Introduction

Antithrombin (AT) is a glycoprotein related to the regulation of blood coagulation. AT is a serine protease inhibitor and its effect is derived from the inhibition of thrombin, factor IXa, Xa, XIa, plasmin, and kallikrein. AT is synthesized in the liver and present in human plasma at levels of 0.14 to 0.20 g/L. Its molecular weight is approximately 58000 Da with 432 amino acids. AT contains four N-glycosylation sites at the asparagine residues Asn96, Asn135, Asn155, and Asn192. The glycoprotein has a microheterogeneity based on the diversity of those N-linked oligosaccharides, of which there are two groups of isoforms, namely the α-form of AT (α-AT) and the lower abundant β-form lacks the oligosaccharide on Asn135. The main oligosaccharide structure of human AT consists of biantennary complex-type oligosaccharides lacking a core fucose. Generally, Chinese hamster ovary (CHO) cells produce recombinant human AT (rhAT) with core-fucosylated oligosaccharides. However, rhAT lacking core-fucose oligosaccharides can be produced by POTELLIGENT® technology, which uses FUT8 knockout CHO cells in production. The rhAT has more variable glycans, such as tetra-antennary complex type, high-mannose type, and mannose 6-phosphate species as minor components compared to plasma-derived human AT (phAT). In addition, the site-specific glycan profile was different between two ATs. We evaluated the effect of these properties on efficacy and safety based on a comparison of rhAT made by that technology with phAT in terms of their respective oligosaccharide structures, site-specific oligosaccharide profiles, and the ratio of α- and β-forms. Although some structural differences were found between the rhAT and phAT, we concluded that these differences have no significant effect on the efficacy and safety of rhAT.

Keywords Antithrombin, glyco-engineering, oligosaccharide profile, fucose

(Received May 17, 2019; Accepted August 6, 2019; Advance Publication Released Online by J-STAGE August 16, 2019)
α-AT by column chromatography purification.

To compare the efficacy and safety of rhAT to phAT, an open-label, randomized, phase 3 study was conducted. This study showed that the efficacy and safety were similar for 36 IU/kg/day rhAT and 30 IU/kg/day phAT from the clinical point of view.

In this report, we present a comparison between rhAT and phAT in terms of the oligosaccharide structure, site-specific oligosaccharide profile, and the ratio of α- and β-AT, and consider the effect on efficacy and safety derived from the structural differences between rhAT and phAT.

Materials and Methods

Materials
RhAT was produced at Kyowa Kirin (Tokyo, Japan), phAT was purchased from Japan Blood Products Organization (Tokyo, Japan), N-glycosidase F (PNGase F) was purchased from New England Biolabs (NEP) (Ibpsi, MA), 2-aminobenzamide (2-AB) and tris(hydroxymethyl)aminomethane (Tris) were purchased from Nacalai Tesque (Tokyo, Japan), NaBH₃CN, dimethylsulfoxide (DMSO) and iodoacetamide (IAM) were purchased from Sigma-Aldrich (St. Louis, MO), acetic acid, acetonitrile, sodium acetate, sodium hydroxide, sodium dodecyl sulfate (SDS), disodium hydrogenphosphate, citric acid, and sodium bicarbonate were purchased from Wako Pure Chemical Industries (Tokyo, Japan), guanidine HCl was purchased from MP Biomedicals (Santa Ana, CA), 2,5-dihydroxybenzoic acid (DHB) was purchased from Waters (Milford, MA), peptidyl-Asp metalloendopeptidase (Asp-N) was purchased from Promega (Fitchburg, WI), neuraminidase was purchased from Roche Diagnostics (Risch-Rotkreuz, Switzerland), β-galactosidase was purchased from Calbiochem (Hayward, CA), SDS gel buffer, bare fused-silica capillary and 5-carboxytetramethylrhodamine succinimidyl ester (5-TAMRA SE) were purchased from Beckman Coulter, Inc. (Fullerton, CA), NAP-5 columns was purchased from GE Healthcare Life Sciences (Piscataway, NJ), diithiothreitol (DTT) was purchased from Life technologies (Carlsbad, CA).

