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Effect of Depolymerized Holothurian Glycosaminoglycan (DHG) on the Activation of Factor VIII and Factor V by Thrombin

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Our previous study has shown that depolymerized holothurian glycosaminoglycan (DHG) has two different inhibitory activities in the blood coagulation cascade: heparin cofactor II-dependent thrombin inhibition; and antithrombin III- and heparin cofactor II-independent inhibition of the intrinsic factor Xase complex [Nagase *et al.* (1995) *Blood* 85, 1527-1534]. In the present study, the effect of DHG on the activation of factor VIII and factor V by thrombin was examined with purified human components. DHG inhibited the activation of factor VIII by thrombin at concentrations exceeding 80 nM, but not the activation of factor V by thrombin at concentrations of up to 8 μM. On Western blot analysis, DHG inhibited the cleavage of factor VIII light chain at concentrations exceeding 0.8 μM. The interaction between DHG and factors VIII and V and thrombin was examined with a DHG-cellulofine column. DHG had strong affinity for factor V and thrombin, but slight affinity for factor VIII. The interaction of DHG with thrombin was analyzed, using fluorescein isothiocyanate-labeled DHG. One mole of DHG bound 2 mol of thrombin, with a dissociation constant (K_d) of 3.04×10^{-6} M. These results suggest that DHG interferes with the interaction between thrombin and factor VIII, probably by making a binary complex through the anionic binding exosite II of thrombin.

Key words: anticoagulant, factor VIII, glycosaminoglycan, holothurian, thrombin.

DHG is a depolymerized glycosaminoglycan extracted from the sea cucumber, *Stichopus japonicus* Selenka. We have demonstrated that DHG has antithrombotic and anticoagulant activities distinct from those of heparin (*1, 2*). In one of these studies (*2*), we showed that DHG had two different inhibitory activities in the blood coagulation cascade: HCII-dependent inhibition of thrombin; and ATIII- and HCII-independent inhibition of factor X activation by factor IXa-factor VIIIa complex. The anticoagulant effect of DHG was very similar to that of PPS, which has been shown to inhibit thrombin HCII-dependently and to prolong APTT, ATIII- and HCII-independently (*3, 4*). Wagenvoord *et al.* (*5*) have reported that PPS inhibits both factor VIIIa formation (activation of factor VIII by thrombin) and the function of factor VIIIa, probably by the formation of a factor VIII(a)-PPS complex. Recently, heparin has been reported to inhibit the thrombin-catalyzed activation of factor VIII and also the activation of factor X by the intrinsic factor Xase in the absence of ATIII and HCII (*6*). Based on these published reports, we were prompted to further characterize the anticoagulant activity of DHG and to investigate the effect of DHG on the activation of factor VIII and factor V by thrombin in the absence of ATIII and HCII. In this study, we present evidence that DHG inhibits the activation of factor VIII by thrombin, making a binary complex with thrombin. The mechanism of the inhibition is discussed and compared with that of heparin.

MATERIALS AND METHODS

Materials—Fluorogenic substrate, Boc-Val-Pro-Arg-MCA, was purchased from the Peptide Institute (Osaka). Cephalin® from bovine brain was purchased from Sigma Chemical (St. Louis, MO, USA) and dissolved according to the manufacturer's instructions. BSA was purchased from Sigma Chemical. DHG, a sulfated fucose-branched chondroitin 4,6-sulfate with an average molecular weight of 12,500, was prepared by depolymerization of the extracts of *S. japonicus* Selenka (*7*). Human thrombin, human factor V, and recombinant human factor VIII (Kogenate®) were purchased from Sigma Chemical, Diagnostica Stago (Asnieres, France), and Bayer (Bayerwerk, Germany), respectively. Thrombin gave a single band with a molecular weight of 37,000 on SDS-PAGE, and its 13 amino-terminal amino acid residues were identical with those of a heavy chain and a light chain of α-thrombin, as determined by gas phase sequencer. Plasma-derived human factor VIII-vWF

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Abbreviations: AMC, 7-amino-methylcoumarin; ATIII, antithrombin III; APTT, activated partial thromboplastin time; Boc, *t*-butoxycarbonyl; BSA, bovine serum albumin; DHG, depolymerized holothurian glycosaminoglycan; FITC, fluorescein isothiocyanate; HCII, heparin cofactor II; MCA, methylcoumarin-7-amide; PPS, pentosan polysulfate; PT, prothrombin time; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate; TBS, Tris-buffered saline; vWF, von Willebrand factor.

complex was kindly supplied by The Chemo-Sera-Therapeutic Foundation (Kumamoto). Fibrinogen in this preparation was removed by using a heparin cellulofine column (Chisso). APTT reagent (Actin®) and PT reagent (activated thromboplastin) were purchased from American Dade (Aguada, Puerto Rico) and International Reagents (Kobe), respectively. Factor VIII-deficient plasma and factor V-deficient plasma were purchased from George King Bio-Medical (Overland Park, KS, USA). FITC was purchased from Wako Chemical Industries (Osaka). The molar concentrations of human factors VIII and V and thrombin were calculated from concentration units, using their specific activities and molecular weights (*8-10*).

