**ORIGINAL ARTICLE**

# Protective effect of a newly developed fucose-deficient recombinant antithrombin against histone-induced endothelial damage

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## Abstracts

Antithrombin is expected to modulate both prothrombotic and proinflammatory reactions in sepsis; vascular endothelium is the primary target. In the present study, we sought to evaluate the protective effects of a newly developed fucose-deficient recombinant antithrombin. Endothelial cells were treated in vitro with histone H4 to induce cellular damage. Low to high doses of either plasma-derived antithrombin or recombinant thrombomodulin were used as treatment interventions. Morphological change, apoptotic rate, cell viability, cell injury, and syndecan-4 level in the medium were evaluated. Immunofluorescent staining with anti-syndecan-4 was also performed. Both types of antithrombin reduced cellular damage and apoptotic cell death. Both plasma-derived and recombinant antithrombin improved cell viability and reduced cellular injury when administered at a physiological concentration or higher. Syndecan-4 staining became evident after treatment with histone H4, and both antithrombins suppressed the staining intensity at similar levels. The syndecan-4 level in the medium was significantly decreased by both antithrombins. None of the indicators showed a significant difference between plasma-derived and recombinant antithrombin. In conclusion, both recombinant and plasma-derived antithrombin can protect vascular endothelial cells. Recombinant antithrombin may represent a useful new therapeutic agent for sepsis-associated vascular damage.

**Keywords** Antithrombin · Oligosaccharide · Vascular endothelial cell · Syndecan · Histone

## Introduction

Antithrombin, a naturally derived serine protease inhibitor, consists of 432 amino acids [1]. Though anticoagulation targeting thrombin and other coagulation factors is its major activity [2], antithrombin is also known to exert anti-inflammatory actions through both anticoagulation-dependent and -independent mechanisms [3, 4]. Previous studies have revealed that both the number and the type of N-linked oligosaccharides attached to antithrombin affect

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the anticoagulant activity [5, 6]. Two antithrombin isoforms, α and β, exist in plasma, and each form has either four or three oligosaccharides. α-antithrombin accounts for 90–95% of the total antithrombin in plasma, and this form has oligosaccharides at each of the four glycosylation sites. The remainder consists of β-antithrombin and Asn135 is unoccupied by an oligosaccharide in this form [7]. The absence of an oligosaccharide at Asn135 contributes to the higher heparin-binding affinity of the β-form, suggesting that the molecular structure hampers the binding of antithrombin to heparin [8–10].

The anticoagulant activity of antithrombin is well known to increase to a thousand-fold or even more by binding to heparin. Independent of the occupation of Asn135, heparin binding is also affected by a core fucose at the reducing end of the attached oligosaccharides [11, 12]. The oligosaccharide in human antithrombin does not contain a fucose, and it has been reported that the fucosylation of the oligosaccharide at Asn155 contributes to a reduction in heparin binding affinity. As a matter of fact, the fucosylation of the oligosaccharide was a serious unsolved defect of previously developed forms of recombinant antithrombin [12, 13]. Recently, an antithrombin that has four oligosaccharides but that lacks a core fucose was produced using recombination by Kyowa-Kirin (Tokyo, Japan). They used CHO DG44 cell lines deficient in α-1,6-fucosyltransferase (*FUT8*) [14] and *N*-acetylglucosaminyltransferase I (*GnT-I*) [15], and this novel antithrombin was named antithrombin-γ (AT-γ). Another unique point of AT-γ is its high-mannose type oligosaccharide, which also contributes to an increase in heparin affinity [16]. A clinical study demonstrated that AT-γ and pd-AT had equivalent effects, and AT-γ has been approved for the treatment of thrombosis in patients with congenital antithrombin deficiency and disseminated intravascular coagulation (DIC) in Japan since 2015. In addition to its anticoagulant effect, however, antithrombin is also expected to exhibit an anti-inflammatory effect when it is applied for the treatment of sepsis-associated DIC [3]. Therefore, antithrombin was recommended to use in the practice guidelines for septic DIC. [17]. Since whether AT-γ exerts an anti-inflammatory effect has not been previously reported, we planned to examine the protective effect of this new agent on vascular endothelial cell injury in the present study.

