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Interactions between depolymerized fucosylated glycosaminoglycan and coagulation proteases or inhibitors

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ABSTRACT

Fucosylated glycosaminoglycan (FG) is a structurally novel glycosaminoglycan derivative, and it has potent anticoagulant activity. Depolymerized FG (dFG) is a selective factor Xase (FXase, FIXa-FVIIIa complex) inhibitor and it has antithrombotic action without major bleeding risks. In this study, we report the effects of dFG-3 (Mw ~14 kDa) on the catalysis rates of factor IIa (FIIa), factor Xa (FXa) and factor IXa (FIXa) inhibition by antithrombin (AT), and the kinetic of the interactions between coagulation proteases or inhibitors and dFG-3 were also studied using biolayer interferometry (BLI) technology. We found that dFG-3 had much weaker catalysis activity of coagulation proteases inhibition by AT compared with heparin (UFH). The binding affinity of AT bound to dFG-3 was lower than UFH, and the UFH-AT interaction fitted well with biphasic-binding model while dFG-3-AT interaction was monophasic-binding, suggesting dFG-3 might not have allosteric activation effect on AT. The results are consistent with AT-independent inhibitory activities of dFG-3. dFG-3 could strongly bind to FIXa with much higher affinity than UFH, further explained the reason for its potent FXase inhibitory activity. Additionally, the binding ability of dFG-3 and FIXa decreased with decreasing molecular, and the fucose side chains and carboxyl groups of dFG-3 might be required for its high affinity binding with FIXa. Our data supports further the investigation of dFG-3 as a promising anticoagulant drug inhibiting the intrinsic FXase by binding to FIXa.

1. Introduction

Venous thromboembolism is the third leading cause of cardiovascular-associated death, and anticoagulants are used to treat a wide variety of conditions that involve arterial or venous thrombosis [1]. Unfractionated heparin (UFH) and low molecular weight heparins (LMWHs) have been the clinical cornerstones of antithrombotic treatment and prophylaxis for the past 70 years, but the risk of hemorrhagic complications is still a major concern with their use [2,3]. UFH has multiple potential anticoagulant mechanisms, mainly antithrombin (AT) dependent inhibition of coagulation proteases by conformational activation of serpin and template mechanisms [4,5]. In recent years, a series of studies has shown that inhibitors of the intrinsic coagulation pathway could prevent thrombosis with negligible bleeding risks [1,6–8].

Fucosylated glycosaminoglycan (FG) is a glycosaminoglycan derivate with chondroitin sulfate like backbones and fucose side chains from marine sea cucumber [9]. FG has potent anticoagulant and antithrombotic activities [9], and the mechanisms include: inhibition of factor Xa (FXa) generation by the intrinsic tenase complex, heparin cofactor II (HCII) dependent inhibition of thrombin (FIIa), AT dependent inhibition of FIIa, inhibition of factor VIII (FVIII) activation by FIIa [9–13]. Depolymerized FG (dFG) has been demonstrated to inhibit plasma thrombin generation primarily by reducing factor X activation [14], and a depolymerized FG has been found to exert an antithrombotic effect with less bleeding than UFH and LMWH in rats and dogs [15,16]. Recently, studies in our group also showed that depolymerized FG retained the anticoagulant activity and could inhibit venous thrombosis without causing side effects [13,17,18]. Thus, for the depolymerized FG

Abbreviations: FG, fucosylated glycosaminoglycan; dFG, depolymerized fucosylated glycosaminoglycan; UFH, unfractionated heparin; LMWH, low molecular weight heparin; FXase, factor Xase; AT, antithrombin; FIIa, thrombin; FXa, factor Xa; FIXa, factor IXa; BLI, biolayer interferometry; HCII, heparin cofactor II; FVIII, factor VIII; Mw, molecular weight; Fpx, fondaparinux; FXIa, factor XIa; k_1, pseudo-first order rate constant; k_2, second-order rate constant.

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as a promising antithrombotic candidate drug, it is very valuable to study its anticoagulant mechanism related to its low bleeding tendency.

In our previous study, we found that dFG had significantly reduced AT-dependent anti-FIIa activity, but its FXase inhibition activity and HCII-dependent anti-FIIa activity remain strong potency [13,19]. The anticoagulant mechanism of dFG was obviously different from that of UFH. Thus, further studies should be needed to elucidate the differences between dFG and UFH. In this study, we further investigated the interactions between coagulation proteases or inhibitors (AT, HCII) and a depolymerized FG (dFG-3, Mw ~14 kDa) and compared with UFH. We firstly reported the effect of dFG-3 on the second-order rate constants of proteases (FIIa, FXa, FIXa) inhibition by AT. And then, the kinetic and structural features of the interactions between coagulation proteases or inhibitors and dFG-3 were studied using biolayer interferometry (BLI) technology, and competitive binding assays were performed to elucidate the effects of molecular weight and chemical modifications on the interaction between dFG-3 and FIXa.

2. Methods

2.1. Materials

UFH (212 USP U/mg) were purchased from Sigma (USA). LMWH (Enoxaparin, 0.4 mL × 4000 AXaIU) was from Sanofi-Aventis (France). Fondaparinux sodium (Fpx) was from GSK (UK). Human HCII, AT, thrombin, human factor IXa, human factor Xa, thrombin chromogenic substrate CS-01(38), factor IXa chromogenic substrate CS-51(09) and Heparin Anti-Xa kits were all from Hyphen Biomed (France). Human factor XIa was from Assaypro (USA). EZ-Link Amine-PEG_3-Biotin and Zeba Spin desalting columns (>7 kDa) were purchased from Thermo Scientific (USA). SA and SSA biosensors were purchased from Fortebio (USA). All other chemicals were of reagent grade and obtained commercially.