Activation of Factor VIII and Factor V by Thrombin—Recombinant factor VIII (41 nM), plasma-derived factor VIII (10 nM), or factor V (140 nM) was activated with thrombin (1.5 nM) in the absence or presence of various amounts of DHG in TBS-BSA (20 mM Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl, 0.1 mg BSA/ml, and 0.05% NaN_3) at 37°C. At various intervals, a 10-μl sample was diluted in TBS-BSA at 1:100. The diluted sample (10 μl) containing factor VIIIa or factor Va was added to a mixture of factor VIII-deficient plasma (100 μl) and APTT reagent (100 μl) or to a mixture of factor V-deficient plasma (100 μl) and PT reagent (100 μl), respectively. After 2 min of incubation at 37°C, we measured clotting time using a coagulometer (Amelung KC1, Lehbrinksweg, Germany), by adding 100 μl of 25 mM $CaCl_2$. A calibration curve was established with normal human plasma.

SDS-PAGE Analysis of Factor V Activation by Thrombin—Factor V (140 nM) was activated with thrombin (1.5 nM) for 30 s at 37°C in the absence or presence of various amounts of DHG. Fifty microliters of the mixture was added to 50 μl of 0.125 M Tris-HCl buffer, pH 6.8, containing 4% SDS, 30% glycerol, and 0.005% Bromophenol blue, and the mixture was incubated for 10 min at 95°C. Samples of 20 μl were subjected to SDS-PAGE with gradient gels (2-15%) and the buffer system of Laemmli (*11*). Following electrophoresis, proteins were visualized by staining the gels with silver staining reagent (*12*). Molecular weight markers were purchased from Daiichi Pure Chemicals, Tokyo; myosin (200 kDa), β galactosidase (116 kDa), bovine serum albumin (66 kDa), and aldolase (42 kDa).

Western Blot Analysis of Factor VIII Activation by Thrombin—Recombinant factor VIII (41 nM) was activated with thrombin (1.5 nM) for 30 s at 37°C in the absence or presence of various amounts of DHG. Fifty microliters of the mixture was mixed with 50 μl of the sample buffer for SDS-PAGE, as described above, and the mixture was incubated for 10 min at 95°C. Samples of 20 μl were subjected to SDS-PAGE with gradient gels (4-20%). Following electrophoresis, the gels were overlaid with a presoaked nitrocellulose membrane and electroeluted in a Bio-Rad Trans Blot cell at 50 V for 20 h in 25 mM Tris-HCl buffer, pH 8.3, containing 192 mM glycine and 20% (v/v) methanol. The nitrocellulose membrane was then removed and soaked in blocking buffer (Block Ace, Snow Brand Milk Products, Tokyo) for 6 h at 4°C, then incubated with anti-factor VIII C2 domain monoclonal antibody (NMC-VIII/5, 20 μg/ml) (*13*) for 12 h at 4°C. Following a 1-h wash in washing buffer (20 mM Tris-HCl buffer, pH 8.0, containing 0.5 M NaCl and 0.05% Tween 20), the membrane was incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad Laboratories, Tokyo) for 1 h. The final washing procedure consisted of three changes of washing buffer. Horseradish peroxidase was detected with an enhanced chemiluminescence (ECL) Western blotting detection kit (Amersham, Buckinghamshire, England). The membrane was incubated in ECL solution for 1 min and exposed to X-ray film for 30 min.