## Materials and methods

### Endothelial cell culture

Rat aortic endothelial cells were purchased from Cell Applications, Inc. (San Diego, CA, USA), and their uptake of DiI-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 tissue culture plates and grown to confluence.

### Histone-induced cytotoxicity

Histone H4 was purchased from New England Biolabs (Ipswich, MA, USA). 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, CA, USA) without FBS at 2 h before the addition of histone H4. Histone H4 was added to 0.2 mL of Opti-MEM and the concentration was adjusted. Eight hours after the treatment with histone H4 and antithrombins, the media were collected and used for the assessment of cellular damage.

### Treatment with pd-AT or AT-γ

Pd-AT and AT-γ were provided by Japan Blood Products Organization (Tokyo, Japan). The culture medium was changed to the serum-free medium, and either pd-AT or antithrombin AT-γ was added to Opti-MEM containing histone H4. The cell viability changed depending on the culture period and dose of histone H4 (Suppl. 1). The final concentrations of both antithrombins were 75, 150 and 300 µg/mL, and the concentration of histone H4 was 50 µg/mL. For the control, only histone H4, and not antithrombin, was applied. After 8 h, the media were harvested from the cultures and stored at − 70 °C until assay. Each experiment was done three times in duplicate.

### Microscopic observations and detection of apoptotic cells

To evaluate the morphological changes, the endothelial cells were observed using the Eclipse Pol microscopic system (Nikon, Tokyo, Japan) at 8 h after histone H4 administration. In each well, total of six fields were selected randomly by two individuals, and each field was photographed (EOS 5D Mark III, Canon, Tokyo, Japan). For the detection of apoptosis, the cells were stained with Apoptosis/Necrosis Detection Kit (ENZ-51002–25, Enzo Life Sciences, NY, USA) according to the manufacturer's instructions. Six fields in each sample were randomly selected under a fluorescent microscope by two blinded individuals, and the mean ratio of the apoptotic cells (late phase apoptotic cell count/total cell count) was calculated.

## Measurement of CCK-8, LDH and syndecan-4 in the medium

Cell viability and injury were evaluated using the Cell Counting Kit 8 (CCK-8; Dojindo, Kumamoto, Japan) and a lactate dehydrogenase (LDH) assay kit (TAKARA Bio, Shiga, Japan), respectively. The assay kits were used according to the manufacturers' instructions. Basically, CCK-8 detects the activity of dehydrogenases in the cells [18]. The concentration of syndecan-4 was measured using the Syndecan-4 ELISA kit (IBL International GmbH, Hamburg, Germany), according to instructions of the manufacturer.

## Immunofluorescent staining for syndecan-4

Immunofluorescent staining for syndecan-4 was performed according to the manufacturer's instructions using anti-syndecan-4 antibody (ab24511; Abcam PLC, Cambridge, UK). 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 h. After incubation, the cells were washed five times with BSS and then used for the immunofluorescent microscopic examination.

## Statistical analysis

Statistical analysis was performed using a one-way ANOVA with the Dunnett post hoc test using statistical software (StatView IITM). Data were presented as the mean ± standard deviation (SD). Differences were considered statistically significant at $P < 0.05$.

## Results

Figure 1 is a representative image of the experiment repeated three times in duplicate and upper left panel shows a phase-contrast image of normal vascular endothelium. In the control (upper right panel), the addition of histone H4 at a concentration of 50 µg/mL resulted in apparent shrinkage and separation of the cells at 8 h. The endothelial cells shrunk along with a decrease in cell number and exhibited disrupted nuclei and an unclear membrane boundary. As shown in the lower panels, 300 µg/mL of pd-AT or AT-γ suppressed the aforementioned morphological changes induced by histone H4.

