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Structure and anticoagulant properties of sulfated glycosaminoglycans from primitive Chordates*

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ABSTRACT
Dermatan sulfates and heparin, similar to the mammalian glycosaminoglycans, but with differences in the degree and position of sulfation were previously isolated from the body of the ascidian Styela plicata and Ascidia nigra. These differences produce profound effects on their anticoagulant properties. S. plicata dermatan sulfate composed by 2-O-sulfated α-L-iduronic acid and 4-O-sulfated N-acetyl-β-D-galactosamine residues is a potent anticoagulant due to a high heparin cofactor II activity. Surprisingly, it has a lower potency to prevent thrombus formation on an experimental model and a lower bleeding effect in rats than the mammalian dermatan sulfate. In contrast, A. nigra dermatan sulfate, also enriched in 2-O-sulfated α-L-iduronic acid, but in this case sulfated at O-6 of the N-acetyl-β-D-galactosamine units, has no in vitro or in vivo anticoagulant activity, does not prevent thrombus formation but shows a bleeding effect similar to the mammalian glycosaminoglycan. Ascidian heparin, composed by 2-O-sulfated α-L-iduronic acid, N- and 6-O-sulfated glucosamine (75%) and α-L-iduronic acid, N- and 6-O-sulfated glucosamine (25%) disaccharide units has an anticoagulant activity 10 times lower than the mammalian heparin, is about 20 times less potent in the inhibition of thrombin by antithrombin, but has the same heparin cofactor II activity as mammalian heparin.

Key words: ascidian, heparin, dermatan sulfate, heparin cofactor II, anticoagulant, antithrombotic.

INTRODUCTION
Heparin and dermatan sulfate polymers have recently been isolated from test cells and tissues of ascidians (Chordate – Tunicate), and characterized in our laboratory. These glycosaminoglycans possess unique structural features, when compared to their mammalian counterparts. The ascidian heparin has the same basic structure of mammalian heparin, but with less 2-O-sulfation. On the other hand, the ascidian dermatan sulfate contains the same backbone structure but with different patterns of sulfation. All of the ascidians dermatan sulfates had a high content of iduronic acid 2-sulfate residues, but they differed in the pattern of sulfation of the N-acetylglactosamine units. These differences produce profound effects on the anticoagulant and antithrombotic activities of the ascidian glycosaminoglycans. In the present review we describe our findings about the structure and the anticoagulant prop-

*Invited paper.
properties of these compounds.

MAMMALIAN GLYCOSAMINOGLYCANS

The ability of certain glycosaminoglycans to interfere with blood coagulation has been known for over 50 years (McLean 1916), as illustrated by the extensive use of heparin as an antithrombotic agent (Rodén 1989). The effect of heparin is to accelerate the inactivation of plasma coagulation enzymes by the serpins (serine protease inhibitor) antithrombin (Bourin and Lindahl 1993) and heparin cofactor II, another serpin, which is also activated by dermatan sulfate, and which selectively inhibits thrombin (Tollefsen et al. 1982).

HEPARIN

Heparin is a highly sulfated glycosaminoglycan composed by disaccharide repeats of hexuronic acid (α-L-iduronic acid or β-D-glucuronic acid) linked 1,4 to D-glucosamine. The heparin molecules isolated from highly vascularized tissues, such as beef lung and hog mucosa are made up of a heterogeneous mixture of polymers with a similar backbone. The heterogeneity results from variations of sulfation on the D-glucosamine (N-acetylated, N-sulfated, O-sulfated at C6 and/or C3) and on the uronic acid residue (O-sulfated at C2). Because of its unique binding to antithrombin (ATIII), involving a specific pentasaccharide sequence, heparin is endowed of a potent anticoagulant activity (McLean 1916). In the presence of heparin, the rates of inhibition of thrombin, factor Xa, and factor IXa by AT III are increased ~1000-fold (Jordon et al. 1980) so that inhibition is essentially instantaneous. AT III inhibits the target protease by forming a 1:1 stable complex with the protease (Rosenberg and Domus 1973). The AT III-binding pentasaccharide has been identified as GlcNAc(6SO₄)−GlcA−GlcNS(3-SO₄)−IdoA(2-SO₄)−GlcNS(6-SO₄) (Conrad 1998). The O-sulfate group at C3 of the glucosamine residue is always present, and represents a unique structure found only in the ATIII-binding sequences of heparin. The induction of a conformational change in ATIII appears to be sufficient for the acceleration of the inactivation of factor Xa (Rosenberg and Domus 1973). In contrast, acceleration of the thrombin inhibition reaction by AT III requires an interaction between heparin and thrombin as well as heparin and ATIII (Griffith 1983, Oosta el al. 1981, Laurent et al. 1978).

