Antithrombotic and Anticoagulant Activities of a Low Molecular Weight Fucoidan by the Subcutaneous Route


From Laboratoires Fournier, Dijon, FREMER, Nantes, Laboratoire d’Hématologie, CHU Necker-Enfants Malades, Université Paris V and Université Paris-Nord, Villetaneuse, France

Summary

Fucoidans (high-molecular-weight sulfated polysaccharides extracted from brown seaweeds) have anticoagulant and antithrombotic effects. They inhibit thrombin by catalyzing both serpins (antithrombin and heparin cofactor II) according to their chemical structures and origins. In this study, a low-molecular-weight (LMW) fucoidan of 8 kDa was obtained by chemical degradation of a high-molecular-weight fraction. The antithrombotic and anticoagulant activities of this new compound were compared to those of a low-molecular-weight heparin (LMWH), dalteparin, following subcutaneous administration to rabbits. This LMW fucoidan exhibited dose-related venous antithrombotic activity, with an ED$_{50}$ of about 20 mg/kg, 2 h after a single subcutaneous injection. Its activity was comparable to that of dalteparin (close to 200 anti-Xa IU/kg) and was maximal 30 min after a single subcutaneous injection. The activity remained stable (about 70%) from 1 to 4 h after injection, but disappeared by 8 h. The antithrombotic activity was not associated with either a prolongation of the thrombin clotting time (TCT) or an increase in anti-Xa activity, contrary to dalteparin. A slight prolongation of APTT occurred with both compounds. This venous antithrombotic activity was associated with a decrease in ex vivo thrombin generation and with a significant increase in the lag phase in a thrombin generation test. LMW fucoidan thus has potent antithrombotic activity and a potentially weaker haemorrhagic effect (i.e. a smaller effect on coagulation tests and a smaller prolongation of the bleeding time) than dalteparin.

Introduction

In a preliminary study, we isolated a fucoidan fraction from the brown seaweed Ascophyllum nodosum by using a previously reported procedure (1). This fraction, with a molecular weight of about 20 kDa, showed interesting antithrombotic properties (1), after intravenous administration to rabbits, relative to unfractionated heparin (UFH).

Recent studies on low-molecular-weight heparins (LMWH) have shown that these compounds are at least as effective and safe as UFH, while being more convenient, in the prophylaxis and treatment of deep venous thrombosis (2). Another glycosaminoglycan, dermatan sulfate, has also been tested (3). More recently, a new low-molecular-weight dermatan sulfate with better bioavailability than high-molecular-weight dermatan sulfate was also found to be a potent antithrombotic agent in animals after subcutaneous administration (3). In a pilot study, this compound was active in the treatment of deep venous thrombosis (4).

On the basis of these studies, we isolated a fucoidan fraction with a molecular weight of about 8 kDa and tested it in rabbits. The purpose of this work was to compare the antithrombotic activity of this new fucoidan fraction (LMW fucoidan) with a LMWH, dalteparin, when administered subcutaneously to rabbits. The anticoagulant activities, haemorrhagic effects and effects on thrombin generation of ex vivo plasma samples were also compared.

Materials and Methods

Reagents

A new low-molecular-weight fucoidan was obtained by acid hydrolysis of a high-molecular-weight fucoidan by using a procedure adapted from Colliec et al. (5). The molecular weight, as determined by high-performance steric exclusion chromatography (HPSEC), was 8 ± 1 kDa. The chemical composition as previously determined was as follows: 48 ± 4% fucose, 4.5 ± 1.8% uronic acid, 9.4 ± 0.3% sulfur and 0.25 ± 0.07% nitrogen (6). Dalteparin (Fragmin®) (2500 anti-Xa IU/0.2 ml, 160 anti-Xa IU/mg) was from Kabi Pharmacia (St Quentin, France). Purified human thrombin (920 NIH U/vial), cephalin and kaolin (C. K. Prest®,) and Reptilase® were from Diagnostica Stago (Asnières, France). Bovine factor Xa (71 nkat) and the chromogenic substrate S-2238 were from Biogenic (Montpellier, France). Thromborel (human thromboplastin without Polybrene) and Neothromtin® were from Behring (Marburg, Germany). APTT was from Organon Technika (Fresnes, France).