Figure 2 shows the levels of cell viability (CCK-8, left panel) and injury (LDH, right panel) in cultured endothelial cells treated with histone H4. The CCK-8 level decreased to 48.3 ± 8.91% of the normal condition after the treatment with histone H4. The decrease in cell viability was
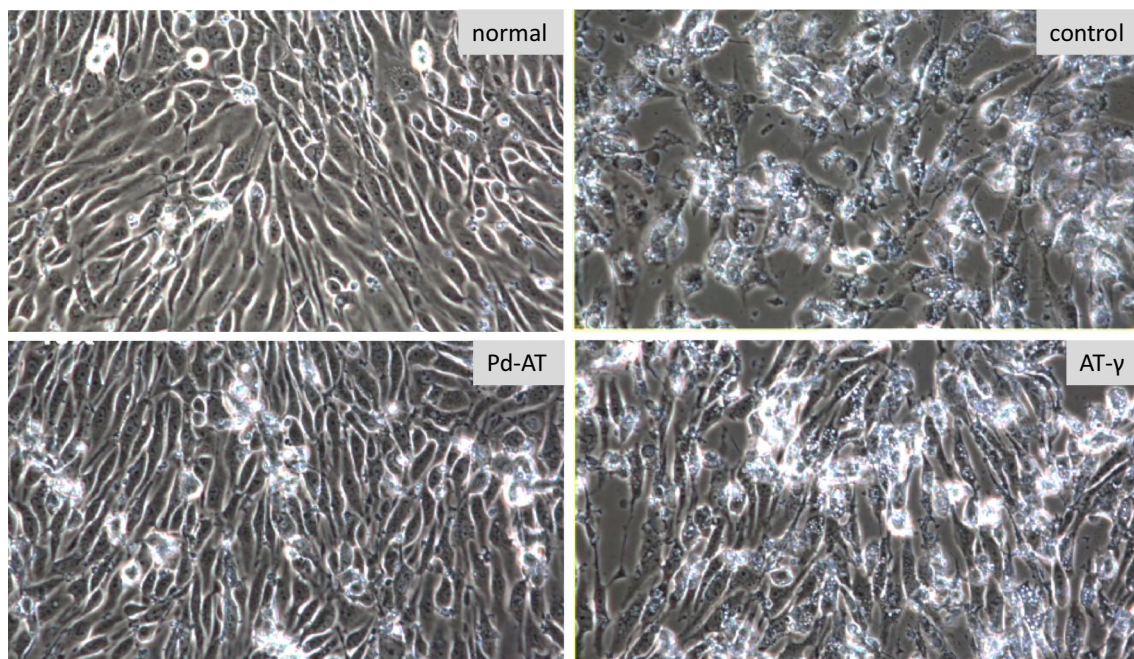