DERMATAN SULFATE

Dermatan sulfate is an anticoagulant glycosaminoglycan consisting of alternating disaccharide units of hexuronic acid (iduronic acid or glucuronic acid) 1, 3 linked to N-acetylgalactosamine. Variations in the degree of sulfation on both hexuronic acid (2-O-sulfated) and N-acetylgalactosamine (4-O- or/and 6-O-sulfated) are responsible for the extensive heterogeneity of this polymer (Kjellén and Lindahl 1991). The anticoagulant activity of dermatan sulfate is due to the binding of a specific region within the molecule, consisting of [IdoA(2SO₄)−GalNA(4SO₄)] n (n ≥ 3) (Scully et al. 1988, Maimone and Tollefsen 1990, Pavão et al. 1998) to a glycosaminoglycan binding site in heparin cofactor II (HCII), a serine protease inhibitor from plasma (Tollefsen et al. 1982). The binding of dermatan sulfate increases by 1000 fold the specific inhibition of thrombin by HCII (Tollefsen et al. 1983). Since [IdoA(2SO₄)−GalNA(4SO₄)] comprises only 5% of the disaccharides present in dermatan sulfate, clustering of this subunit to form the high affinity hexasaccharide must not be a random biosynthetic event (i.e., the random probability of three such disaccharides occurring together would be 0.000125).

ANTITHROMBOTIC USE OF MAMMALIAN GLYCOSAMINOGLYCANS

Heparin has been used clinically for almost 70 years. It is an important therapeutic agent in the treatment of patients with thrombosis or patients at risk to develop it. Heparin is highly used in the prevention of thromboembolic events frequently observed after some kind of surgery, especially pelvic and orthopedics (Leyvraz et al. 1983, Poller 1985). How-
ever, the unrestricted use of heparin as an antithrombotic agent is limited due to its various side effects, such as thrombocytopenia, osteoporosis, and hemorrhagic complications (Ockelford et al. 1982, Carreras 1980, Mätzsch et al. 1986). More recently, heparin has been gradually replaced by its low molecular weight derivative (less than 3,000 and ~9,000, comparing to ~13,000 of native heparin), obtained by partial fragmentation and fractionation of native heparin (Hamulyák et al. 1995, Meyer et al. 1995). Fragments below 16 to 20 monosaccharide units per heparin molecule (MW < 5,000), while containing the specific pentasaccharide sequence essential for binding AT III, are not long enough to permit binding to thrombin; they therefore inhibit only activated factor X (Choay et al. 1981). Low molecular weight heparins prolong moderately the clotting time (indicating no thrombin inhibition) but are still capable of potentiating the inhibition of activated factor X. This fact at first raised the hope of dissociating the antithrombotic property (anti-Xa) from the anticoagulant (inhibition of thrombin), which then would avoid the hemorrhage-inducing effect of unfractionated heparin. However, it has been shown more recently in animal experiments, that anti-Xa activity is a prerequisite, although not sufficient in itself, for a thrombosis-preventing effect. Heparin molecules, large enough to retain some thrombin-blocking action are indeed also necessary (Holmer et al. 1982, Thomas et al. 1982, Thomas et al. 1989).