Animals

All experiments were done on male New Zealand rabbits weighing between 2 and 2.5 kg and obtained from Elevage des Pins (Epeigné sur Deme, France). The animals were used after one week of quarantine in a room maintained at 20°C, with food and water ad libitum. The animal experiments were approved by Laboratoires Fournier’s ethics committee (registration # 6305/006 and 6305/017).

Antithrombotic Effects

The technique used was that of Wessler (7, 8), with bovine factor Xa as the thrombogenic stimulus (1, 9).

To evaluate the dose relationship of antithrombotic activity, LMW fucoidan and dalteparin in saline (0.9%), or 0.9% saline (control), were administered by subcutaneous injection (s.c.) in a volume of 1 ml/kg body weight at various doses (5-40 mg/kg LMW fucoidan and 50-200 anti-Xa IU/kg dalteparin), 2 h before induction of thrombosis according to a previously described method (1). The antithrombotic effect was estimated by the weight reduction of the wet thrombus (mg) after polysaccharide injection. To assess the kinetics of antithrombotic activity, the compounds were administered subcutaneously at doses close to their respective ED$_{50}$ values (dose reducing the wet clot weight by 80%), 0.5, 1, 2, 4, 6 and 8 h before thrombus induction.

Following thrombosis induction, blood samples from treated and control animals were collected in 3.8% sodium citrate (9 vol blood/1 vol citrate) to determine ex vivo anticoagulant activity and ex vivo thrombin generation.
Platelet-poor plasma (PPP) was obtained by centrifugation at 2400 g for 10 min at 20° C and stored at -70° C until use.

**Anticoagulant Activity**

In vitro assays, i.e. the activated partial thromboplastin time (APTT), thrombin clotting time (TCT), reptilase time and prothrombin time, were performed as previously described (1). LMW fucoidan and dalteparin were diluted in human PPP at concentrations ranging from 20 to 100 µg/ml LMW fucoidan and from 1 to 6 µg/ml dalteparin. These concentrations were chosen to obtain comparable prolongations of the APTT with the two products.

Ex vivo anticoagulant activities of LMW fucoidan and dalteparin were evaluated by APTT (with kaolin as activator) and TCT from rabbit blood collected at each point of the kinetic study of antithrombotic activity (from 0.5 to 8 h).

**Ex Vivo Thrombin Generation Test**

Rabbit PPP was collected at various times during the kinetic study of antithrombotic activity (from 0.5 to 8 h). The intrinsic pathway of thrombin generation was assessed with a modification of Ofosu’s method (10) as previously described (11). Briefly, thrombin generation was triggered by adding a mixture of cephalin and ellagic acid (Neothromtin®) to defibrinated rabbit PPP. At various times, thrombin generation was stopped by adding 100 µl of activated PPP to 400 µl of 0.01 M EDTA in 0.03 M sodium barbiturate buffer pH 8.35 containing 0.15 M NaCl and 0.1 g/l BSA preincubated at 4° C. The generated thrombin was quantified by adding 50 µl of EDTA-containing sample to 450 µl of 1 mM S-2238. The peak value of generated thrombin and the lag phase (time between contact activation and detection of the first traces of thrombin) obtained with plasma from rabbits treated with polysaccharides were compared with those obtained with control rabbit plasma.

**Haemorrhagic Potential**

This study was performed with rabbits as described by Carter et al. (12) and modified by Doutremepuich et al. (13). As previously described (1), wounds were made on the left marginal vein of the rabbit ear. The bleeding time (expressed in min) was measured as the time between wounding and the cessation of bleeding from all wounds.

Both polysaccharides were injected at 5 × ED50 in a volume of 1 ml/kg body weight, subcutaneously. LMW fucoidan (100 mg/kg) and dalteparin (1000 anti-Xa IU/kg) were administered 2 h before measuring the bleeding time.