**Fig. 1** Morphological changes in the endothelial cells after treatment with histone H4 and the effects of pd-antithrombin and antithrombin-γ. After exposure to 50 µg/mL of histone H4 for 8 h, a phase contrast view of the endothelial cells showed shrunken cells, the disruption of the intercellular junction, rupture of the plasma membrane, and an increase in cellular debris. The above changes were attenuated by either 300 µg/mL of pd-AT or AT-γ at a similar level. *pd-AT* plasma-derived antithrombin, *AT-γ* antithrombin-γ
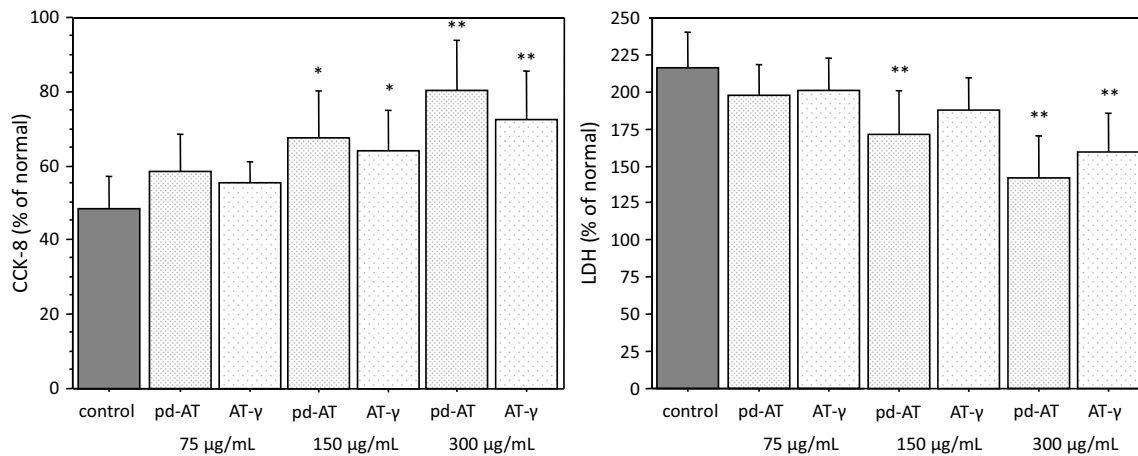
**Fig. 2** Cell viability and injury after treatment with histone H4. Cell viability was measured based on the CCK-8 level, while cellar injury was represented by the LDH in the culture medium. Cell viability was decreased to less than half of the normal condition. The decrease was significantly suppressed by pd-AT and AT-γ. The elevation of LDH was significantly suppressed by both antithrombins. Data are expressed as the percentage of the normal value (mean ± standard deviation). *CCK-8* cell counting kit-8, *LDH* lactate dehydrogenase. *$P < 0.05$, **$P < 0.01$, compared with the control. *pd-AT* plasma-derived antithrombin, *AT-γ* antithrombin-γ

significantly suppressed by the treatment with 150 and 300 μg/mL of pd-AT ($P = 0.014, 0.0005$, respectively). Treatment with 150 and 300 μg/mL of AT-γ also suppressed the decrease in cell viability ($P = 0.041, 0.005$, respectively). The LDH levels in the medium increased to $216.7 \pm 23.2\%$ of normal in the control. No significant effect was seen when the pd-AT or AT-γ concentration was 75 μg/mL, whereas 150 and 300 μg/mL of pd-AT ($P = 0.010, 0.0037$, respectively) and 300 μg/mL of AT-γ ($P = 0.014$) significantly suppressed the increase in LDH. Regarding comparisons between pd-antithrombin and AT-γ, no significant differences were observed at any of the doses.

As for cell death, endothelial cells during the early phase of apoptosis were stained by Annexin V (yellow), while the nuclei of cells during the late phase of apoptosis were stained using 7-amino-actinomycin D (7-AAD, red) (Fig. 3). The ratio of apoptotic cells to total cells was significantly suppressed by the treatment with 150 μg/mL of pd-AT ($P = 0.014$) and 300 μg/mL of pd-AT and AT-γ ($P = 0.0045, 0.021$, respectively). No significant effect was seen when the pd-AT or AT-γ concentration was 75 μg/mL. Regarding comparisons between pd-antithrombin and AT-γ, no significant differences were observed at any of the doses ($P = 0.34$ [75 μg/mL], 0.18 [150 μg/mL], 0.29 [300 μg/mL]).

Figure 4 shows representative images of syndecan-4 staining. Either 300 μg/mL of pd-AT or AT-γ attenuated the intensity, compared with the control. Normal confluent endothelial cells did not exhibit syndecan-4 on their surfaces; however, after treatment with histone H4, syndecan-4 was visualized mainly in the periphery and the adhesion sites of the damaged cells. The right panel shows the syndecan-4 levels in the medium. The syndecan-4 levels were significantly decreased by the treatment with 150 μg/mL of pd-AT ($P = 0.036$) and AT-γ ($P = 0.013$) and with 300 μg/mL of pd-AT and AT-γ ($P = 0.0003, 0.0001$, respectively). No difference was seen between pd-AT and AT-γ.

