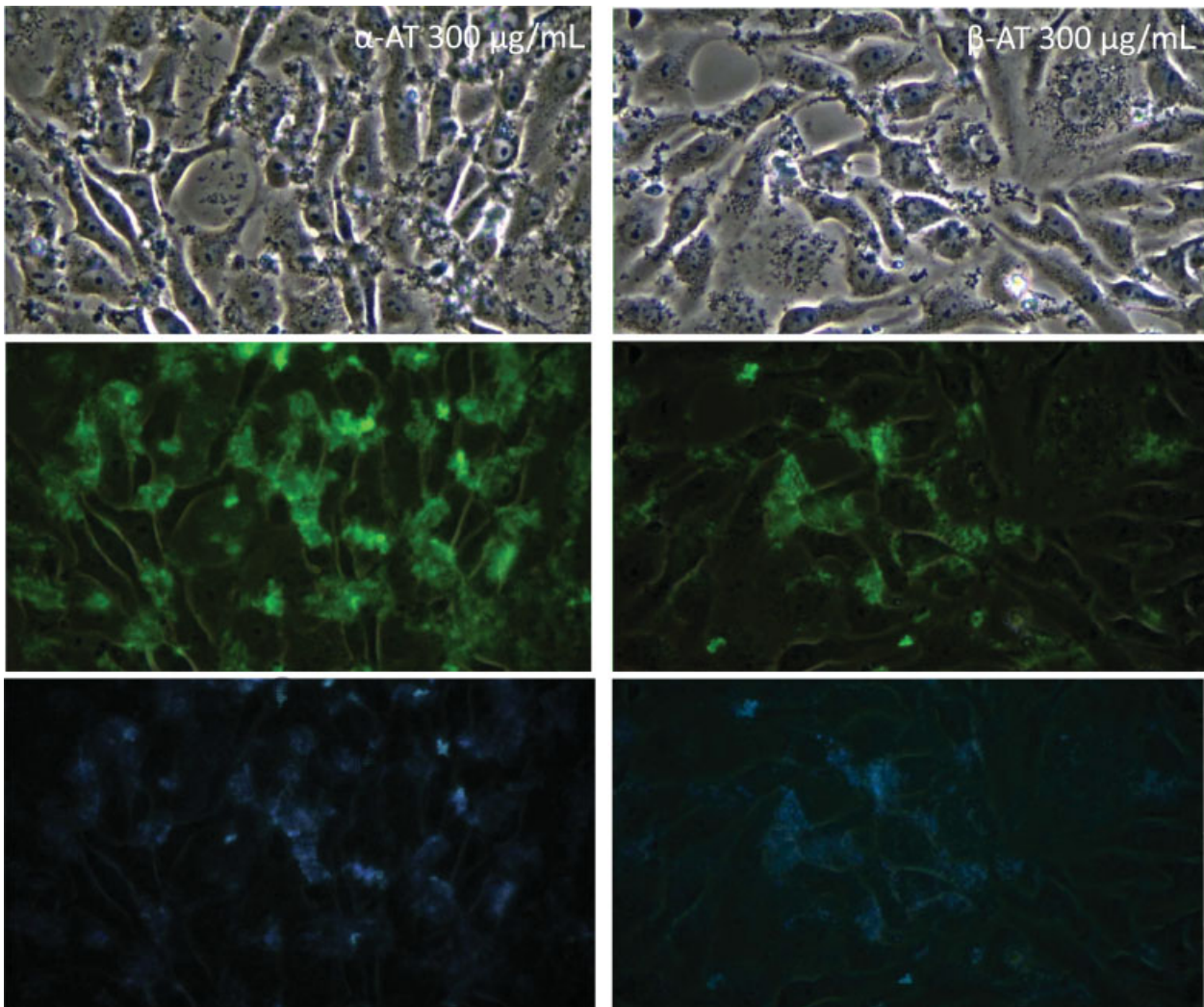


Fig. 4 Phase-contrast and immunofluorescent views of staining for syndecan-4 and antithrombin. Cultured endothelial cells were treated with 35 $\mu\text{g}/\text{mL}$ of histone H4 and 300 $\mu\text{g}/\text{mL}$ of either α - or β -antithrombin. The upper panels show representative images of a phase-contrast view of the endothelial cells at 8 hours. The middle and lower panels show endothelial cells stained with either anti-syndecan-4 antibody or anti-antithrombin antibody. Syndecan-4 and antithrombin were located at the same position. Syndecan-4 staining was more intense with the treatment of α -antithrombin, while the antithrombin staining seemed almost equal between α - and β -antithrombin. AT, antithrombin.