**Analysis of Data**

Results of in vitro tests are given as means ± SD. In both in vivo and ex vivo experiments, the thrombus wet weight (in mg), bleeding time (in min) and coagulation time (in s) in the treated and control groups were submitted to analysis of variance. Significant differences between groups were identified with Student’s t-test. Values are given as means ± SEM, ED50 or ED80 (dose producing a 50% or 80% effect) with the corresponding 95% confidence limits.

**Results**

**In Vitro Anticoagulant Activity**

The LMW fucoidan did not prolong the reptilase time or prothrombin time at the concentrations tested (20-100 µg/ml in human PPP) (not shown). As indicated in Table 1, an increase in the APTT was observed at all concentrations of both compounds. However, a higher concentration of fucoidan than dalteparin (on a weight basis) was required for an equivalent prolongation of the APTT. For TCT, a prolongation of the coagulation time was observed with all tested concentrations of dalteparin, but only at the highest concentration of LMW fucoidan.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Thrombus wet weight (mg)</th>
<th>Inhibition (%)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>130.9 ± 15.4</td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td>LMW fucoidan</td>
<td>5</td>
<td>104.8 ± 16.2</td>
<td>19.9</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>50.1 ± 16.3</td>
<td>65.7***</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>14.9 ± 9.3</td>
<td>88.6***</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>5.0 ± 3.6</td>
<td>96.2***</td>
<td>5</td>
</tr>
<tr>
<td>Dalteparin</td>
<td>50</td>
<td>104.8 ± 20.3</td>
<td>19.9</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>30.9 ± 12.8</td>
<td>76.4***</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>14.4 ± 9.9</td>
<td>89.0***</td>
<td>5</td>
</tr>
</tbody>
</table>

Dose-effect of LMW fucoidan and dalteparin (with a saline control) administered subcutaneously 2 hours before thrombus induction in Wessler’s model with bovine factor Xa as procoagulant stimulus.

Data are mean ± SEM thrombus wet weight. %: percentage decrease in thrombus weight, calculated as treated/control values. N: number of animals in each group.

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**Table 1** In vitro anticoagulant activity of LMW fucoidan and dalteparin

<table>
<thead>
<tr>
<th>LMW fucoidan (µg/ml PPP)</th>
<th>APTT (sec)</th>
<th>TCT (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>38 ± 3</td>
<td>23 ± 2</td>
</tr>
<tr>
<td>20</td>
<td>51 ± 0.9**</td>
<td>24.5 ± 0.6</td>
</tr>
<tr>
<td>30</td>
<td>60 ± 0.8**</td>
<td>25 ± 1.3</td>
</tr>
<tr>
<td>50</td>
<td>74 ± 1.5***</td>
<td>26 ± 1.1</td>
</tr>
<tr>
<td>100</td>
<td>105 ± 1.9***</td>
<td>35 ± 2.5*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Dalteparin (µg/ml PPP)</th>
<th>APTT (sec)</th>
<th>TCT (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>46.5 ± 1.2**</td>
<td>32 ± 1.5**</td>
</tr>
<tr>
<td>2</td>
<td>57.5 ± 1.7**</td>
<td>128 ± 32.8**</td>
</tr>
<tr>
<td>4</td>
<td>79 ± 2.8***</td>
<td>&gt;180***</td>
</tr>
<tr>
<td>6</td>
<td>107 ± 2.5***</td>
<td>&gt;180***</td>
</tr>
</tbody>
</table>

Activated partial thromboplastin time (APTT) performed with the Organon® APTT kit. Thrombin coagulation time (TCT) determined with purified human thrombin (5 NIH U/ml). LMW fucoidan and dalteparin were tested after dilution in human platelet-poor plasma (PPP), at various concentrations.

Data are means ± SD in sec. * p ≤ 0.05 ** p ≤ 0.01 *** p ≤ 0.001 vs control.
Dose-effect studies. The dose-effect relationship of the LMW fucoidan fraction was evaluated 2 h after subcutaneous injection at doses from 5 to 40 mg/kg. In these conditions, the mean thrombus wet weight in control animals (n = 11) was 130.9 ± 15.4 mg (Table 2). Two hours after s.c. injection of the LMW fucoidan or dalteparin, the thrombus wet weight fell in a dose-related manner (Table 2). The ED50 values of LMW fucoidan and dalteparin, calculated by logarithmic regression, were 8.6 (6.1-12.1) mg/kg and 76.2 (54.8-105.9) anti-Xa IU/kg, respectively. The ED80 values were 19 (13.9-26.1) mg/kg LMW fucoidan and 139.2 (98.6-196.6) anti-Xa IU/kg dalteparin.