Analysis of Affinities of Factors VIII and V and Thrombin to DHG-Cellulofine Column—Recombinant factor VIII (5 μg), factor V (13 μg), or thrombin (6 μg) was applied to a DHG-cellulofine (Chisso, Chiba) column (0.8× 7.5 cm) in 20 mM Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl, 5 mM $CaCl_2$, 0.01% Tween 80, and 0.05% NaN_3 at room temperature. The columns were washed free of unbound proteins with the same buffer. Elution was accomplished with a 60-ml linear NaCl gradient (0.15-1 M). One milliliter fractions were collected at a flow rate of 1 ml/min at 4°C. The activities of factor VIII and factor V of each fraction were measured with fluorogenic APTT in factor VIII-deficient plasma and with fluorogenic PT in factor V-deficient plasma, respectively, using a fluorogenic thrombin substrate, Boc-Val-Pro-Arg-MCA. In these experiments, the samples from each fraction were diluted more than 100-fold to eliminate the effect of high salt concentrations. Thrombin activity was measured using the fluorogenic thrombin substrate. AMC liberated was measured, as described previously (*14*), with a centrifugal autoanalyzer (Cobas Bio, Roche Products, Welwyn, England) equipped with a fluorescence unit. In the control experiment, an acetylated amino cellulofine (Chisso, Chiba) column was used instead of the DHG-column.

Analysis of the Interaction of DHG with Thrombin—DHG was labeled with FITC by the method of Nagasawa and Uchiyama (*15*). Excess FITC in the mixture was removed by gel-filtration with a Sephadex G-25 (Pharmacia Biotech, Uppsala, Sweden) column (3×30 cm) equilibrated with 0.1 M sodium phosphate buffer, pH 6.2, containing 1 M NaCl. The passed-through fraction was dialyzed against distilled water and lyophilized. The preparation was then dissolved in 0.05 M sodium phosphate buffer, pH 6.2, containing 3 M ammonium sulfate, and applied to a HiTrap Phenyl Sepharose HP column (bed volume: 5 ml, Pharmacia Biotech) equilibrated with the same buffer. FITC-DHG was separated from non-labeled DHG by elution with a 30-ml linear ammonium sulfate gradient (3-0 M). The FITC/DHG ratio of the purified conjugates was found to be approximately 0.7 : 1. Measurement of the fluorescence intensity was performed with a fluorescence spectrophotometer (model F-450; Hitachi, Tokyo). The interaction of FITC-DHG with thrombin was examined by monitoring the enhanced fluorescence (ΔF) of the FITC-DHG as a function of thrombin concentration at excitation and emission wavelengths of 494 and 505 nm, respectively. The competitive binding of FITC-DHG and nonlabeled DHG to thrombin was measured by monitoring ΔF after the addition of thrombin (2.5 μM) to FITC-DHG or nonlabeled DHG. The stoichiometry of the interaction was determined by adding various concentrations of thrombin, ranging from 2.5×10^{-7} to 4×10^{-5} M. The dissociation constant (K_d) for the thrombin-DHG complex was determined by adding various concentrations of thrombin to 1.3×10^{-7} M FITC-DHG. Since the change of the fluorescence intensity of FITC-DHG upon the binding of thrombin

was mono-phasic and the binding stoichiometry was calculated to be 1:2 (DHG:thrombin) as described in "RESULTS," the K_d was estimated by nonlinear least squares fit of the data to the equation, as described by Jordan *et al.* (*16*):

$$\frac{\Delta F}{\Delta F_{max}} = \frac{K_d + (D) + (T)}{2(D)} - \sqrt{\left[\frac{K_d + (D) + (T)}{2(D)}\right]^2 - \frac{(T)}{(D)}}$$

where (D) and (T) are the total concentrations of DHG (total concentrations of binding sites for thrombin) and thrombin, respectively, and ΔF_{max} and K_d are the fluorescence change of the FITC-DHG saturated with thrombin and the dissociation constant of the complex, respectively.

RESULTS

Effect of DHG on the Activation of Factor VIII and Factor V by Thrombin—Figure 1 shows the time course of the activation of recombinant factor VIII (A), plasma-derived factor VIII (B), and factor V (C) by thrombin in the absence or presence of various amounts of DHG. In these experiments, a mixture containing DHG and factor VIII or factor V was diluted 1:1,100 to eliminate the effect of DHG on the assay of factor VIII or factor V. DHG retarded the activation of recombinant factor VIII by thrombin at concentrations exceeding 80 nM. At concentrations of 0.8 to 8 μM, DHG retarded both the initial rate of factor VIII activation and the rate of the inactivation of factor VIII (Fig. 1A). The inhibition of factor VIII activation by DHG was also observed when the plasma-derived factor VIII, or factor VIII-vWF complex, was used instead of the recombinant factor VIII (Fig. 1B). This effect of DHG on factor VIII was very similar to that of heparin (*6, 17*). On the other hand, the effect of DHG in inhibiting the activation of factor V by thrombin was negligible at concentrations of up to 8 μM (Fig. 1C).

