Thrombosis Research (2007) **119**, 631–641

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THROMBOSIS RESEARCH

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REGULAR ARTICLE

Recombinant human antithrombin expressed in Chinese hamster ovary cells shows *in vivo* efficacy on rat DIC model similarly to plasma-derived antithrombin regardless of different *N*-glycosylation

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Received 18 October 2005; received in revised form 10 April 2006; accepted 22 May 2006
Available online 17 July 2006

KEYWORDS
Antithrombin;
DIC;
N-Glycosylation;
β-Isoform

Abstract Plasma-derived human antithrombin (pAT) is used for the treatments of disseminated intravascular coagulation (DIC) and hereditary antithrombin deficiencies. We expressed recombinant human antithrombin (rAT) in Chinese hamster ovary (CHO) cells. The purified rAT is composed of 55% α-isoform and 45% β-isoform. The structure of the N-linked oligosaccharides of rAT is the same biantennary complex type as previously found in pAT with less sialylated on the non-reducing ends. Most of the oligosaccharides of rAT are fucosylated at the reducing ends of *N*-acetylglucosamine, while those of pAT are not fucosylated. Despite of the difference in sialylation and fucosylation of the oligosaccharide units, rAT and pAT showed indistinguishable heparin cofactor and progressive activities, and they bound to thrombin in a one-to-one stoichiometric manner. In lipopolysaccharide (LPS)-induced and thromboplastin-induced DIC rat models, rAT reduced fibrinogen and platelet

Abbreviations: rAT, recombinant human antithrombin; pAT, plasma-derived human antithrombin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BHK, baby hamster kidney; CHO, Chinese hamster ovary; DIC, disseminated intravascular coagulation; ELISA, enzyme-linked immunosorbent assay; GlcNAc, *N*-acetylglucosamine; HPLC, high performance liquid chromatography; LPS, lipopolysaccharide; NMR, nuclear magnetic resonance; PA, pyridylaminated; RP-HPLC, reverse phase high performance liquid chromatography; SD, Sprague-Dawley; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SEM, standard error of the mean; TAT, thrombin antithrombin complex.

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0049-3848/$ - see front matter
doi:10.1016/j.thromres.2006.05.010

consumption to a similar extent with pAT. In LPS-induced DIC model, both ATs similarly restrained the increase of alanine aminotransferase and aspartate aminotransferase activities. Finally, pharmacokinetic analysis showed that both ATs had similar half-lives in the circulation of normal rats. Together, the present study demonstrated that rAT prepared in CHO cells has potential for a substitute of pAT in therapeutic use.

Introduction

Plasma-derived human antithrombin (pAT) is a 58 kDa glycoprotein circulating in blood at a concentration of 125 mg/l [1] and plays a significant role in controlling hemostasis by preventing excessive fibrin formation. It belongs to the serine proteinase inhibitor family [2], inhibiting several coagulation factors, such as thrombin and activated factor X in a progressive manner termed "progressive activity". Its inhibitory activity is greatly enhanced, ~1000-fold, in the presence of heparin. This activity is called "heparin cofactor activity" [3]. It contains 15% carbohydrate with sialylated biantennary complex types of oligosaccharide [4,5], which attach to four Asn residues at positions 96, 135, 155 and 192 [6]. There are two isoforms of pAT in terms of glycosylation at Asn^{135} [7]; glycosylated α-isoform and non-glycosylated β-isoform. β-Isoform exhibits a higher heparin-binding affinity than α-isoform because the attached sugar chain at Asn^{135} hinders the binding of heparin [8].

pAT, a biopharmaceutical, is used as for the treatments of disseminated intravascular coagulation (DIC) and hereditary AT deficiencies [9,10]. Therapeutic trials for the treatment of severe sepsis and septic shock were also conducted with pAT [11]. Animal studies and clinical trials have suggested other potential applications of AT such as burn shock [12], hepatopathy [13], subarachnoid hemorrhage [14], hematopoietic stem-cell transplantation [15], and pre-eclampsia [16], but these applications have not yet been clinically approved.

Recombinant human antithrombin (rAT) is produced in a high yield in transgenic goats [17] and it has been used in a clinical trial for the aftercares of heparin-resistant patients who received bypass surgery [18]. rAT was also expressed in mammalian cells such as Chinese hamster ovary (CHO) [19,20], COS-1 [20,21], and baby hamster kidney (BHK) cells [22]. However, the methods for large-scale production have not been established. We have developed an expression system of rAT in CHO cells, and recently the production level reached 1 g/l by fed-batch fermentation [23]. The purified rAT was found to have an equal specific activity to pAT and contained N-linked oligosaccharides. It is less glycosylated and has a higher heparin binding affinity than pAT [24]. In this study, the structures of five N-linked oligosaccharide of rAT were resolved, and *in vitro* and *in vivo* efficacies of rAT were found to be comparable to those of pAT.

Materials and methods

Materials

rAT was prepared as described previously. Briefly, rAT secreted into a serum-free media was purified by successive chromatography on heparin-affinity, hydroxyapatite, anion-exchange and hydrophobic interaction columns using alkaline buffers (pH 8.0) [24]. The specific activity of rAT was 7.3 IU/mg and the purity was over 99%. A commercial pAT, Neuart, was obtained from Mitsubishi Pharma Corporation (Osaka, Japan). PNGase F, cellulose cartridges, pyridylamination kit and pyridylaminated (PA)—sugar chain-023 were from Takara Bio (Otsu, Japan). Plasma-derived human thrombin was purchased from Roche Diagnostics (Basel, Switzerland). Lipopolysaccharide (LPS) (*Escherichia coli* O127:B8) was from Difco Laboratories (Detroit, MI, USA). Thromboplastin (thromboplastin C plus) was purchased from Dade Behring (Deerfield, IL, USA). Specific pathogen-free rats were obtained from Keari (Osaka, Japan). The rats were housed under the following conditions; 12-h light—dark cycle, 22 ± 2 °C, and humidity of 50 ± 10%. They were fed a standard diet and water ad libitum. The animal study protocols were approved by the Mitsubishi Pharma Corporation Animal Care and Use Committee.

Methods

N-linked oligosaccharide analysis

To release N-linked oligosaccharides, 0.25 mg of rAT was digested at 37 °C for 16 h with 1 mU of PNGase F in 0.2 M Tris—HCl, pH 8.6, 0.5% 2-mercaptoethanol, 0.2% sodium dodecyl sulfate, 1% Nonidet P-40. The complete conversion of rAT to the 49 kDa core