Depolymerized Holothurian Glycosaminoglycan With Novel Anticoagulant Actions: Antithrombin III- and Heparin Cofactor II-Independent Inhibition of Factor X Activation by Factor IXa-Factor VIIIa Complex and Heparin Cofactor II-Dependent Inhibition of Thrombin

By Hideki Nagase, Kei-ichi Enjoji, Kazuhisa Minamiguchi, Keiko T. Kitazato, Kenji Kitazato, Hidehiko Saito, and Hisao Kato

The inhibition mechanism of a polysaccharide anticoagulant, depolymerized holothurian glycosaminoglycan (DHG), was examined by analyzing its effects on the clotting time of human plasma depleted of antithrombin III (ATIII), of heparin cofactor II (HCII), or of both heparin cofactors. The effect exerted by this agent on the activation of prothrombin and factor X in purified human components were also examined and all effects were compared with those of other glycosaminoglycans (GAGs). The capacity of DHG to prolong activated partial thromboplastin time was not reduced in ATIII-depleted, HCII-depleted, or ATIII- and HCII-depleted plasma, whereas its capacity to prolong prothrombin time and thrombin clotting time was reduced in HCII-depleted plasma. DHG inhibited the amidolytic activity of thrombin in the presence of HCII with a second order rate constant of $1.2 \times 10^4$ (mol/L)$^{-1}$ min$^{-1}$. These results indicated that DHG has two different inhibitory activities, one being an HCII-dependent thrombin inhibition and the other an ATIII- and HCII-independent inhibition of the coagulation cascade. The heparin cofactors-independent inhibitory activity of DHG was investigated in the activation of prothrombin by factor Xa and in the activation of factor X by tissue factor-factor VIIa complex or by factor IXa. DHG significantly inhibited the activation of factor X by factor IXa in the presence of factor VIIa, but not in the absence of factor VIIa. The interaction between DHG and factors IXa, VIIIa, and X was investigated with a DHG-cellulofine column, on which DHG had strong affinity for factors IXa and VIIIa. These findings show that the heparin cofactors-independent inhibition exhibited by DHG was caused by inhibition of the interaction of factor X with the intrinsic factor Xase complex, probably by binding to the factor IXa-factor VIIIa complex.

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**Subjects, Materials, and Methods**

Subjects and materials. The following materials were purchased from the listed companies: UFH (porcine mucosal origin, 187.5 IU/mg; Naculai tesque, Kyoto, Japan), LMWH (porcine intestinal mucosa origin, average molecular weight 4,000 to 6,000; Sigma Chemical Co, St Louis, MO), and DS (porcine skin origin, average molecular weight 11,000 to 25,000; Seikagaku Kogyo, Co, Tokyo, Japan). The chromogenic substrates, S-2238 and S-2222, and the fluorescent substrates, Z-Pyr-Gly-Arg-MCA and Boc-Val-Arg-MCA, were purchased from Kabi Vitrum (Stockholm, Sweden) and Biopharm. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

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from Peptide Institute (Osaka, Japan), respectively. Bovine brain cephalin was purchased from Sigma Diagnostics (St Louis, MO) and dissolved according to the manufacturer’s instruction. Bovine serum albumin (BSA), phosphatidylserine (PS), and phosphatidylcholine (PC) were purchased from Sigma Chemical Co. DHG (average molecular weight 10,000 to 15,000) was prepared by depolymerization of extracts of _Stichopus japonicus_ Selenka. Its structure was determined to be a fucose-branched chondroitin 4-sulfate by means of chemical degradation and 2D-NMR spectroscopy (Fig 1).

Pooled human plasma was obtained from 10 healthy donors with informed consent and stored at −70°C until use. Human ATIII was purchased from Cosmo Bio Co (Tokyo, Japan). Human HCII was purified as described by Tran et al. Its homogeneity was confirmed with each protein. The IgG were isolated using a column conjugated with an anti-ATIII IgG column. ATIII activity measured according to Odegard et al was less than 2%. The total protein concentration in each of the depleted plasmas was unchanged.