Effect of DHG on the Cleavage of Factor VIII by Thrombin—We investigated the effect of DHG on the thrombin-catalyzed cleavage of factor VIII by Western blot analysis, using an anti-factor VIII C2 domain monoclonal antibody (Fig. 2). The recombinant factor VIII used was a heterodimer of a heavy chain (A1-A2-B) and a light chain. The activation of factor VIII by thrombin is associated with cleavage at Arg^{1689} in the light chain to produce A3-C1-C2 (*18, 19*). This cleavage is necessary for the dissociation of factor VIII from vWF (*20*). In the absence of DHG, the light chain of factor VIII was cleaved partially by 1.5 nM thrombin (Fig. 2, lane 2) and completely by 15 nM thrombin (Fig. 2, lane 9). DHG obviously inhibited the cleavage of factor VIII light chain at concentrations exceeding 0.8 μM. This result was consistent with the results obtained from the measurement of factor VIII coagulant activity, as described above. These results indicate that DHG inhibited the cleavage of Arg^{1689} in the light chain by thrombin. Since the monoclonal antibody used in this experiment recognized factor VIII light chain, the effect of DHG on the cleavage of Arg^{376} and Arg^{740} in the heavy chain of factor VIII was not examined.

Effect of DHG on the Cleavage of Factor V by Thrombin—We examined the effect of DHG on the thrombin-catalyzed cleavage of factor V by SDS-PAGE, followed by silver staining of the proteins, as described under "MATERIALS AND METHODS" (Fig. 3). It has been established that human thrombin cleaves human factor V (A1-A2-B-A3-C1-C2) at the carboxyl terminals of Arg^{709}, Arg^{1018}, and Arg^{1545}, generating a heavy chain (A1-A2) and a light chain (A3-C1-C2) of factor Va (*21*). As shown in Fig. 3 (lanes 2 and 10), human thrombin cleaved human factor V into three fragments (210, 110, and 73/76 kDa) after 30 s of incubation and into two fragments (110 and 73/76 kDa) after 10 min of incubation. Factor V in lane 1 was faintly stained, whereas factor V fragments were strongly stained. These results indicate that the fragments of 110 and 73/76 kDa were the heavy chain and the light chain of factor Va, generated by the cleavage of Arg^{709} and Arg^{1545}, respectively. The result also indicates that a fragment with molecular

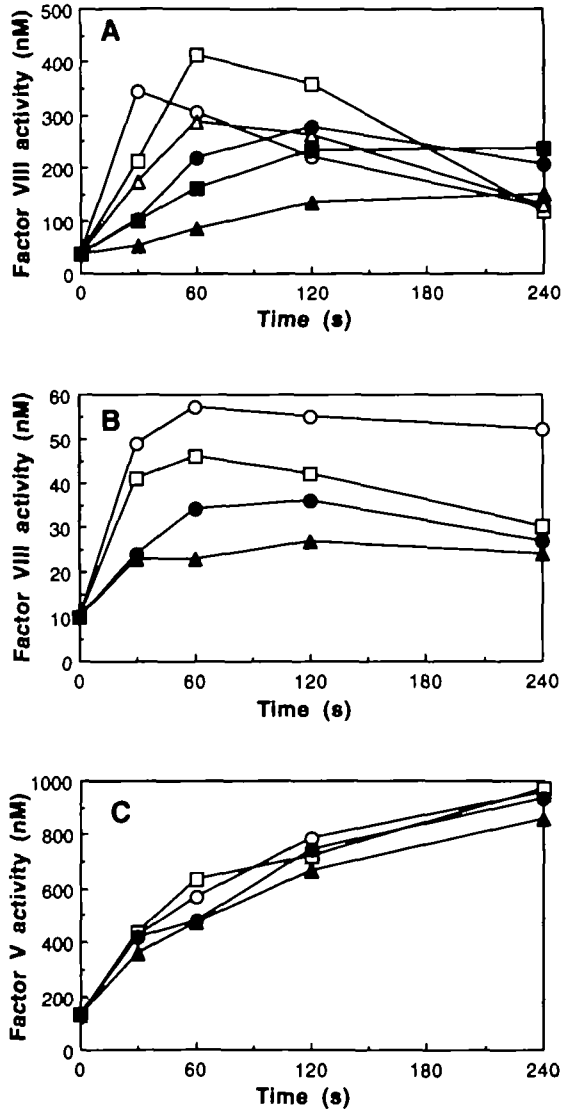

Fig. 1. **Effect of DHG on the activation of recombinant factor VIII (A), plasma-derived factor VIII (B), and factor V (C) by thrombin.** Recombinant factor VIII (41 nM), plasma-derived factor VIII (10 nM), or factor V (140 nM) was activated with thrombin (1.5 nM) in the presence of DHG. At various time intervals, samples were diluted, and factor VIII and factor V activities were measured as described under "MATERIALS AND METHODS." ○, in the absence of DHG; □, DHG (80 nM); △, DHG (240 nM); ●, DHG (800 nM); ■, DHG (2.4 μM); ▲, DHG (8 μM).