Release and fluorescence derivatization of N-linked oligosaccharide from antithrombin
N-linked oligosaccharides were released enzymatically with PNGase F; 0.6 mg protein was mixed with 6 × 10⁷ U PNGase F and the mixture was incubated at 37°C overnight. After digestion, ice-cold ethanol was added and the mixture was centrifuged to remove proteins. The supernatant was dried using a centrifugal vacuum evaporator. Oligosaccharides were labeled according to a previously reported procedure with slight modification. A labeling reagent (0.7 M 2-AB, 1.6 M NaBH₃CN in 70:30 (v/v) DMSO/acetic acid) was added to the released and purified N-glycan samples. The reaction mixtures were incubated at 37°C overnight. The excess labeling reagent was removed by an HLB column (10 mg sorbent per cartridge, 30 μm particle size) (Waters). The cartridge was first washed with 1 mL of 5% acetonitrile. The reaction mixture was then loaded on the column and washed with 1 mL of 5% acetonitrile, twice. After washing, the 2AB-labeled N-glycans were eluted with 1 mL of 20% acetonitrile. The elution from the column was dried with a centrifugal vacuum evaporator, and the residue was dissolved in water for HPLC analysis.

HPLC analysis of 2AB-labeled oligosaccharides and fraction
The HPLC system was an Alliance2695 system (Waters). The 2AB-labeled N-glycans were separated on an anion-exchange column (CarboPac PA-1, 4 × 250 mm, Thermo Scientific). The mobile phases were water (A), 0.5 M sodium acetate (B) and 0.5 M sodium hydroxide (C). The column was equilibrated with 10% C. After holding for 15 min in 10% C, the 2AB-labeled N-glycans were eluted using a gradient of 0.36% B/min for 97 min and 2.5% B/min under 10% C. The flow rate was 0.5 mL/min, and fluorescence detection was performed with excitation at 330 nm and emission at 420 nm. Each oligosaccharide peak was fractionated and desalted using a reverse-phase solid phase extraction column (Sigma-Aldrich, St. Louis, MO).

Matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF/MS)
Mass spectrometry analysis was conducted using MALDI-TOF/MS (AutoflexII, Bruker Daltonics). Samples were dissolved with water and then mixed with a DHB matrix solution. One microliter of the mixture was spotted on a stainless-steel sample target board. The sialylated oligosaccharide samples were measured in the linear negative ion mode. The neutral oligosaccharides samples were measured in the reflector positive ion mode.

Site-specific N-glycosylation analysis
For analysis of site-specific oligosaccharides profile, Asp-N peptide mapping was employed. Sample preparation was performed following the manufacturer’s protocol. Antithrombin was denatured by adding 0.13 M Tris, 7 M guanidine HCl, pH 8.0. For reduction, 40 mg/mL DTT was added, followed by incubation at 37°C for 1 h. Then, for alkylation, 100 mg/mL IAM was added, followed by incubation at 37°C for 1 h. As a next step, the buffer solution was exchanged to a digestion buffer (0.1 M Tris, pH 8.0) using NAP5 column (GE Healthcare Life Sciences, Piscataway, NJ). The elution of NAP-5 filtration was mixed with Asp-N solution at a 1:250 enzyme:substrate ratio and incubated at 37°C for 16 h.

The Asp-N digested peptide mixture was separated by RP-HPLC (Agilent 1200) on a C18 column (YMC-Pack Pro C18, 4.6 × 250 mm) and fractionated into four glycopeptide peaks. The mobile phases consisted of 0.1% TFA in water (mobile phase A) and 0.1% TFA in acetonitrile (mobile phase B). After holding for 20 min in 10% B, the peptides were eluted using a gradient of 0.41% B/min for 140 min with a flow rate of 0.5 mL/min. UV absorption was measured at a wavelength of 214 nm.

Analysis of the β-AT contents using capillary electrophoresis-SDS (CE-SDS)
CE-SDS analysis was performed using a Proteomelab PA800 system with a LIF detector using 488 nm of an argon laser as a light source and an emission band-pass filter of 560 nm (Beckman Coulter, Inc., CA) with a fused silica capillary (Beckman Coulter, Inc.) of 50 μm i.d., a total length of 40 cm and effective length of 30 cm. Neuraminidase and galactosidase were added to an antithrombin sample and incubated at 37°C overnight. The sample solution was equilibrated with a buffer by passing through a NAP-5 column previously equilibrated with 0.1 M sodium bicarbonate, pH 8.3. A 10-μL portion of 5-TAMRA SE dissolved in DMSO at a concentration of 0.3 mM was added to 190 μL of the sample solution. Then, the solution was incubated at 30°C for 5 min. Excess dye was removed by NAP-5 and the buffer exchanged into 85 mM citrate-phosphate buffer (pH 6.5). The labeled sample was mixed with a SDS solution containing DTT, and incubated at 90°C for 5 min.