Coagulation assay. Activated partial thromboplastin time (APTT) and thrombin clotting time (TCT) were determined by using a commercially available APTT reagent (Platelin Excel LS; Organon Teknika Co, Durham, NC) and human thrombin (3,000 U/mg protein; Sigma Chemical Co). Prothrombin time (PT) was determined by a standard method using tissue thromboplastin extracted from rabbit brain (Simplastin; Organon Teknika Co). The PT reagent was diluted in 0.025 mol/L CaCl2 at 1:30, so that the PT of normal plasma was 30.4 seconds. The effects of the GAGs on APTT, TCT, and PT were tested by the addition of 10 μL of sample to 100 μL of plasma. Prothrombin activation in contact-activated normal plasma and factor Xa generation in glass surface-activated factor VIII-deficient plasma (George King Bio-Medical, Inc, Overland Park, KS) were measured as described previously.

Antithrombin and anti-factor Xa chromogenic assay. Antithrombin and anti-factor Xa activities in the presence of ATIII were measured with S-2238 and S-2222, respectively, as described previously. Antithrombin activity in the presence of HCII was measured with S-2238, as follows. A mixture containing 200 μL of HCII (480 nmol/mL) and 200 μL of various amounts of each GAG was incubated at 37°C for 2 minutes. A 200-μL aliquot of thrombin (55 nmol/L) was then added. After incubation at 37°C for 1 minute, the residual thrombin activity was measured by adding 200 μL of 1 mol/L S-2238 solution containing polybrene (1 mg/mL). The reaction was stopped after 1 minute by the addition of 200 μL of 50% (wt/vol) acetic acid, and the absorbance at 405 nm was measured.

Kinetic analysis of antithrombin activity. Thrombin (33 pmol/L) and HCII (6.5 nmol/L) were incubated with various amounts of GAGs in 200 μL of TBS-BSA (20 mmol/L Tris-HCl buffer, pH 8.0, containing 150 mmol/L NaCl, 0.1 mg BSA/mL, and 0.05% NaN3). At various time intervals, portions were removed and residual thrombin activity was determined by the addition of Boc-Val-Pro-Arg-MCA (final concentration, 100 μmol/L). The AMC liberated was measured as described previously by using a fluorescence spectrophotometer (model F-4500; Hitachi Ltd, Tokyo, Japan). The second order rate constant was determined according to the equation, kobs = -ln(Eo/En)/t, where t is the reaction time, Eo and En are the remaining thrombin activity at time t and initial thrombin activity, respectively, and [Eo] is the initial inhibitor (HCII) concentration.

Activation of factor X by factor IXa. Ten microliters of 9.1 mmol/L factor VIIIa was mixed with 965 μL of TBS-BSA containing 0.18 mmol/L factor IXa, Cephalin (55 μL), 5 mmol/L CaCl2 and various amounts of GAGs. After 30 seconds, factor X activation was initiated by adding 25 μL of 2.8 μmol/L factor X to the mixture (final concentrations: factor IXa, 180 pL/mL; factor VIIIa, 91 pL/mL; factor X, 70 pL/mL). After incubation at 37°C for 1 minute, the factor Xa generated was measured by the addition of 1 mL of a mixture of 2-Pyr-Gly-Arg-MCA (final concentration, 100 μmol/L) and EDTA (final concentration, 10 mmol/L). When factor VIIIa was excluded, the reaction mixture containing 34 mmol/L factor IXa and 270 mmol/L factor X was incubated for 15 minutes. The AMC liberated was measured using a fluorescence spectrophotometer (model F-4500; Hitachi Ltd) or a centrifugal autoanalyzer (Cobas Bio) equipped with a fluorescence unit.