Kinetic studies. Maximal inhibitory activity (95% reduction in thrombus wet weight) was observed 30 min after a single subcutaneous injection of LMW fucoidan (20 mg/kg). This activity remained stable and close to 70% from 1 to 4 h and disappeared by 8 h. Subcutaneous administration of 200 anti-Xa IU/kg dalteparin significantly reduced thrombus wet weight up to 2 h after administration, with maximal inhibition (100%) at 1 h. After 2 h the activity fell in a time-related manner and disappeared by 8 h (Fig. 1).

Ex Vivo Coagulation Parameters

The antithrombotic activity of the LMW fucoidan was related to mild anticoagulant activity (Fig. 2A). Whatever the test (APTT or TCT), a slight but significant increase in anticoagulant activity was observed from 0.5 to 4 h after treatment (<40% for APTT and ≤30% for TCT). No significant anticoagulant activity was detected after 6 h. Furthermore, no change in anti-Xa activity was observed relative to control animals (not shown).

In contrast, a strong time-related increase in both TCT and anti-Xa activity was observed with dalteparin. Moreover, the profiles of the anticoagulant and antithrombotic activities were similar (Fig. 2B). A marked increase (100% and 80% respectively) was observed in TCT and anti-Xa activities for 1-2 h after subcutaneous injection; these activities then fell but remained significantly above control values at 4 h (p <0.01), and disappeared by 8 h (the increase in TCT was close to 40% but was not statistically significant). However, APTT was only slightly significantly increased from 1 to 6 h after dalteparin injection (~20%).

Ex Vivo Thrombin Generation Test

Plasma samples were obtained from the kinetic study in which both products were used at their respective antithrombotic ED80 values. Two hours after s.c. injection, both compounds (Fig. 3A) inhibited thrombin generation by about 50% (relative to the thrombin peak value).
The LMW fucoidan (Fig. 3B) significantly increased the lag phase of thrombin generation from 0.5 to 4 h, contrary to dalteparin. The LMW fucoidan (Fig. 3B) significantly increased the lag phase of thrombin generation from 0.5 to 4 h, contrary to dalteparin.

Haemorrhagic Potential

The effects of LMW fucoidan and dalteparin on the bleeding time 2 h after subcutaneous injection at approximately 5 times their ED_{80} values were evaluated. At this dose, the percentage increase in the bleeding time was lower with fucoidan (47%) than with dalteparin (96%), corresponding to a bleeding time with drug/control bleeding time ratio of 1.47 for LMW fucoidan and 1.96 for dalteparin. However, owing to individual variations in bleeding times, this difference was not significant (Table 3).

Discussion

Unfractionated heparin (UFH), given by continuous intravenous infusion or by the subcutaneous route, is considered an antithrombotic drug of choice for the initial treatment of acute deep vein thrombosis. However, the narrow efficacy-to-safety ratio, the need for close laboratory monitoring, the weak activity on clot-bound thrombin, and the potential for serious side effects (bleeding, thrombocytopenia, etc.), have led to the development of new antithrombotic agents (14).

In recent years, low-molecular-weight heparins (LMWH) have been preferred for their better efficacy-to-safety ratio (2). These new compounds also have a longer plasma half-life and less variability in their anticoagulant profile. It is well known (15) that both UFH and LMWH mainly exert their antithrombotic activities by potentiating the antithrombin (AT) effect.

Studies on dermatan sulfate and LMW dermatan sulfate (Desmin), which act via heparin cofactor II (HCII), have shown that these compounds are powerful inhibitors of thrombus formation (3, 4, 16, 17). Desmin has a weak anticoagulant effect and does not induce bleeding complications. Moreover, the antithrombotic activity of LMW dermatan sulfate lasts longer than that of native dermatan sulfate in several species (18).