mass of 210 kDa was generated by the cleavage of Arg^{1018}, and that this consisted of a part of the B domain and A3-C1-C2. With increases in the amount of DHG, generation of the light chain was inhibited, but generation of the 210-kDa fragment and the heavy chain was not significantly inhibited, as shown in Fig. 3 (lanes 3-9). These results indicate that DHG did not inhibit the cleavage of Arg^{709} and Arg^{1018}, but that it inhibited further cleavage of Arg^{1545} by the action of thrombin. As described in the previous section, DHG did not inhibit the activation of factor V in terms of the measurement of factor Va cofactor activity. Therefore, our results demonstrate that the generation of factor Va activity was not inhibited by DHG despite the inhibition of the generation of light chain. This result is consistent with the observation of Monkovic and Tracy (*22*) and with the recently published report of Keller *et al.* (*23*) that the cleavage at Arg^{1018} was sufficient for the expression of factor Va cofactor activity.

Affinity of Factors VIII and V and Thrombin to DHG-Cellulofine—Recombinant factor VIII, factor V, and thrombin were applied, respectively, to a DHG-cellulofine column to examine the interaction with DHG (Fig. 4). They did not bind to a control column (acetylated amino cellulofine) at a physiological salt concentration (0.15 M NaCl), as indicated by the open circles in Fig. 4. Thrombin and factor V bound to DHG-cellulofine under the same conditions and were eluted at 0.37 and 0.55 M NaCl, respectively (Fig. 4, A and C). Recombinant factor VIII did not bind to DHG-cellulofine, but it had slight affinity for DHG, as shown by the peak of the passed-through fraction being broader than that of the control column (Fig. 4B). These results indicate that DHG has strong affinity for factor V and thrombin but slight affinity for recombinant factor VIII.

Interaction of DHG with Thrombin—DHG was labeled

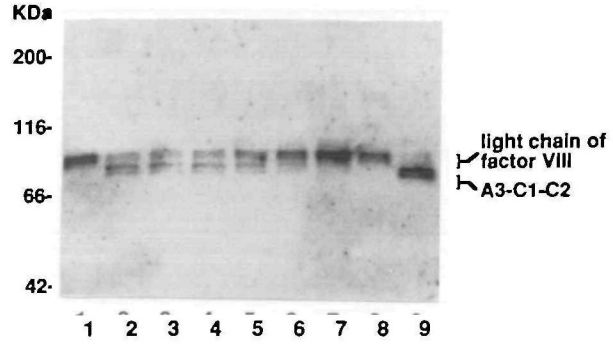

Fig. 2. **Effect of DHG on the cleavage of the factor VIII light chain by thrombin.** Recombinant factor VIII (41 nM) was activated with thrombin (1.5 nM) for 30 s at 37°C in the absence or presence of various amounts of DHG. Samples were then prepared for SDS-PAGE, followed by Western blotting as described under "MATERIALS AND METHODS." Lane 1, factor VIII only; lane 2, in the absence of DHG; lane 3, DHG (8 nM), lane 4, DHG (80 nM); lane 5, DHG (240 nM); lane 6, DHG (800 nM), lane 7, DHG (2.4 μM); lane 8, DHG (8 μM); lane 9, factor VIII (41 nM) was activated with thrombin (15 nM) for 10 min in the absence of DHG

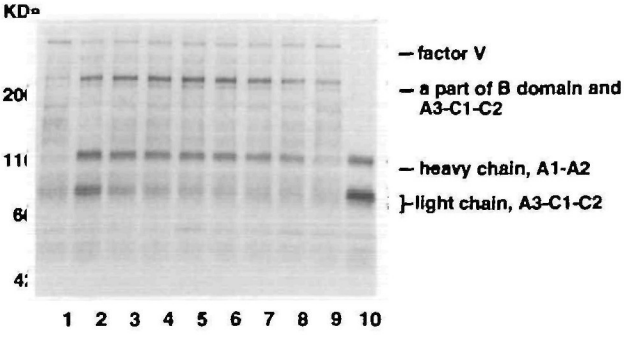

Fig. 3. **Effect of DHG on the cleavage of factor V by thrombin.** Factor V (140 nM) was activated with thrombin (1 5 nM) for 30 s at 37°C in the absence or presence of various amounts of DHG. Samples were then prepared for SDS-PAGE, followed by silver staining, as described under "MATERIALS AND METHODS." Lane 1, factor V only; lane 2, absence of DHG; lane 3, DHG (80 nM); lane 4, DHG (240 nM); lane 5, DHG (800 nM); lane 6, DHG (2.4 μM); lane 7, DHG (8 μM); lane 8, DHG (24 μM); lane 9, DHG (80 μM); lane 10, factor V (140 nM) was activated with thrombin (15 nM) for 10 min in the absence of DHG.