## Discussion

AT-γ is a newly developed recombinant antithrombin. The unique point of this recombinant antithrombin is the structure of the oligosaccharide. Though AT-γ is a mixture of several glycoforms (Suppl. 2), the α-isoform is the major component. Oligosaccharides are reported to be nonfucosylated and to contain a higher volume of mannose; consequently, its heparin-binding affinity is almost the same as that of pd-AT [16]. High-mannose oligosaccharide is less negatively charged and thus, less repulsive to the heparan sulphate of syndecans [19]. In vitro studies have revealed that antithrombin structured with this type of glycoform has a heparin-binding affinity and anticoagulant activity similar to that of pd-AT [16]. Following preclinical and early-phase clinical studies, a randomized comparative phase III study of AT-γ (36 IU/kg for 5 days) versus pd-AT (30 IU/kg for 5 days) in 222 patients with sepsis-associated DIC was performed, and the result showed the comparable DIC resolution rate and the similar survival rate. Based on this result, AT-γ has been approved for use in patients with DIC in Japan. Needless to say, recombinant antithrombin is safer than plasma-derived products. In addition, it contributes to the stable supply regardless of the decreasing blood donation. Moreover, it may reduce the medical cost since the expense is depending on the consumption.
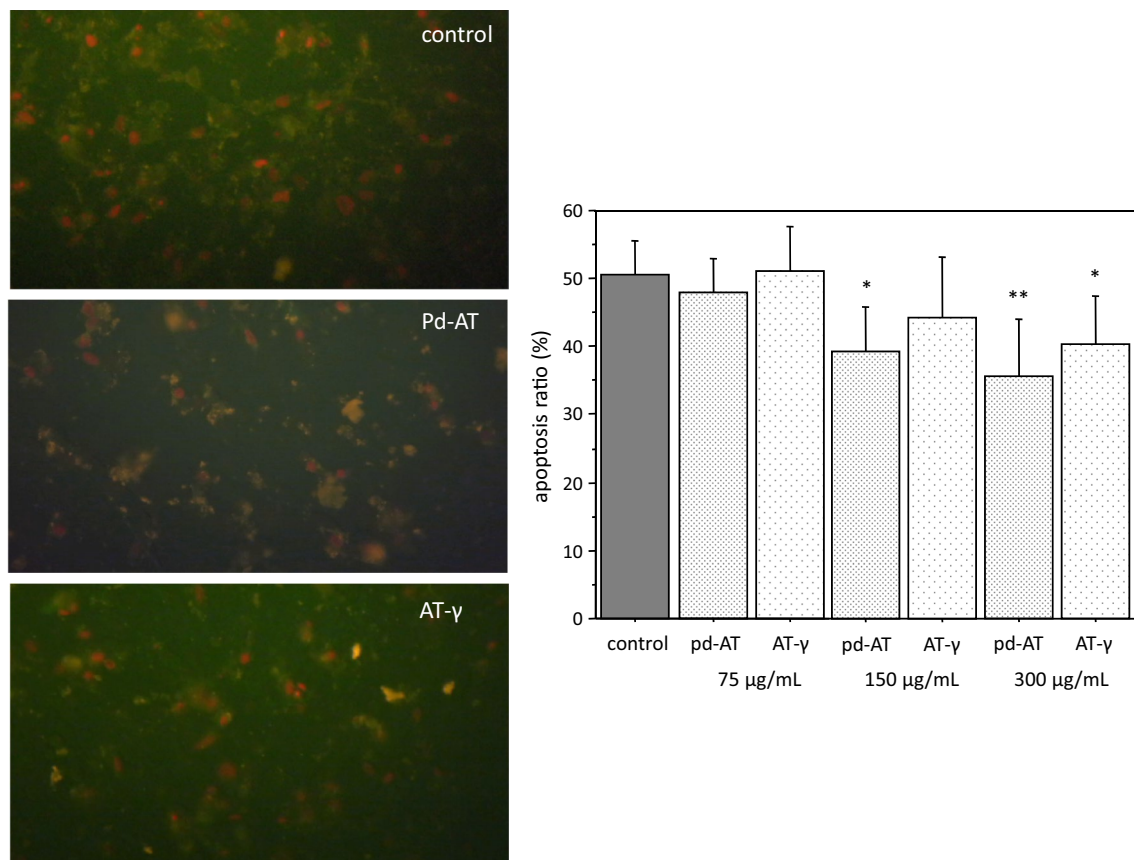
**Fig. 3** Apoptosis ratio after treatment with histone H4, and the effects of pd-AT and AT-γ. Apoptotic cell death was induced by histone H4. Early-phase apoptosis was visualized using Annexin V (yellow), while late-phase apoptosis was visualized as red nuclei stained by 7-amino-actinomycin (red). The representative images of the control, 300 μg/mL of pd-AT and 300 μg/mL of AT-γ were shown. The ratio of late-phase apoptotic cells to the total cells (apoptosis ratio) was significantly suppressed by the treatment with pd-AT or AT-γ. Data are expressed as the mean ± standard deviation. *$P < 0.05$, **$P < 0.01$ compared with the control. *pd-AT* plasma-derived antithrombin, *AT-γ* antithrombin-γ