In a previous paper (1), we compared the venous antithrombotic (according to Wessler’s model) and anticoagulant activities of a 20 kDa fucoidan fraction with those of UFH in rabbits. The same antithrombotic effect (ED_{80}) was obtained with 1.8 mg/kg fucoidan fraction and 0.1 mg/kg for UFH injected intravenously. The antithrombotic effect of fucoidan persisted longer than that of UFH. However, the bleeding time and the ex vivo anticoagulant effect (APTT and TCT) were slightly higher after fucoidan injection.

We thus prepared a LMW fucoidan and compared its anticoagulant and antithrombotic activities to those of a LMWH (dalteparin) after a single s.c. administration to rabbits. The LMW fucoidan had a dose-dependent antithrombotic action when tested in the Wessler stasis model, with an ED_{80} of about 20 mg/kg compared to 200 anti-Xa IU/kg (corresponding to 1.25 mg/kg) dalteparin. Thus, on a weight basis, the ratio i.v. UFH (or s.c. LMW heparin)/i.v. fucoidan (or s.c. LMW fucoidan) necessary to obtain the same antithrombotic effect is similar (1/17). Following LMW fucoidan s.c. injection at a dose close to its antithrombotic ED_{80}, a significant and stable reduction in thrombus wet weight (around 70%) persisted for 4 h. After LMWH injection, the reduction in thrombus wet weight was stable for 2 h and fell in time-related manner until 8 h. Nevertheless, contrary to UFH, the antithrombotic and anticoagulant effects of LMW fucoidan can be dissociated. As with LMWH, the antithrombotic activity of LMW fucoidan is not associated with a marked prolongation of the APTT (less than 40% compared to the control time). The antithrombotic activity of LMW fucoidan was not associated with a prolongation of the TCT, which remained close to control values. In contrast, dalteparin strongly increased TCT and anti-Xa activity (100% and 80%, respectively) when an antithrombotic effect was observed. Numerous data on heparins and other heparin-like oligosaccharides suggest that plasma anti-Xa activity does not correlate with antithrombotic activity (19, 20).

Another mechanism proposed by Ofosu et al. (21) to explain the antithrombotic activity of heparin is inhibition of prothrombinase complex formation or expression of its activity. Thomas et al. (22) reported that inhibition of thrombin generation would be a more effective way of preventing thrombosis than inhibition of prothrombinase. Thus, inhibition of thrombin generation by heparin and LMWH is an important parameter which relates to their anticoagulant actions in vivo (23). Fucoidan is able, like heparin, to inhibit thrombin generation, although it does not have strong anticoagulant activity. Fucoidan acts as a catalyst for thrombin inhibition by HC II (24-26) and thereby affects thrombin generation. The profile of thrombin generation in plasma shows significant differences in the mode of action of various anticoagulants (27). Fucoidan, like hirudin (28), delays contact-induced thrombin generation, but neither product prevents the subsequent explosive appearance of thrombin. Other mechanisms could provide alternative explanations for the delaying effect of fucoidan on thrombin generation. Inhibition of initial trace amounts of generated thrombin inhibits prothrombinase complex formation by reducing factor V activation by
thrombin; this latter mechanism may be important in the case of fucoidan. Despite their comparable antithrombotic activity, dalteparin and LMW fucoidan seem to act by different mechanisms. The differences between the two compounds can be detected in global clotting assays, as dalteparin, but not fucoidan, prolonged the TCT at its antithrombotic ED_{50}. Moreover a strong increase (about 100%) in the lag phase of thrombin generation tests was observed only with LMW fucoidan, whereas inhibition of peak thrombin generation was similar with the two compounds.

Thus, LMW fucoidan exhibited antithrombotic activity after s.c. injection, with only a slight increase in the bleeding time (47%, compared to 96% with dalteparin); this difference was not significant between the two compounds because of the limited number of animals tested. Moreover, like dalteparin, LMW fucoidan slightly prolonged the APTT, whereas contrary to dalteparin it did not prolong the thrombin clotting time. These characteristics should confer a low bleeding risk.

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References


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