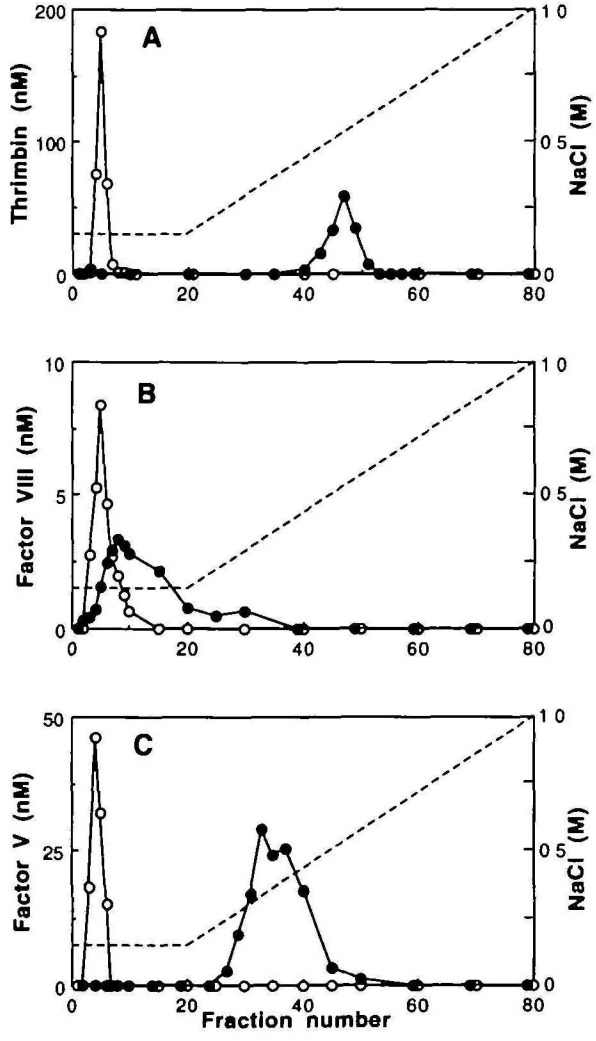

Fig 4 **Elution profiles of thrombin (A), recombinant factor VIII (B), or factor V (C) from DHG-cellulofine column.** Thrombin (6 μg), factor VIII (5 μg), or factor V (13 μg) was applied to the DHG-cellulofine column or acetylated amino cellulofine column and eluted with a 60-ml linear NaCl gradient. The activities of thrombin, factor VIII, and factor V of each fraction were measured, as described under "MATERIALS AND METHODS." ●, DHG-cellulofine; ○, acetylated amino cellulofine; · · · , NaCl concentration.

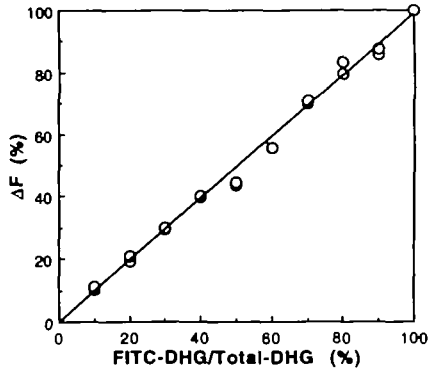

Fig. 5. **Competitive binding of FITC-DHG and non-labeled DHG to thrombin, monitored by fluorescence spectroscopy.** FITC-DHG and/or non-labeled DHG (total 0.13 μM) was added to thrombin (2.5 μM) in TBS buffer (20 mM Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl and 0.05% NaN_3) at 25°C. The extent of fluorescence enhancement of FITC-DHG, ΔF, was determined as described under "MATERIALS AND METHODS." Fluorescence enhancement (ΔF) was plotted against the ratio of FITC-DHG to total DHG. —, linear least squares fit of the data.