Dermatan sulfate was initially shown to have antithrombotic activity in the rabbit jugular venous stasis model (Fernandez et al. 1986). The amount of bleeding from a standardized incision was much greater with heparin than with dermanat sulfate at equivalent antithrombotic doses, suggesting that dermanat sulfate might be a safer therapeutic agent. The relative hemorrhagic effects of the two glycosaminoglycans appeared to be correlated with the ability to inhibit collagen-induced platelet aggregation in vitro. Recent studies suggest that dermanat sulfate is a more potent inhibitor than heparin of clot-bound thrombin (Fernandez et al. 1986, Buchanan et al. 1986) and that dermanat sulfate in combination with low molecular weight heparin may produce synergistic antithrombotic effect (Cosmi et al. 1993). Human clinical trials have shown that dermanat sulfate prevents thrombosis during hemodialysis (Lane et al. 1992) and that in the post-operative setting (Agnelli et al. 1992, Prandoni et al. 1992). However, the mechanism of antithrombotic activity of dermanat sulfate has not been clearly established yet. The antithrombotic mechanism of dermanat sulfate is a matter of controversy. Fernandez et al. (1987) showed that there is a good correlation between antithrombotic potency and HCII activity of dermanat sulfate. However, Dol et al. (1988) reported that enhance of the catalytic activity of dermanat sulfate fail to increase its antithrombotic activity. More recently, Sie et al. (1993) showed that the antithrombotic effect of dermanat sulfate is essentially dependent of HCII binding and activation.

ASCIDIAN GLYOSAMINOGLYCANS

HEPARIN

A heparin with similar structure and lower anticoagulant properties to the mammalian heparin has been identified in the test cells and tissues of the ascidian *Styela plicata* (Cavalcante et al. 2000). An extensive structural analysis of the polymer indicated that the invertebrate heparin is composed mainly by the disaccharide \([\alpha-L-IdoA(2SO_4)-1 \rightarrow 4\beta-D-GlcN(SO_4)(6SO_4)-1]\). About 25% of the disaccharide \([\alpha-L-IdoA-1 \rightarrow 4\beta-D-GlcN(SO_4)(6SO_4)-1]\) is also present. This molecule was shown to occur in cytoplasmic granules of test cells, and also inside granules of epithelial cells forming a layer along the lumen of intestine and pharynx of the ascidian *Styela plicata* (Cavalcante et al. 2000).

The anticoagulant properties of the ascidian heparin (Table I) show that the polymer has an anticoagulant activity 10 times lower than the mammalian heparin. In addition, it is about 20 times less potent in the inhibition of thrombin by antithrombin, when compared to the mammalian counterpart (Table I). However, it activates heparin cofactor II
Dermatan Sulfate

Dermatansulfates,similartothemammalianglycosaminoglycan, but withdifferencesinthedegree and position of sulfation was previously isolated from the body of the ascidian *Ascidia nigra* (Pavão et al. 1995) and *Styela plicata* (Pavão et al. 1998) (Table II). The dermatan sulfate in the tissues of the ascidian occurs in the extracellular matrix of intestine and pharynx (Gandra et al. 2000). The *A. nigra* dermatan sulfate, composed predominantly by \[4\alpha-L-IdoA(2SO_4)-1 \rightarrow 3\beta-D-GalNAc(6SO_4)-1\]_n, where n ≥ 3, has no discernible aPTT activity and has very low ability to potentiate heparin cofactor II, as indicated by the IC50 for thrombin inhibition (Table II). On the other hand, the dermatan sulfate from *S. plicata*, composed by \[4\alpha-L-IdoA(2SO_4)-1 \rightarrow 3\beta-D-GalNAc(4SO_4)-1\]_n, where n ≥ 3, has an aPTT activity 4 times higher than mammalian dermatan sulfate and is a potent activator of heparin cofactor II, possessing a HCII activity 10 times higher when compared to the mammalian counterpart (Table II).

**In Vivo Anticoagulant Activity of Ascidian Dermatan Sulfates**

We have started to investigate the effect of ascidian and mammalian dermatan sulfates on coagulation, using an experimental model in rats. 4 mg/kg of the dermatan sulfate samples were injected intravenously as a bolus in the carotid artery of anesthetized Wistar rats. Blood was collected at different times after administration of the glycans for aPTT analysis (Vicente et al. 2001). We observed an increase in the aPTT up to ~ 3-fold during the first 10 min, following injection of the *S. plicata* dermatan sulfate, returning to basal levels after 20 min. Bovine lung dermatan sulfate induced a less intense change in the aPTT values, with a maximum increment of 1.5-fold at 15 min, returning to normal values after 20 min. *A. nigra* dermatan sulfate did not produce any significant change in the aPTT during the 60-min experiment. These results show a good correlation with the experiments obtained *in vitro*, where the aPTT was measured in human plasma. Intravascular administration in rats of the low HCII active *A. nigra* dermatan sulfate, which showed a discernible aPTT activity *in vitro* (Table II), also did not produce a significant change in the aPTT in the experiment *in vivo*. Mammalian dermatan sulfate, which has an intermediate HCII activity, produced a mild pronounced change in the aPTT in the experiments *in vivo* (Vicente et al. 2001).