of α -isoform, we hypothesized that β -isoform was more suitable for the treatment of endothelial damage in sepsis. For that purpose, the vascular endothelial cell damage was induced by histone H4. Histones leak out from the dead cell and mediate the inflammation as a damage-associated molecular marker (DAMP). Circulating histones are also known to damage the endothelial cell directly during sepsis. Recent studies have repeatedly demonstrated a positive relationship between the histone level and the severity of sepsis.^{15–17} Other than DAMPs, histones are expelled from the neutrophils adhere to the vascular endothelium as a component of neutrophil extracellular traps (NETs).¹⁸ In this article, we used histone H4 to induce endothelial damage, and as this event plays a major role in the progression of sepsis,¹³ we confirmed the cellular damage both morphologically and biochemically. As shown in **Fig. 1**, histone H4 damaged the endothelial cells in a dose-dependent manner, and the effect was significant when the histone H4 dose was more than 30 $\mu\text{g}/\text{mL}$. Thus, the concentration of H4 was set

just above this level. Under this condition, we next examined the effects of α - and β -antithrombin on “protecting” the endothelial cells. We observed that cell viability, as evaluated using CCK-8, significantly improved with both α - and β -antithrombin in a dose-dependent manner, while cellular injury, as measured using the LDH level, was significantly suppressed only by a high dose of β -antithrombin.

During sepsis, the glycocalyx is a major target of histones.¹⁹ The endothelial glycocalyx is an extracellular layer that is composed of syndecan, heparan sulfate, and hyaluronan.²⁰ The glycocalyx contributes to the smooth circulation of the blood by suppressing cell attachment, acting as a transducer of shear stress to intracellular vascular signals²¹ in addition to acting as a barrier for plasma proteins, such as albumin and antithrombin.²² Thus, the degradation of the glycocalyx increases neutrophil adhesion and further accelerates cellular injury.²³ Several experimental and clinical studies have revealed that the degree of glycocalyx shedding depends on the severity of septic shock and trauma.^{24–26} As the fundamental

role of NETs is thought to be host defense,^{18,27} it seems reasonable that NETs and its major component histone damage vascular endothelial cells to prevent microbial dissemination. In this article, fewer morphological changes were seen in the endothelial cells that were treated using β -antithrombin, indicating that the protective effect was more prominent for the β -isoform. To be more specific for the endothelial glycocalyx, the staining of syndecan-4 was compared between the two isoforms. The intensity was higher after treatment with the α -isoform, suggesting that the protective effect was less with this isoform. Intact cells stained negative for syndecan-4. After treatment with histone H4, syndecan-4 staining became visible at the cellular margin and at adhesion sites, suggesting that syndecan-4 can be used as a marker of cell damage *in vitro*. This finding is in accordance with results from previous studies,^{28,29} reporting that endothelial cells did not express syndecans on their surface under static conditions; however, once the damage was induced and the gap opened, the syndecans between the cells and the basal area were exposed and syndecan-4 attached there became visible after staining. In this article, antithrombin staining was observed at the same site, indicating that it might have been attached to syndecan-4. De Jong and Walstra³⁰ also reported that antithrombin was concentrated at the level of the vascular endothelial basement membrane. Interestingly, the intensity was similar for β -antithrombin, suggesting that the connection could be tighter between syndecan-4 and β -antithrombin. While the mechanism by which antithrombin protects endothelial cells remains to be clarified, it is speculated that since antithrombin is an inhibitor of various inflammatory proteases, it prevents the enzymatic attack by binding to the glycocalyx.^{31,32} This hypothesis is supported by clinical evidence showing that a beneficial effect of antithrombin was recognized only when it was administered without heparin.¹¹ Thus, the concomitant use of heparin might induce unfavorable interference by binding to antithrombin and abrogating its attachment to syndecan.