The SDS gel buffer was loaded into the capillary at 70 psi for 15 min from the outlet side. A sample solution was injected
electrokinetically at 5 kV for 20 s. Separation was conducted in the negative polarity mode at 15 kV for 70 min.

**Results and Discussion**

**Oligosaccharide profile of rhAT and comparison with phAT**

2-AB labeled N-linked oligosaccharides from rhAT were analyzed by anion-exchange HPLC and the structure of each peak was identified by MALDI-TOF/MS. Figure 1A shows a chromatogram of the oligosaccharide profile of the rhAT and Table 1A gives the assigned carbohydrate structure of each peak. Most of the glycans in the rhAT are terminal-sialylated and non-core fucosylated complex type glycans, and the most abundant structure is two sialylated, bi-antennary, and non-fucosylated complex type N-glycan in rhAT. As minor structures, high mannose type, sialylated tri-antennary, and tetra-antennary N-glycans were also detected. In addition, some peaks of mannose 6-phosphate (M6P) or sulfate glycans were detected. The presence of M6P glycans were confirmed by the shift of the retention time of their peaks in HPLC by phosphatase digestion (data not shown), and the presence of sulfate glycans were confirmed by MALDI-TOF/MS analysis. M6P is known to allow the transport of proteins to the lysosome by interacting with the M6P receptor\(^{25,26}\) and sulfate glycan is reported to have important functions concerning inflammation\(^{27,28}\). But, it is unclear why the rhAT molecules having these oligosaccharides were expressed. There is no core-fucosylated glycan species because of the potelligent technology. Hence, there is no trace of O-linked saccharide using LC/MS analysis of the rhAT peptide mapping (data not shown).

Figure 1B shows a chromatogram of the oligosaccharide profile of phAT and Table 1B gives the carbohydrate structure of each peak. Most of the glycans in the phAT are terminal-sialylated and non-core fucosylated complex type glycans, and the most abundant structure is two sialylated, bi-antennary, and non-fucosylated complex type N-glycans in phAT, the same as in the rhAT. The structure of two peaks (No. g and i) was not
### Table 1A Oligosaccharide structures of rhAT

| Peak No. | Structure | Peak No. | Structure |
|----------|-----------|----------|-----------|
| 1        | ![Structure](image1) | 17       | ![Structure](image2) |
| 2        | ![Structure](image3) | 18       | ![Structure](image4) |
| 3        | ![Structure](image5) | 19       | ![Structure](image6) |
| 4        | ![Structure](image7) | 20       | ![Structure](image8) |
| 5        | ![Structure](image9) | 21       | ![Structure](image10) |
| 6        | ![Structure](image11) | 22       | ![Structure](image12) |
| 8        | ![Structure](image13) | 23       | ![Structure](image14) |
| 9        | ![Structure](image15) | 24       | ![Structure](image16) |
| 10       | ![Structure](image17) | 25       | ![Structure](image18) |
| 11       | ![Structure](image19) | 26       | ![Structure](image20) |
| 13       | ![Structure](image21) | 27       | ![Structure](image22) |
| 15       | ![Structure](image23) | 28       | ![Structure](image24) |
| 16       | ![Structure](image25) | 29       | ![Structure](image26) |

Peaks No. 7, 12, and 14 were not identified by mass spectrometry. The presence of a phosphate group in M6P species was confirmed by alkaline phosphatase digestion.

### Table 1B Oligosaccharide structures of phAT

| Peak No. | Structure | Peak No. | Structure |
|----------|-----------|----------|-----------|
| a        | ![Structure](image27) | c        | ![Structure](image28) |
| b        | ![Structure](image29) | f        | ![Structure](image30) |
| c        | ![Structure](image31) | h        | ![Structure](image32) |
| d        | ![Structure](image33) |          |           |

Peaks No. g and i were not identified by mass spectrometry.
Molecules are known to be abundant in human plasma. Oligosaccharide linked glycoproteins and sulfated glycan related caused by high-mannose type and M6P type oligosaccharides contained in phAT. It is reported that there is no adverse event M6P type, and sulfated oligosaccharides, which are not RhAT has high-mannose type, sialylated tetra-antennary type, particular, rhAT has more variable oligosaccharide structures. Though the main peaks of phAT and rhAT have the same two sialylated, bi-antennary, and non-fucosylated complex-type structure, the elution time is slightly different. This difference is assumed to be due to the difference in the sialic acid binding position (Terminal sialic acids of phAT are α2-6 linkages, while that of rhAT are presumed to be α2-3 linkages from the literature). As minor structures, sialylated tri-antennary type, core-fucosylated type, and Lewis x or Lewis a antigen structures were detected. However, the M6P and sulfate glycan species detected in the rhAT were not detected in the phAT.