The glycocalyx covering the endothelial surface of the vascular bed is an important interface where inflammation and coagulation interplay [20]. The glycocalyx is composed of glycoproteins including syndecans and proteoglycans [21] and contributes to the smooth circulation of the blood by suppressing cell attachment [22]. For example, Ishiguro et al. [23] reported that syndecan-4-null mice exhibit severe malcirculation and easily succumb to lipopolysaccharide injection. In fact, the glycocalyx is an essential target of inflammatory mediators such as histones and proteases [24]. Physiologic anticoagulants such as antithrombin, thrombomodulin and activated protein C, which contribute to anti-coagulation and anti-inflammation, are all connected to the endothelium, and antithrombin is known to bind to the heparan sulphate of syndecan-4 [25]. In a previous study, we demonstrated that the distribution of antithrombin as visualized using immunostaining was the same as that of syndecan-4 (unpublished data), and the syndecan-4 level in the culture medium is known to increase in accordance

with the cellular damage [26]. In the present study, there was no difference in the fluorescent intensity of syndecan-4 staining or the syndecan-4 levels in the media between AT-γ and pd-AT. Since syndecan-4 expression as visualized using immunostaining is an indicator of endothelial damage, we think that the protective effects might be similar. The similar levels of syndecan-4 in the media help to confirm this result.