**TABLE I**

Values of plasma aPTT and IC50 for thrombin inhibition by AT and HCII in the presence of bovine or *S. plicata* heparin.

<table>
<thead>
<tr>
<th></th>
<th>aPTT Units/mg</th>
<th>IC50 (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine</td>
<td>193</td>
<td>0.0005</td>
</tr>
<tr>
<td><em>S. plicata</em></td>
<td>19.3</td>
<td>0.01</td>
</tr>
</tbody>
</table>

**In Vivo Antithrombotic Activity of Ascidian Dermatan Sulfates**

The effect of ascidian and bovine lung dermatan sulfate samples on thrombosis was investigated preliminarily, using an experimental venous thrombosis model. A single injection of *S. plicata* dermatan sulfate, given 5 min before the thrombogenic stimulus with brain thromboplastin caused a dose-dependent inhibition of thrombus formation. With the dosage of 1.6 mg/kg body weight, a 50% reduction was observed and with 4 mg/kg body weight, thrombosis was not observed in any of the animals. Surprisingly, mammalian dermatan sulfate was more effective than *S. plicata* dermatan sulfate in dosages up to 3 mg/kg body weight. A 50% reduction was observed at the dosage of 0.9 mg/kg body weight and with 4 mg/kg, mammalian dermatan sulfate totally...
TABLE II

Major repetitive disaccharide units of bovine dermatan sulfate, *Styela plicata* dermatan sulfate and *Ascidia nigra* dermatan sulfate, and their anticoagulant properties in vitroa.

<table>
<thead>
<tr>
<th>DS sample</th>
<th>Major disaccharide units</th>
<th>aPTT (units/mg)b</th>
<th>HCII activity IC50 (µg/ml)c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine lung</td>
<td>[4-α-L-IdoA-1 → 3-β-D-GalNAc(4SO4)-1]n</td>
<td>2</td>
<td>3.0</td>
</tr>
<tr>
<td><em>Styela plicata</em></td>
<td>[4-α-L-IdoA(2SO4)-1 → 3-β-D-GalNAc(4SO4)-1]n</td>
<td>8</td>
<td>0.31</td>
</tr>
<tr>
<td><em>Ascidia nigra</em></td>
<td>[4-α-L-IdoA(2SO4)-1 → 3-β-D-GalNAc(6SO4)-1]n</td>
<td>0.5</td>
<td>320</td>
</tr>
</tbody>
</table>

aThese glycosaminoglycans have the same backbone structure [4-α-L-IdoA-1 → 3-β-D-GalNAc-1]n, but possess different patterns of sulfation. The repetitive disaccharide units of bovine dermatan sulfate are sulfated at carbon 4 of the galactosamine moiety; small amounts (< 5%) of 2-O-sulfated α-L-iduronic acid residues are also present. The *S. plicata* dermatan sulfate is sulfated at both 2-O-position of the α-L-iduronic acid and the 6-position of the N-acetyl-β-D-galactosamine units. On the *A. nigra* dermatan sulfate most of the α-L-iduronic units are 2-O-sulfated and the N-acetyl-β-D-galactosamine are 6-O-sulfated. These differences produce profound effects on their anticoagulant properties. The *S. plicata* dermatan sulfate has the higher aPTT and HCII activities, whereas the *A. nigra* dermatan sulfate has the lowest anticoagulant properties. Bovine lung dermatan sulfate possesses intermediate aPTT and HCII activities. HCII, heparin cofactor II. b aPTT activity is expressed in units/mg, using a standard curve based on the international Heparin standard (193 units/mg). c Values obtained from Pavão et al. 1998.