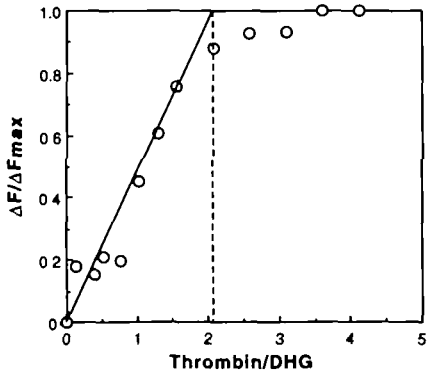

Fig. 6. **Stoichiometry for the interaction of thrombin and DHG.** FITC-DHG (9.7 μM) was added to various concentrations of thrombin in TBS buffer at 25°C. The extent of fluorescence enhancement of FITC-DHG, ΔF, was determined as described under "MATERIALS AND METHODS." The values were normalized to the observed maximal increase ($\Delta F/\Delta F_{max}$) and plotted against the molar ratio of thrombin to FITC-DHG.

with FITC to monitor the interaction with thrombin, as described under "MATERIALS AND METHODS." To demonstrate that the labeling procedure did not affect the binding activity of DHG, we measured the competitive binding of FITC-DHG with nonlabeled DHG to thrombin. As shown in Fig. 5, the change in fluorescence was proportional to the percentage of FITC-DHG to total DHG, that is, 50% substitution of FITC-DHG with nonlabeled DHG caused a 50% change in fluorescence. This result indicates that the affinity of FITC-DHG for thrombin was the same as that of nonlabeled DHG. Moreover, APTT prolongation by FITC-DHG was almost the same as that brought about by nonlabeled DHG (data not shown). These results suggest that the functional activity of DHG was not changed by the labeling procedure. Figure 6 shows the stoichiometry for the interaction of DHG and thrombin. The ratio of thrombin to DHG was calculated to be 2.1 to 1, indicating that 1 mol of DHG bound 2 mol of thrombin. The dissociation constant for the thrombin-DHG complex was determined

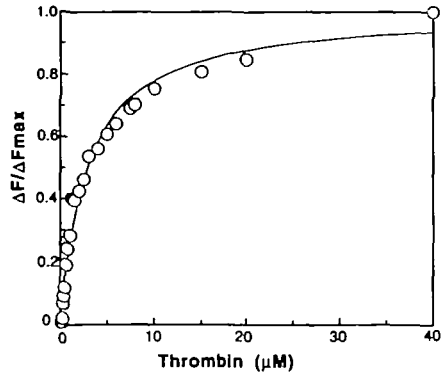

Fig. 7. **The binding of FITC-DHG to thrombin.** FITC-DHG (0.13 μM) was added to various concentrations of thrombin in TBS buffer at 25°C. The extent of fluorescence enhancement of FITC-DHG, ΔF, was determined as described under "MATERIALS AND METHODS." The values were normalized to the observed maximal increase ($\Delta F/\Delta F_{max}$) and plotted against the concentration of thrombin added. —, nonlinear least squares computer fit of the data to the equation, as described under "MATERIALS AND METHODS."

by adding various concentrations of thrombin to a fixed amount of FITC-DHG (Fig. 7) and estimated by nonlinear squares fit of the data to the equation as described in "MATERIALS AND METHODS." The best statistical fit was achieved by employing the value $K_d = 3.04 \times 10^{-6}$ M, assuming that 2 mol of thrombin bound to 1 mol of DHG.

DISCUSSION

The major effect of glycosaminoglycans such as heparin and dermatan sulfate as antithrombotic agents has been established: namely, they accelerate the inactivation of thrombin by ATIII or HCII, forming a ternary complex through their specific interaction (*24*). However, recent studies have demonstrated that heparin also has ATIII-independent inhibitory activity toward the activation of factor X by the factor IXa-factor VIIIa complex (*25*) and toward the activation of factor VIII by thrombin (*6, 17*). In our previous study, we demonstrated that a glycosaminoglycan from the sea cucumber, DHG, had inhibitory activity toward the activation of factor X by the factor IXa-factor VIIIa complex, in addition to showing HCII-dependent inhibition of thrombin (*2*). The intrinsic factor Xase inhibition exerted by DHG was more potent than that of heparin and dermatan sulfate (*2, 25*). In the present study, we have presented evidence that DHG has inhibitory activity toward the activation of factor VIII by thrombin. The effect was confirmed by the measurement of factor VIIIa activity and by monitoring the limited cleavage of factor VIII on SDS-PAGE. The generation of factor VIIIa activity from factor VIII and the cleavage of factor VIII light chain by thrombin were inhibited with increasing amounts of DHG. Although the effect of DHG on the cleavage of factor VIII heavy chain was not examined, the results indicated that DHG had the inhibitory activity toward factor VIII activation. Therefore, together with the previous findings (*2*), the present paper indicates that DHG has the three different inhibitory activities toward blood coagulation cascade reactions: (a) inhibition of factor VIII activation by thrombin, (b) inhibition of factor X activation by factor IXa-factor VIIIa complex, and (c) the HCII-dependent inhibi-

tion of thrombin activity.