The mechanism responsible for the protective effect has not been fully elucidated; however, the binding of antithrombin to syndecan-4 is thought to be involved. The observation that β-antithrombin, which has a higher affinity for heparin, has a stronger effect than α-antithrombin supports this idea [9]. Kaneider et al. [26] revealed that the protective effect of antithrombin was abolished by the concomitant incubation with pentasaccharide, and their report also supports the above hypothesis. As a matter of fact, syndecans play a major role in regulating cell morphology, since they bind to and regulate the distribution of the cytoskeleton [28]. Furthermore, syndecan-4 is reportedly concentrated at the
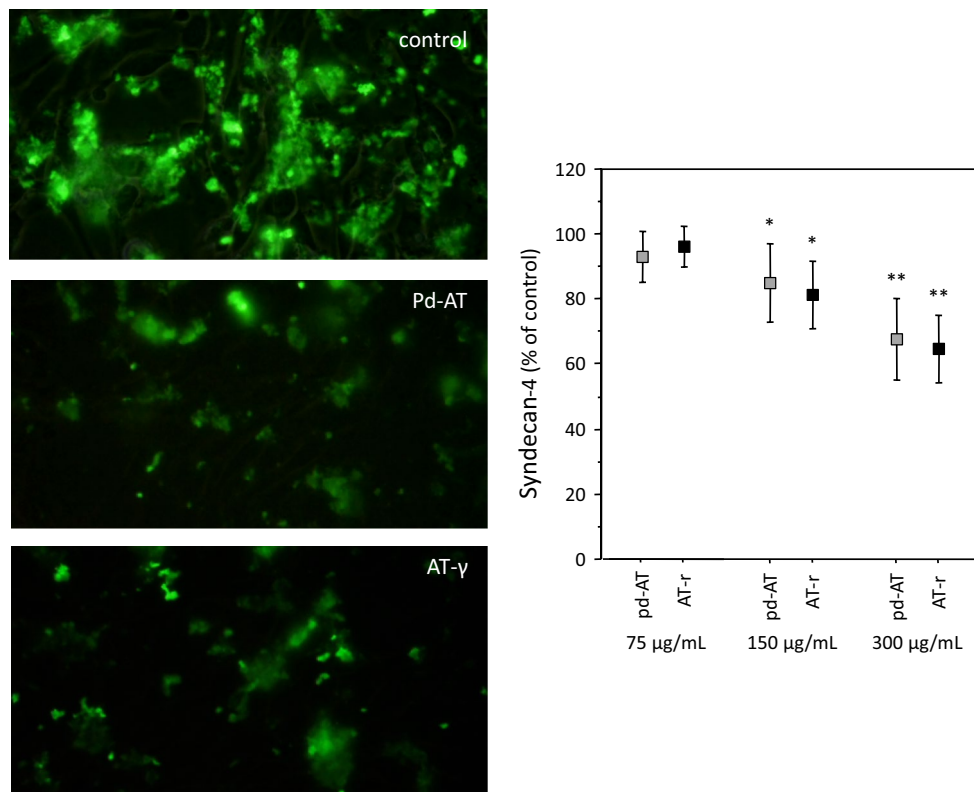
**Fig. 4** Immunofluorescent views of staining for syndecan-4 and syndecan-4 level in the culture medium. Cultured endothelial cells were treated with histone and either 300 μg/mL of pd-AT or AT-γ. The upper panel show-suppressed by both antithrombins in a concentration-dependent manner. The syndecan-4 level did not differ between the antithrombins. Data are expressed as the mean ± standard deviation. *$P < 0.05$, **$P < 0.01$ compared with the control. *pd-AT* plasma-derived antithrombin, *AT-γ* antithrombin-γ

focal adhesion site and potentiates cellular stability [29]. If antithrombin can stabilize syndecan-4 by binding to heparan sulphate, this could be one possible explanation for the protective effect.

Other reports have speculated that since antithrombin is an inhibitor of various inflammatory proteases, it prevents enzymatic attacks by binding to the glycocalyx [25, 27]; however, this mechanism of action is irrelevant to the current model.

Finally, the presently reported study has some limitations. First, though the main targets of histones were considered to be the microvascular endothelial cells, we used aortic endothelial cells in this study to obtain consistent results. Microvascular endothelial cells are more sensitive to histone and affected by the non-FBS condition. The studies should be repeated using microvascular endothelial cells in the future. Second, the evaluation of apoptosis was not objective enough and would be problematic. More sophisticated procedure is required for the precise evaluation. Finally, the expression of syndecan differs between physiological conditions and static conditions. Since syndecan functions as a transducer of shear stress to intracellular vascular signals [30], cells cultured under static conditions do not express

syndecan on its surface [31]. On the other hand, syndecans exist between the cells (endothelial cleft) and also at the basement of the cells [31, 32]. Therefore, once damage has been induced and the gap has opened, the cells detach from the basement and the syndecans are exposed and stained. Therefore, the findings obtained in this study should be confirmed in an in vivo study.

## Conclusions

The protective effects of novel recombinant AT-γ on vascular endothelial cells were comparable to those of pd-AT. AT-γ can be expected as a new therapeutic agent for sepsis-associated vascular damage.

## Compliance with ethical standards

**Conflict of interest** The authors have no competing interests to declare.

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