The main oligosaccharides of rhAT and phAT were the same (two sialylated, bi-antennary, and non-fucosylated complex type), but their minor oligosaccharides were not similar, and in particular, rhAT has more variable oligosaccharide structures. RhAT has high-mannose type, sialylated tetra-antennary type, M6P type, and sulfated oligosaccharides, which are not contained in phAT. It is reported that there is no adverse event caused by high-mannose type and M6P type oligosaccharides in enzyme replacement therapy for lysosomal storage disease, i.e., agalsidase beta contains substantial amounts high-mannose and M6P oligosaccharides. Sialylated tetra-antennary type oligosaccharide linked glycoproteins and sulfated glycan related molecules are known to be abundant in human plasma. Overall, the impact of those glycan species detected only in rhAT on clinical safety is assumed to be low.

**Site-specific oligosaccharide profile of rhAT**

Sample preparation involved the digestion of AT with Asp-N and fractionation of the resulting peptides using RP-HPLC. The chromatogram of Asp-N peptide map is shown in Fig. 2, with the glycopeptide peaks identified and separated from each other. Oligosaccharide profiles of these collected fractions were acquired. Table 2 shows the ratio of each peak area sorted by the sialic acid number, M6P, and sulfated glycan residue in each glycopeptide.

Asn135 residue of the rhAT has a higher level of M6P type oligosaccharides than other glycan-linked sites. From the percentage of the N0 peak, Asn155 residue has a higher level of immature processing oligosaccharides than others. The reason why the N-glycan at Asn155 is much less completely processed is unclear. But the Asn155 glycans of the rhAT were immature as well as rhAT produced in BHK cells. The glycan of Asn192 residue is the most sialylated in four glycosylated sites because of the highest SA/N value in Table 2.

The site-specific oligosaccharide manner of phAT was confirmed in some reports. Asn96 and Asn192 have mono- or bi-sialylated, biantennary, and non-fucosylated glycans. Asn135 additionally has tri-sialylated, triantennary, and core fucosylated glycans. Asn155 has the most variable glycans which include bi-sialylated, biantennary, and core-fucosylated glycans. All glycans other than bi-sialylated, biantennary, and non-fucosylated glycans are reported to be minor components.

The glycan structure of rhAT and phAT has nothing in common, except that all glycosylated Asn has the main glycan structures bi-sialylated, biantennary, and non-fucosylated. The Asn showing the most variability in phAT is the Asn155 residue, but Asn135 has the most variable glycan structures in rhAT. The cause of the difference between the two molecules is unclear.

There are some reports that glycosylation differences at Asn155 cause the overall affinity of AT. The negative charge of sialylated species in AT glycans may reduce the binding affinity to the negatively charged heparin molecule. As regards the pharmacokinetics, a multiple-dose study showed that 72 IU/kg rhAT is bioequivalent to 60 IU/kg phAT in healthy volunteers (data on file; Kyowa Kirin Co., Ltd., Tokyo, Japan). This is mainly due to the fact that the clearance of rhAT is a

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**Table 2 Relative abundance of oligosaccharides for each N-glycosylation site on rhAT**

| Sample | N-Glycosylation site | Peak area, % | SA/N, mol/molN0 N1 N2 N3 N4 M6P N2S |
|--------|----------------------|-------------|------------------|
| rhAT   | Asn96                | 1.6         | 19.5             | 69.6 | 6.0 | 0.5 | 2.8 | 0.0 | 1.79 |
|        | Asn135               | 2.0         | 16.7             | 40.4 | 14.8| 4.7 | 21.3| 0.1 | 1.65 |
|        | Asn155               | 8.1         | 33.5             | 55.9 | 1.4 | 0.1 | 1.0 | 0.0 | 1.50 |
|        | Asn192               | 3.6         | 24.0             | 47.1 | 19.8| 2.3 | 3.2 | 0.0 | 1.88 |