The interaction of DHG with thrombin was examined qualitatively with a DHG-cellulofine column and quantitatively by monitoring the change of the fluorescence intensity of FITC-DHG. The results indicated that 2 mol of thrombin bound 1 mol of DHG, with a K_d of 3.04×10^{-6} M. Heparin has been shown to bind with an anionic binding exosite II of thrombin, with a K_d of $6\text{-}10 \times 10^{-6}$ M (*26-29*). The oligosaccharide chain of a 3-disaccharide moiety of heparin has been shown to behave equivalently (*29*). Therefore, we conclude that DHG, with an apparent molecular weight of 12,500, bound 2 mol of thrombin through its anionic binding exosite II with affinity equal to that of heparin. The affinity of factor VIII toward DHG-cellulofine was very weak, as has been demonstrated with heparin-Sepharose (*6*). DHG did not inhibit the activity of thrombin to hydrolyze synthetic substrate (*2*). Based on these findings, we speculate that the formation of a binary complex of DHG with thrombin interferes with the interaction of an active site of thrombin with a specific cleavage site of factor VIII. vWF did not affect the inhibitory activity of DHG toward the activation of factor VIII by thrombin, consistent with the results reported by Barrow *et al.* (*6*), who showed that vWF did not affect the heparin-mediated inhibition of the activation of factor VIII by thrombin at physiological concentrations.

In contrast to its effect on the activation of factor VIII by thrombin, DHG did not inhibit the activation of factor V by thrombin. Nonetheless, factor V showed high affinity to DHG-cellulofine and DHG inhibited the cleavage of one of three arginyl peptide bonds of factor V (Arg^{1545}) by thrombin. From these results, we speculate that a ternary complex of thrombin, DHG and factor V was formed, but that the active site of thrombin was accessible to two of three arginyl peptide bonds of factor V (Arg^{709} and Arg^{1018}), leading to the generation of factor Va activity.

The findings of recent reports and of this study show that sulfated polysaccharides have multiple functions in the blood coagulation cascade: first, inhibition of the interaction of the factor IXa-factor VIIIa complex with factor X; second, inhibition of the interaction of thrombin with factor VIII; and third, acceleration of complex formation of ATIII with thrombin, factor Xa, or factor IXa and of HCII with thrombin. Heparin, dermatan sulfate, DHG, and pentosan polysulfate (PPS) affect these three reactions to different degrees. Heparin strongly interacts with ATIII, in contrast to the other three sulfated polysaccharides (*30*). The inhibitory effect of heparin on the thrombin-mediated feedback reaction has been demonstrated to be of crucial importance (*31, 32*). DHG and PPS show characteristic features in their strong interaction with the factor IXa-factor VIIIa complex and with thrombin in the absence of ATIII. On the other hand, the major effect of heparin is thought to be its acceleration of the inactivation of thrombin by ATIII, due to the strong affinity of heparin for ATIII, through a unique pentasaccharide sequence (*33*), and due to the high concentration of ATIII in plasma. These multiple functions of sulfated polysaccharides are presumed to be caused by a binary or ternary complex formed through the specific binding sites of these proteins. It is clear that the different potency of these glycosaminoglycans is due to the different structures of the oligosaccharide unit. The potency of DHG, a sulfated fucose-branched chondroitin sulfate E, is clearly different from that of dermatan sulfate (chondroitin sulfate B) (*2*) and chondroitin sulfate E (*34*), whose major action is the inactivation of thrombin through HCII; these characteristics suggest the important role of the sulfated fucose residue of DHG. The degree of sulfation (mean number of sulfate groups per saccharide) of the polysaccharides is also important for their function. The degree of sulfation of DHG was 1.3, which is higher than those of dermatan sulfate and chondroitin sulfates (*7*). Thus, it is likely that the charge density of the sulfated polysaccharides may be important for the binding of the factor IXa-factor VIIIa complex and thrombin, and for determining the capacity to inhibit intrinsic factor Xase complex and factor VIII activation by thrombin. Although the function of DHG is qualitatively the same as that of PPS *in vitro*, DHG has been shown to be a more promising antithrombotic drug, with minimized hemorrhagic side effects (Kitazato *et al.*, manuscript submitted), than PPS and other sulfated polysaccharides (*35-37*). It remains to be elucidated why DHG, unlike PPS, exerts an antithrombotic effect without producing any significant hemorrhagic effect.

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