2.2. Fucosylated glycosaminoglycan

The native FG was extracted and purified from the body walls of the sea cucumber *Thelenota ananas* as previously described [20]. Depolymerized FGs (dFG-1, dFG-2, dFG-3, dFG-4, dFG-5, dFG-6) were obtained by controlled chemical depolymerization [19]. Carboxyl-reduced dFG-3 (dFG-a), carboxylic ethyl ester of dFG-3 (dFG-b), carboxylic benzyl ester of dFG-3 (dFG-c), carboxylic 1-butenyl ester of dFG-3 (dFG-d), partially deacetylated dFG-3 (dFG-e), and partially defucosylated FG (dFG-f) were developed as previously described [13].

2.3. Effect of glycosaminoglycans on the rates of FIIa, FXa and FIXa inhibition by AT

Reactions of AT and coagulation proteases (FIIa, FXa, FIXa) were measured in a discontinuous assay under pseudo-first order rate conditions at room temperature in TS/PEG buffer (0.02 M Tris-HCl, pH 7.4, 0.15 M NaCl, 0.1% PEG8000, 2 mM $CaCl_2$) [21,22]. The concentrations of AT ranging from 20 to 8000 nM and were at least ten fold higher than that of protease (2 to 40 nM). AT and protease were incubated in a 20 μL reaction volume in a 96-well plate at various time intervals, then 80 μL chromogenic substrate (1 mg/mL, containing 1 mg/mL Polybrene) were added simultaneously. Additionally, 30% ethylene glycol was contained for the chromogenic substrate of FIXa. The absorbance at 405 nm was measured for 5–30 min to determine the rate of substrate cleavage (V). The pseudo-first order rate constant (k_1) was determined by plotting ln (V/V_0) versus time, where V_0 is the rate of substrate cleavage by the protease in the absence of AT. The apparent second-order inhibition rate (k_2) was determined by dividing k_1 by the AT concentration.

To determine the effect of glycosaminoglycans (dFG, UFH, Fpx) on the k_2 of FIIa, FXa or FIXa inhibition by AT, various concentrations of glycosaminoglycans were premixed with AT before incubation with protease.

2.4. Biotinylation of dFG-3 or UFH

dFG-3 or UFH was biotinylated by using Amine-PEG_3-biotin as previously described [23,24]. And the reaction mixture was desalted with the Zeba Spin desalting columns. Then the biotinylated compounds were immobilized onto the surface of SA or SSA biosensors.

2.5. BLI kinetic measurements of coagulation proteases or inhibitors binding to immobilized dFG-3 and UFH

Increasing concentrations of coagulation proteases (FIIa, FIXa, FXIa) or inhibitors (AT, HCII) were allowed to interact with immobilized dFG-3 and UFH. All interaction experiments were conducted at 30 °C in PBSB (0.05 M sodium phosphate at pH 7.2, 0.15 M NaCl, 0.1% BSA) or HEPES buffer (0.15 M NaCl, 20 mM HEPES, pH 7.4, 2 mM $CaCl_2$, and 0.05% Tween 20) using an Octet Red 96 instrument (Fortebio, USA). Final volume for all the solutions was 200 μL. Assays were performed in black solid 96-well flat bottom plates with agitation set to 1000 r/min. A 600–900 s biosensor washing step was applied prior to the analysis of the association of the ligand on the biosensor to the analyte in solution for 600–1200 s. Finally the dissociation was followed for 900–1800 s. Dissociation wells were used only once to ensure buffer potency. After dissociation, the sensor surface was regenerated in 4 M NaCl or 2 M NaCl in HEPES buffer. Correction of any systematic baseline drift was done by subtracting the shift recorded for a sensor loaded with ligand but incubated with no analyte.

2.6. Solution competition BLI study

To assess the relative ability of soluble UFH or dFG-3 to compete with the immobilized dFG-3 or UFH for binding to coagulation proteases or inhibitors, a competition binding assay was performed to determine their respective EC_{50} values. AT (1000 nM), HCII (500 nM) or FIXa (50 nM) was preincubated with increasing concentrations of UFH or dFG-3 prior to interact with immobilized dFG-3. After each run, dissociation and regeneration were performed as described above.

To determine the structure-activity relationship of dFG-3-FIXa interaction, gradient concentrations of dFG-3 derivatives were preincubated with FIXa (50 nM) prior to interact with immobilized dFG-3. Association, dissociation and regeneration process were performed as described above.

2.7. Date analysis

For BLI kinetic assays, data were analyzed using the Octet software version 7.0 and the binding curves were globally fitted using a 1:1 or 2:1 model [25]. For the competition assays, the response at the end of the association step for each compound concentration was plotted as the relative proportion of remaining free AT, HCII or FIXa, with response of AT, HCII or FIXa alone (no compounds) normalized to 1. The EC_{50} was determined by fitting the data to the following equation using the Origin 8.0 software (OriginLab, USA):

$$B = \frac{{EC_{50}}^n}{{EC_{50}}^n + [I]^n} \quad (1)$$

Where B represents the fractional specific binding, [I] represents the concentration of compounds used as a competitor, EC_{50} represents the concentration of compounds that causes a 50% reduction in the BLI response, and n represents the pseudo-Hill coefficient [26,27].