N0: Non-sialylated N-glycans (including high-mannose type, hybrid type and complex type glycans), N1: mono-sialylated N-glycans, N2: bi-sialylated N-glycans, N3: tri-sialylated N-glycans, N4: tetra-sialylated N-glycans, M6P: mannose 6-phosphate N-glycans, N2S: bi-sialylated and sulfated N-glycans. SA/N: number of sialic acid links per N-glycan (SA/N = N1% + N2% × 2 + N3% × 3 + N4% × 4 + M6P (mono-sialylated)% + N2S% × 2)/100.
The sialic acid content per AT molecule of rhAT and phAT, calculated from oligosaccharide profiles, was 6.7 and 7.9 mol/mol, respectively. Since it is reported that the number of sialic acid residues at the non-reducing end of N-linked oligosaccharides affects the half-life in plasma, their difference in the clearance of AT might be due to the difference in their sialic acid content. In addition, AT bearing the mannose residues at the nonreducing end of N-linked glycan only in rhAT may affect the plasma circulation time through mannose receptor-mediated uptake in the liver and macrophages.

Although there is a difference in the clearance between rhAT and phAT, it is confirmed that the difference of the site-specific oligosaccharide profile has no effect on their efficacy because 36 IU/kg/day rhAT and 30 IU/kg/day phAT showed the same efficacy in a phase 3 study.

**β-AT content of rhAT and phAT**

The β-AT lacks an N-linked oligosaccharide on Asn135 residue. The β-AT is known to bind heparin with higher affinity than α-AT, and the β-AT bearing the complex type N-linked glycan is reported to show a much shorter half-life than the α-AT. If the contents of α-AT and β-AT are different from phAT, rhAT will not show the equivalency with blood products of phAT in efficacy and pharmacokinetics (PK). The α-AT and β-AT were separated using CE-SDS based on the difference of their molecular weight. In addition, to reduce the heterogeneity of oligosaccharides on AT causing broad shapes in CE-SDS analysis, neuraminidase and galactosidase digestion was employed for sample preparation. Though the non-reducing ends of the oligosaccharide linked to rhAT are sialic acid or galactose residue, except for high-mannose species, the digestion of these glycosidases makes the non-reducing ends of the oligosaccharides to be the N-acetyl glucosamine residue.

The electropherogram of the rhAT and phAT is shown in Fig. 3. The β-AT peak appeared in front of the main peak, which is the α-AT peak. These peaks were assigned from an experiment using rhAT with different linkage numbers of

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**Fig. 3** Electropherogram of neuraminidase and galactosidase digested rhAT (A) and phAT (B). IS, Internal Standard. The upper figure shows a full-scale view; the lower figure shows an enlarged view.
Among them, and there is no report that the difference shows a difference in biological activity between them. Currently, some α-AT and β-AT is very low in both rhAT and phAT, it is thought that there is no α-AT. But, since the content of α-AT is higher than that of the β-AT in rhAT with α-AT and β-AT, it is important to for a manufacturer to reconcile the content of α-AT in rhAT with that in phAT.

It is reported that the binding strength of β-AT to heparin is higher than that of α-AT and the biological activity of the β-AT is higher than that of the α-AT. But, since the content of β-AT is very low in both rhAT and phAT, it is thought that there is no difference in biological activity between them. Currently, some AT drugs are commercially available in Japan. The difference between the contents of α-AT and β-AT is thought to be small among them, and there is no report that the difference shows any effect on their clinical effectiveness.

Conclusions

Several phAT drugs are on the market for the treatment of DIC. Though phAT drugs are manufactured with safety measures to prevent infectious diseases, there still may be unknown infectious agents. RhAT drug product can provide clinical benefits compared with phAT from the viewpoint of the risk of infectious agents. Potentially, an AT drug used for DIC patients can provide clinical benefits compared with phAT from the viewpoint of the risk of infectious agents. RhAT drug product can provide clinical benefits compared with phAT from the viewpoint of the risk of infectious agents. RhAT drug product can provide clinical benefits compared with phAT from the viewpoint of the risk of infectious agents. RhAT drug product can provide clinical benefits compared with phAT from the viewpoint of the risk of infectious agents. RhAT drug product can provide clinical benefits compared with phAT from the viewpoint of the risk of infectious agents. 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