Activation of factor X by factor VIIa-tissue factor. Factor X activation was initiated by mixing 240 μL of TBS-BSA containing recombinant tissue factor (1.3 ng), a mixture of 25% PC and 75% PS, 66 pmol/L factor VIIa, and 5 mmol/L CaCl2, with 20 μL of a mixture of 360 nmol/L factor X and various amounts of GAGs (final concentration, 200 μmol/L, TBS-BSA, pH 8.0). The reaction was initiated by mixing 200 μL of the factor X mixture with 200 μL of the GAG mixes for 1 minute and the residual factor Xa activity was measured with S-2238, as follows. A mixture containing 200 μL of factor Xa (270 nmol/L) and 200 μL of various amounts of each GAG was incubated at 37°C for 1 minute. The factor Xa activity in the presence of ATIII was measured with S-2238 and S-2222, respectively, as described previously. The factor Xa activity in the presence of HCII was measured with S-2238, as follows. A mixture containing 200 μL of ATIII (480 nmol/mL) and 200 μL of various amounts of each GAG was incubated at 37°C for 2 minutes. A 200-μL aliquot of thrombin (55 nmol/L) was then added. After incubation at 37°C for 1 minute, the residual thrombin activity was measured by adding 200 μL of 1 mol/L S-2238 solution containing polybrene (1 mg/mL). The reaction was stopped after 1 minute by the addition of 200 μL of 50% (wt/vol) acetic acid, and the absorbance at 405 nm was measured.
GAG WITH NOVEL ANTICOAGULANT ACTIONS

Table 1. Concentrations of DHG and Other GAGs Required to Double the APTT in Normal, ATIII-Depleted, HCII-Depleted, and Both Heparin Cofactors-Depleted Plasma

<table>
<thead>
<tr>
<th>GAGs</th>
<th>Normal</th>
<th>ATIII-Depleted</th>
<th>HCII-Depleted</th>
<th>ATIII- and HCII-Depleted</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHG</td>
<td>3.8</td>
<td>3.3</td>
<td>3.8</td>
<td>2.7</td>
</tr>
<tr>
<td>UFH</td>
<td>0.8</td>
<td>9.9</td>
<td>0.7</td>
<td>13.1</td>
</tr>
<tr>
<td>LMWH</td>
<td>2.7</td>
<td>22.0</td>
<td>2.6</td>
<td>39.0</td>
</tr>
<tr>
<td>DS</td>
<td>47.4</td>
<td>46.8</td>
<td>219.8</td>
<td>482.0</td>
</tr>
</tbody>
</table>

Concentrations of GAGs are expressed as micrograms per milliliter of plasma. A mixture of 100 μL of plasma and 10 μL of various concentrations of GAGs was incubated with 100 μL of APTT reagent at 37°C for 5 minutes; 100 μL of 25 mmol/L CaCl₂ was then added. APTT for normal, ATIII-depleted, HCII-depleted, and both heparin cofactors-depleted plasma was 33.1, 33.5, 34.0, and 36.3 seconds, respectively.

Activation of prothrombin by factor Xa. Ten microliters of 150 nmol/L factor Va was mixed with 970 μL of TBS-BSA containing 2.2 pmol/L factor Xa, Cephalin (55 μL), 5 mmol/L CaCl₂, and various amounts of GAGs. After 30 seconds, prothrombin activation was initiated by adding 20 μL of 2.4 mmol/L prothrombin to the mixture (final concentrations: factor Xa, 2.2 pmol/L; factor Va, 1.5 nmol/L; prothrombin, 48 nmol/L). After incubation at 37°C for 30 seconds, the thrombin generated was measured by the addition of 1 mL of a mixture of Boc-Val-Pro-Arg-MCA (final concentration, 100 μmol/L) and EDTA (final concentration, 10 mmol/L), as described above. When factor Va was excluded, reaction mixture containing 1.5 nmol/L factor Xa and 120 mmol/L prothrombin was incubated for 5 minutes.

Analysis of affinities of blood coagulation factors to DHG-cellulofine column. Factor IXa (410 μg), factor VIIIa (5 μg), or factor X (820 μg) was applied to a DHG-cellulofine (Chisso Corp, Chiba, Japan) column (0.8 × 7.5 cm) in 20 mmol/L Tris-HCl, pH 8.0, containing 150 mmol/L NaCl, 5 mmol/L CaCl₂, 0.01% Tween 80, and 0.05% NaH₂PO₄ 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 to 1 mol/L). One-milliliter fractions were collected at a flow rate of 1 mL/min at 4°C. In the case of factors IXa and X, the absorbance at 280 nm of each fraction was measured. The factor VIIIa activity of each fraction was measured by determining the APTT of factor VIII-deficient plasma, using human plasma as a standard. In a control experiment, an acetylated amino cellulofine (Chisso Corp) column was used instead of a DHG column.