Effect of Ascidian Dermatan Sulfates on Platelets and Hemorrhagic Effect

To investigate the effect of dermatan sulfate samples on platelet aggregation, ascidian or bovine lung dermatan sulfates were added to a suspension of human washed platelets, and the platelets in solution were counted by a cell counter. Alternatively, platelet aggregation was evaluated in human platelet rich plasma (PRP) by aggregometry. In control experiments ADP was used as an inductor of plasma aggregation. ADP reduced the single platelet count from ~ 300 × 10^6 platelets/ml to ~ 50 × 10^6 platelets/ml. Addition of *S. plicata*, *A. nigra* or bovine lung dermatan sulfate did not produce a reduction in the single platelet count. After addition of ascidian or mammalian dermatan sulfate samples to a human PRP preparation no aggregation was observed during a 20-min experiment. ADP, on the other hand, induced platelet aggregation almost instantaneously.

The hemorrhagic risk of dermatan sulfate samples was assessed based on blood loss from a rat cut-tail, after intravascular administration of the glycans. At the dosage of 4mg/kg body weight, the *S. plicata* dermatan sulfate did not modify the blood loss compared with rats receiving saline. Surprisingly, the blood loss was increased ~ 2-fold in rats receiving the same dosage of *A. nigra* or bovine lung dermatan sulfate (Vicente et al. 2001).

Generally, bleeding effect results from modification of blood coagulation induced by polysaccharides. An increase in plasma aPPT values is usually related with high bleeding effect (Hirsh 1984, Thomas and Roberts 1997). We used a rat cut-tail bleeding experimental model to compare the hemorrhagic effect of the ascidian and mammalian dermatan sulfates. Surprisingly, the *S. plicata* dermatan sulfate at the dosage where the higher antithrombotic effect is observed (4mg/kg body weight), showed the same hemorrhagic effect as that of the control. In contrast, mammalian and *A. nigra* dermatan sulfate, increased blood loss almost 2-fold, compared to the control. The high bleeding effect of mammalian and *A. nigra* dermatan sulfate observed does not seem to be related to an effect of these polysaccharides on platelets, since none of the dermatans induced platelet aggregation (Vicente et al. 2001).

**CONCLUSION**

The data reported in the present review provide important information about the chemical structures in glycosaminoglycans that produce the anticoagulant, antithrombotic and hemorrhagic effects of these polymers. Thus, the anticoagulant activity of dermatan sulfate is related to sulfation at specific positions at carbon 2 of iduronic acid and at carbon 4 of the N-acetylgalactosamine residues, that are required to bind and activate heparin cofactor II. Binding and activation of heparin cofactor II is necessary for the antithrombotic activity of dermatan sulfate, but an enhancement of the HCII activity does not increase the antithrombotic potency of the polymer. Moreover, the hemorrhagic effect of these polymers does not seem to be associated with their anticoagulant effect.

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**RESUMO**

Dermatam sulfato e heparina semelhantes aos glicosaminoglicanos de mamíferos, mas apresentando diferenças no grau e posição de sulfatação foram previamente isolados do corpo das ascídias *Styela plicata* e *Ascidia nigra*. Estas diferenças produzem efeitos profundos nas suas propriedades anticoagulantes. O dermatam sulfato de *S. plicata*, composto por resíduos de ácido *α*-L-idurônico 2-O-sulfatados e N-acetilgalactosamina 4-O-sulfatados é um potente anticoagulante devido a sua alta atividade de cofator II da heparina. Surpreendentemente, este polímero possui uma menor potência na prevenção da formação de trombos e um efeito de sangramento menor, quando comparado com o dermatam sulfato de mamíferos em modelos experimentais em ratos. Por outro lado, o dermatam sulfato de *A. nigra*, também enriquecido em resíduos de ácido idurônico 2-O-sulfatados, mas neste caso sulfatado na posição O-6 das unidades de N-acetilgalactosamina, não apresenta atividade anticoagulante in vitro ou in vivo, não previne a formação de trombos, mas possui um efeito de sangramento semelhante ao glicosaminoglicano de mamífero. A heparina da ascídia, composta por unidades dissacarídeas de ácido *α*-L-idurônico 2-O-sulfatado, glucosamina N- e 6-O-sulfatada (75%) e ácido *α*-L-idurônico, glucosamina N- e 6-O-sulfatada (25%), possui uma atividade anticoagulante 10 vezes menor que a heparina de mamíferos, é aproximadamente 20 vezes menos potente na inibição da trombina por antitrombina, mas possui a mesma atividade de cofator II da heparina de mamíferos.

**Palavras-chave:** ascídias, heparina, dermatam sulfato, heparina cofator II, anticoagulante, antitrombótico.
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