Limitations

First, we used cultured endothelial cells in a static condition for the experiment. Unlike endothelial cells *in vivo*, the cells do not express syndecan on their surfaces. Further experiments should be performed using an *in vivo* model of sepsis.

Second, antithrombin exerts both coagulation-dependent and coagulation-independent anti-inflammatory effects. We focused on the protective effects that were independent from the anticoagulatory effect in the current experiment. Another possible coagulation-independent mechanism is the promotion of endothelial prostacyclin production through an interaction with syndecan-4.³³ Kaneider et al³⁴ reported that antithrombin inhibited the endotoxin-induced adherence of neutrophils to the vascular endothelium through this effect. This finding should be examined in a future experiment.

Third, the supporting data for the link between antithrombin and syndecan-4 are still weak. An upregulation of syndecan-4 and a co-localization with antithrombin do not prove that these factors are mechanistically involved, and further study is required to approach the mechanism of action.

Finally, the direct effect of antithrombin to the histone could not be denied in this study. As some plasma proteins such as albumin, protein C, and pentraxin 3 were known to reduce the toxicity of histone directly,^{35,36} the protective effect could be induced through the independent mechanism from the binding to syndecan. However, as the effect was different between the isoforms, we thought the effect was revealed at least in some part through the binding to syndecan. The direct effect of antithrombin should be examined in another study.

In summary, the protective effect of antithrombin was demonstrated in this study; however, similar findings have already been reported.^{31,37} What was newly discovered in this study was the different effects of the glycoforms. The β -isoform exhibits a profound effect, probably because it binds to syndecan-4 more tightly; however, the actual mechanism of protection could not be clarified in this study. In this regard, Chappell et al³¹ reported that the protective effects were expressed through the maintenance of glycocalyx integrity. It is also reported that antithrombins display potential to attenuate shedding of the glycocalyx. Furthermore, plasma components, especially albumin, are reported to stabilize the glycocalyx and contribute to the endothelial surface layer.³⁸ Interestingly, Nordling et al³⁹ reported that the novel multi-arm heparin immobilized on the endothelial cells could protect the endothelium. All these findings support the idea that the maintenance of the glycocalyx including heparan sulfate-like structures on cell surface protects the endothelial cell. While the details remain unknown, one possible mechanism is that a sufficient level of antithrombin with syndecan may stabilize the glycocalyx and obstruct attacks from histone. This hypothesis clearly warrants further assessment to determine whether the glycocalyx is a suitable therapeutic target with potential benefits from antithrombin treatment in clinical settings. The result of this study indicates that either plasma derived or recombinantly constructed β -antithrombin dominant antithrombin concentrate can be expected as a future therapeutic agent.

Conclusion

The protective effect of antithrombin on vascular endothelial cells was confirmed in this study. This finding suggests that the clinical outcomes may be better if antithrombin is used in the absence of heparin. This study also demonstrated histone-induced endothelial injury was reduced to a larger extent by β -antithrombin, compared with α -antithrombin, which may mean adequate concentrations of former isoform of antithrombin may be more specific for the treatment purposes.

Contributions of Authors

The study design was performed by T.I. and J.T. I.N. and K.S. were involved in the study conduct. Data collection was performed by T.S. and K.O. Data analysis was performed by T.I. and K.S., and I.N., T.I., and J.T. were involved in drafting and revising of the manuscript. All authors read and approved the final manuscript.

Conflict of Interest

T.S. and K.O. are employees of Nihon Pharmaceutical Corporation. The authors declare that they have no other conflict of interests.

Acknowledgments

A part of this study was presented at the 62nd Annual Scientific and Standardization Committee meeting of International Society on Thrombosis and Haemostasis.

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