RESULTS

Effects of DHG and other GAGs on clotting time of normal, ATIII-depleted, HCII-depleted, and both heparin cofactors-depleted plasma. The capacity of GAGs to prolong APTT and PT was expressed as the concentrations required to double APTT and PT (doubling APTT and doubling PT; Tables 1 and 2). In normal plasma, the doubling APTT of DHG was smaller than the doubling PT. These values were smaller than those for the previous DHG preparation, DHG-1. The doubling APTT of DHG in normal plasma was approximately the same as those in ATIII-depleted, HCII-depleted, and both heparin cofactors-depleted plasma, whereas the capacity of DHG to prolong PT was reduced in HCII-depleted and in ATIII- and HCII-depleted plasma, but was not significantly reduced in ATIII-depleted plasma. These results indicate that the prolongation of APTT by DHG is independent of ATIII and HCII, whereas the prolongation of PT depends on HCII. The inhibitory potency of UFH and LMW of APTT and PT was markedly reduced in ATIII-depleted plasma, but not in HCII-depleted plasma, whereas that of DS was reduced in HCII-depleted plasma. These results confirm previous findings that the prolongation of clotting time induced by UFH and LMW depends on ATIII, whereas that induced by DS depends on HCII.

TCT in normal plasma was prolonged most potently by UFH and less potently by LMW, DHG, and DS in that order (data not shown). TCT prolongation by DHG and DS was markedly reduced in ATIII-depleted plasma, whereas the prolongation of TCT by UFH and LMW was reduced in ATIII-depleted plasma. In the plasma depleted of both heparin cofactors, TCT was not significantly prolonged by each

Table 2. Concentrations of DHG and Other GAGs Required to Double the PT in Normal, ATIII-Depleted, HCII-Depleted, and Both Heparin Cofactors-Depleted Plasma

<table>
<thead>
<tr>
<th>GAGs</th>
<th>Normal</th>
<th>ATIII-Depleted</th>
<th>HCII-Depleted</th>
<th>ATIII- and HCII-Depleted</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHG</td>
<td>21.3</td>
<td>37.3</td>
<td>111.2</td>
<td>211.8</td>
</tr>
<tr>
<td>UFH</td>
<td>2.2</td>
<td>59.1</td>
<td>2.8</td>
<td>&gt;1,000</td>
</tr>
<tr>
<td>LMWH</td>
<td>7.7</td>
<td>632.6</td>
<td>8.9</td>
<td>&gt;1,000</td>
</tr>
<tr>
<td>DS</td>
<td>39.7</td>
<td>76.4</td>
<td>&gt;1,000</td>
<td>&gt;1,000</td>
</tr>
</tbody>
</table>

Concentrations of GAGs are expressed as micrograms per milliliter of plasma. A mixture of 100 μL of plasma, 100 μL of 20 mmol/L imidazole buffer (pH 7.4), and 10 μL of various concentrations of DHG was incubated at 37°C for 3 minutes; 100 μL of PT reagent was then added. PT for normal, ATIII-depleted, HCII-depleted, and both heparin cofactors-depleted plasma was 30.4, 31.1, 32.9, and 35.8 seconds, respectively.

Table 3. TCT of Normal, ATIII-Depleted, HCII-Depleted, and Both Heparin Cofactors-Depleted Plasma in the Presence of the Concentrations of DHG and Other GAGs Required to Double the APTT

<table>
<thead>
<tr>
<th>GAGs</th>
<th>Normal</th>
<th>ATIII-Depleted</th>
<th>HCII-Depleted</th>
<th>ATIII- and HCII-Depleted</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>21</td>
<td>20</td>
<td>21</td>
<td>19</td>
</tr>
<tr>
<td>DHG (3.8 μg/mL)</td>
<td>32</td>
<td>31</td>
<td>24</td>
<td>23</td>
</tr>
<tr>
<td>UFH (0.8 μg/mL)</td>
<td>152</td>
<td>21</td>
<td>&gt;200</td>
<td>21</td>
</tr>
<tr>
<td>LMWH (2.7 μg/mL)</td>
<td>&gt;200</td>
<td>22</td>
<td>&gt;200</td>
<td>21</td>
</tr>
<tr>
<td>DS (47.4 μg/mL)</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>22</td>
</tr>
</tbody>
</table>

Values indicate TCT (seconds) of plasma in the presence of GAGs at the concentrations required to double the APTT of normal plasma (shown in parentheses) (see Table 1). A mixture of 100 μL of plasma, 100 μL of 20 mmol/L imidazole buffer (pH 7.4), and 10 μL of each GAG was incubated at 37°C for 1 minute; 100 μL of human thrombin (2.5 NIH U/mL) was then added.
of the GAGs. These results indicate that DHG and DS prolonged TCT HCII dependently and that UFH and LMWH did mainly ATIII dependently. Table 3 shows the TCT of normal, ATIII-depleted, HCII-depleted, and both heparin cofactors-depleted plasma in the presence of GAGs at the concentrations of which they doubled APTT. In normal plasma, DHG at doubling APTT caused slight prolongation of TCT, whereas UFH, LMWH, and DS markedly prolonged TCT at doubling APTT. TCT prolonged by DHG and DS at doubling APTT was reduced in HCII-depleted plasma, whereas TCT prolonged by UFH and LMWH at doubling APTT was reduced in ATIII-depleted plasma. These results suggest that the major inhibitory activity of DHG is the prolongation of APTT, which is independent of ATIII and HCII, although DHG also had HCII-dependent inhibitory activity on thrombin.

Factor IXα generated by glass surface activation of factor VIII-deficient plasma was measured using factor IX-deficient plasma as described in Subjects, Materials, and Methods. The clotting time of factor IX-deficient plasma was described in Subjects, Materials, and Methods. The clotting time of factor IX-deficient plasma was reduced to 71.9 from 557.8 seconds by glass-surface activation for 24 minutes. In the presence of DHG (3.8 μg/mL), it was reduced to 76.8 from 461.7 seconds. These results suggest that factor IXα generation was not inhibited by DHG at doubling APTT.

Figure 2 shows the retardation of prothrombin consumption in contact-activated plasma by GAGs at the concentration of doubling APTT. In normal plasma, all the polysaccharide anticoagulants retarded prothrombin consumption with approximately the same potency (Fig 2A). In ATIII- and HCII-depleted plasma, in contrast, only DHG retarded prothrombin consumption (Fig 2B). The inhibitory effect of UFH and LMWH disappeared in ATIII-depleted plasma, whereas that of DS disappeared in HCII-depleted plasma (data not shown). These findings indicate that the retardation of prothrombin consumption by DHG in contact-activated plasma was not dependent on ATIII and HCII.

**Effects of DHG and other GAGs on the amidolytic activity of thrombin and factor Xa.** None of GAGs tested had any effect on the amidolytic activities of thrombin and factor Xa, at final concentrations of up to 100 μg/mL, in the absence of ATIII or HCII. In the presence of ATIII, DHG showed weak inhibition of thrombin (IC50 = 35.9 μg/mL), but no significant inhibition of factor Xa (IC50 > 100 μg/mL). However, DHG strongly inhibited thrombin in the presence of HCII (IC50 = 0.8 μg/mL). Figure 3A shows the effects of DHG and other GAGs on thrombin in the presence of HCII. At their optimum concentrations, second order rate constants for the inhibition of thrombin were calculated from the data as shown in Fig 3B. The value for DHG was calculated to be 1.2 × 10^8 (mol/L)^-1min^-1. The values for DS and UFH, 2.6 × 10^8 (mol/L)^-1min^-1 for DS and 1.4 × 10^8 (mol/L)^-1min^-1 for UFH, were slightly lower than those reported previously. 

**Effects of DHG and other GAGs on the activation of factor X by factor IXa or factor VIIa and of prothrombin by factor Xa.** As described in the previous section, DHG prolonged APTT in an ATIII- and HCII-independent manner. The inhibition of prothrombin activation in contact-activated plasma was also independent of ATIII and HCII. DHG did not inhibit the generation of factor IXα in contact-activated plasma. To clarify the mechanism of DHG inhibition in the coagulation reactions, we examined the effect of DHG, in comparison with the effects of the other GAGs, on the activation of prothrombin and factor X, using purified coagulation factors.

We first examined the effects of GAGs on the activation of factor X by factor VIIa or factor IXa, measuring the amidolytic activity of factor Xa. The amidolytic activity of the factor VIIa-tissue factor complex and the activation of factor X by the factor VIIa-tissue factor complex in the presence of phospholipid and calcium ion were not inhibited by DHG, UFH, and LMWH (data not shown). On the other hand, all three GAGs inhibited the activation of factor X by factor IXa in the presence of factor VIIa, phospholipid, and calcium ion, and this occurred in a dose-dependent manner (Fig 4A). DHG had the strongest inhibitory activity. The effect of DHG was similar to that when DHG was added...
Effect of DHG and other GAGs on thrombin activity in the presence of HCII. (A) Various amounts of GAGs were mixed with thrombin (33 pmol/L) and HCII (6.5 nmol/L). After 1 minute at 37°C, the remaining thrombin activity was measured using Boc-Val-Pro-Arg-MCA as substrate. (B) Inhibition of thrombin activity in the presence of HCII was measured at the optimum concentrations of DHG (30 μg/mL), DS (3000 μg/mL), and UFH (300 μg/mL). (•) DHG; (○) UFH; (▲) DS.

Affinity of factors IXa, VIIIa, and X to DHG-cellulofine. Factors IXa, VIIIa, and X were applied to the DHG-cellulofine column to examine the interaction between DHG and these coagulation proteins. The proteins did not bind to the control column (acetylated amino-cellulofine) under condition of the physiologic salt concentration. Factors IXa and VIIIa bound to DHG-cellulofine under this condition and were eluted at 0.55 and 0.31 mol/L NaCl, respectively (Fig 6). Although factor X did not bind to DHG-cellulofine, it together with factor X. In contrast, factor X activation by factor IXa was not inhibited in the absence of factor VIIIa (Fig 4B).

We then examined the effects of GAGs on the activation of prothrombin by factor Xa, measuring the amidolytic activity of thrombin. At concentrations exceeding 10 μg/mL, DHG, UFH, and LMWH had inhibitory effects on the activation of prothrombin by factor Xa in the presence of factor Va, phospholipid, and calcium ion (Fig 5A). In the absence of factor Va, the GAGs, at up to 3 μg/mL, did not inhibit the activation of prothrombin. At concentrations exceeding 3 μg/mL, they weakly accelerated thrombin formation (Fig 5B).
Fig 5. Effects of DHG and other GAGs on prothrombin activation by factor Xa in the presence (A) and absence (B) of factor Va. (A) A mixture containing factors Xa and Va, prothrombin, phospholipid, calcium, and various amounts of GAGs was prepared as described under Subjects, Materials, and Methods. After 30 seconds of incubation at 37°C, the thrombin generated was measured using Boc-Val-Pro-Arg-MCA as a substrate. The activity of thrombin generated in the absence of GAGs was taken to be 100%. (B) A mixture containing factor Xa, prothrombin, phospholipid, calcium, and various amounts of GAGs was prepared as described under Subjects, Materials, and Methods. Thrombin activity was measured after 5 minutes of incubation. (○) DHG; (●) UFH; (□) LMWH.

DISCUSSION

DHG, a depolymerized GAG extracted from holothurian, has shown potent antithrombotic effects in both in vitro and in vivo experiments. In thrombin-induced venous thrombus formation in rats, the minimum effective doses of DHG, UFH, LMWH, and DS were 0.3, 0.3, 0.3, and 1 mg/kg, respectively, and the maximum tolerant doses of these GAGs for bleeding after rat tail transection were 3, 0.3, 0.3, and 3 mg/kg, respectively (data not shown; Kitazato et al, manuscript submitted). Together with its relatively long duration of antithrombotic effect, DHG is a promising antithrombotic agent, compared with other GAGs. Both this study and our previous study have shown that the antithrombotic effect of DHG is due to two different anticoagulant activities, one being the HcII-dependent inhibition of thrombin and the
other the heparin cofactors-independent inhibition of factor X activation by the factor IXa-factor VIIIa complex.

The HCII-dependent anticoagulant activity of DHG on thrombin was shown by the following findings: (1) the prolongation of PT and TCT in normal plasma was reduced in HCII-depleted plasma and (2) the amidolytic activity of thrombin was inhibited only in the presence of HCII. However, the prolongation of APTT by DHG in normal plasma was not reduced in heparin cofactors-depleted plasma. Because the retardation of prothrombin consumption by DHG in contact-activated plasma was not dependent on ATIII and HCII and factor IXa generation in plasma induced by the glass surface was not influenced by DHG, it appeared that the heparin cofactors-independent prolongation of APTT by DHG was due to the effects exerted by DHG on the activation of prothrombin or factor X. The second order rate constant for the inhibition of thrombin by DHG in the presence of HCII was comparable with those for the inhibition of thrombin by DS and UFH. Although it is not known which is the major anticoagulant activity of DHG, the HCII-dependent or the two heparin cofactors-independent activity, we suggest that the major anticoagulant activity of DHG may be the heparin cofactors-independent inhibition, this being indicated by our finding that the activity of DHG in the heparin cofactors-dependent prolongations of PT and TCT was weaker than that in the heparin cofactors-independent prolongation of APTT.

To clarify the mechanism responsible for the heparin cofactors-independent anticoagulant activity of DHG, we examined the inhibitory effects of DHG on the activation of factor X and prothrombin using purified human coagulation factors. DHG had a slight inhibitory effect on the activation of prothrombin by the factor Xa-factor Va complex, whereas it had a strong inhibitory effect on the activation of factor X by the factor IXa-factor VIIIa complex. The effect of DHG on the activation of factor X was almost the same when DHG was added after the formation of the intrinsic factor Xase complex. However, DHG did not inhibit the activation of factor X by factor IXa in the absence of factor VIIIa. These findings suggested that DHG inhibited the activation of factor X through its interaction with the factor IXa-factor VIIIa complex. We then examined the interaction between DHG and the coagulation proteins, factors IXa, VIIa, and X, using a DHG-cellulofine column. DHG had strong affinity for factors IXa and VIIIa and slight affinity for factor X. Because DHG showed a strong inhibitory effect of intrinsic factor Xase complex when DHG was added after the formation of the factor IXa-factor VIIIa complex, it is unlikely that DHG interfered with the interaction between factor IXa and factor VIIIa. We therefore presumed that DHG interfered with the interaction between intrinsic factor Xase and factor X by binding to the factor IXa-factor VIIIa complex.

Thus, the anticoagulant effects of DHG were clearly distinguished from those of UFH, LMWH, and DS, which are mediated by ATIII and HCII. In in vivo experiments in rats with thrombin-induced venous thrombus formation (data not shown; Kitazato et al, manuscript submitted), UFH and LMWH significantly prolonged the bleeding time, whereas DHG had no effect on the bleeding time in the tail transection of rats at the minimum dose of each GAG that was effective in the thrombin-induced venous thrombus formation. Thus, it appears that the heparin cofactors-independent inhibition of the intrinsic factor Xase complex may be important in the mechanism whereby the antithrombotic and haemorrhagic properties of DHG are dissociated. Further studies, for example, on the effect of DHG in heparin cofactors-depleted animals, will be required to examine the contribution of this heparin cofactors-independent effect to the in vivo antithrombotic effect of DHG.

The anticoagulant effect of DHG is very similar to that of pentosan polysulfate (PPS), which inhibits thrombin HCII-dependently and prolongs APTT ATIII and HCII independently. Wagenvoord et al have reported that PPS inhibits factor VIIIa formation (activation of factor VIII by thrombin) and that it inhibits the function of factor VIIIa by the formation of a factor VIIIa-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. In light of these findings, it is clear that further investigation of the effects of DHG on the components of intrinsic factor Xase is required to elucidate the heparin cofactors-independent anticoagulant effects of DHG.

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