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Glycosaminoglycan backbone is not required for the modulation of hemostasis: Effect of different heparin derivatives and non-glycosaminoglycan analogs ☆

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ABSTRACT

Heparin and its derivatives are known to regulate a variety of pathophysiological events related to vascular biology. In the present manuscript we examine a variety of heparinomimetics biochemically (electrophoretic behavior and enzymatic degradation) and pharmacologically (in vitro anticoagulant activity and in vivo hemorrhagic and antithrombotic tests) as well as their interactions with cells from the vessel wall using a time resolved fluorometric method and confocal microscopy. Data were determined for unfractionated heparin (UFH), enoxaparin, synthetic heparin pentasaccharide, C3 heparin derived oligosaccharides and phosphosulfomannan (PI-88). While being structurally distinct from UFH, all compounds exhibited anticoagulant, antithrombotic and hemorrhagic activities. In addition, besides the pentasaccharide, they all stimulated the synthesis of an antithrombotic heparan sulfate present at the cell surface and secreted by endothelial cells. Also, like UFH, they interacted with both endothelial and smooth muscle cells and dislodged UFH from its binding sites in a dose dependent manner but, with distinct saturable curves showing that the binding of polymeric structures to extracellular matrix (ECM) proteins does not depend on a glycosaminoglycan backbone. The data also suggest a common pathway, which does not depend on the presence of the conventionally accepted antithrombin binding pentasaccharide, for ECM dependent activity of the heparinomimetic stimulated synthesis of antithrombotic heparan sulfate. Notably, although of similar molecular weight as well as polymeric backbone, the synthetic heparin pentasaccharide showed significant hemorrhagic action and negligible antithrombotic activity in a venous thrombosis model, contrasting with C3, that displayed negligible hemorrhagic effect and potent antithrombotic action. These results provide evidence that structurally unrelated polymers can elicit similar hemostatic activities and show that polymeric sequence is not always crucial for certain activities. The results also suggest that non-GAG structures may provide an alternative route for the pharmaceutical control of hemostasis.

1. Introduction

The search for compounds that may play a role in the prevention and treatment of cardiovascular disorders, thrombosis and neoplasia has been the purpose of several investigations. Heparin is the major antithrombotic drug, widely used for prevention and treatment of venous thrombosis. It was first used in Medicine to prevent blood clot during extracorporeal circulation (Best et al., 1938) and introduced into clinical use in the 1940s to prevent thrombosis in surgical patients (Jorpes, 1952) and in the treatment of deep venous thrombosis (Murray and Best, 1938; Bauer, 1950). However, a number of disadvantages are known to its clinical use. These include extreme structural diversity (Lindahl et al., 1989; Nader et al., 2004), heparin-induced thrombocytopenia and the risk of bleeding (Kelton and Hirsh, 1980; Hirsh, 1984; Arthur et al., 1985; Fareed et al., 1999), poor bioavailability, variable and extremely steep dose–response curve (Hirsh, 1984; Beijering et al., 1996), and more recently deliberate contamination, which led to a worldwide crisis (Blossom et al., 2008; Guerrini et al., 2008; Kishimoto et al., 2008; Lima et al., 2011). Thus, low molecular weight heparins (LMWHs), chemically synthesized or semi-synthetic heparin derivatives and

☆ Dedicated to the memory of Professor Carl P. Dietrich, a pioneer in the studies of structure and biological activities of heparins.

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heparinomimetics have been developed, some of them reaching market approval, in order to overcome some of the clinical disadvantages of unfractionated heparin (UFH).

LMWHs have been introduced as potential replacements for heparin in a variety of thrombotic indications. They exert their different biological activities through interactions with various proteins (Hileman et al., 1998; Mulloy and Linhardt, 2001; Capila and Linhardt, 2002; Sampaio et al., 2006). Among them, antithrombin (AT), heparin cofactor II, tissue factor pathway inhibitor and platelet factor 4 are important determinants for their anticoagulant effects (Lane and Caso, 1989; Tersariol et al., 1992; Sheehan et al., 1993; Weber et al., 1995; Delacoux et al., 1998; Hileman et al., 1998; Berge et al., 1999; Esko, 1999; Mulloy and Linhardt, 2001; Capila and Linhardt, 2002; Sasisekharan et al., 2002; Shinjo et al., 2002; Powell et al., 2004; Coombe and Kett, 2005; Handel et al., 2005; Johnson et al., 2005; Nozik-Grayck et al., 2005; Norrby, 2006; Sampaio et al., 2006; Taylor and Gallo, 2006). The molecular weight, charge density, types of disaccharide present in the chains, pattern of *N*- and *O*-sulfation as well as *N*-acetylation influence the interaction with the latter proteins.

C3, an oligosaccharide mixture produced by γ-irradiation of heparin developed as a potential agent for senile dementia, has been selected for this study due to its ultra low molecular weight (MW 2400 Da) and structural similarity to heparin. C3 shows low anticoagulant effects but, exhibits neuronal action once it passes through the blood–brain barrier. Also, comparative studies showed that C3 was devoid of anti-factor IIa activity and its anti-factor Xa activity was 85% lower than that of UFH (Ma et al., 2002).

The synthetic heparin pentasaccharide (SR90107/Org31540), the first compound in a new class of synthetic oligosaccharides with antithrombotic effects, consists of five saccharide units with sulfate groups strategically positioned, mimicking the UFH motif active for AT, to bind strongly and exclusively to AT, the primary endogenous regulator of blood coagulation (Petitou et al., 1991; Petitou and van Boeckel, 1992), inducing a conformational change that increases the anti-factor Xa activity of antithrombin more than 270 times, without inhibition of factor IIa (Olson et al., 1992).

On the other hand, PI-88 is a highly sulfated oligosaccharide obtained as a randomly, but reproducibly, sulfated mixture with an average of three sulfates per mannose residue bearing one phosphate group at the C-6 position of the non-reducing end and a molecular weight of 2100–2585 Da (Parish et al., 1999; Yu et al., 2002). Thus, the only obvious similarity shared with heparin is its highly polyanionic character. PI-88 also inhibits heparanase activity and competes with heparan sulfate for the binding of growth factors involved in angiogenesis, such as FGF and VEGF. This compound consistently prolongs the activated partial thromboplastin time (APTT) through activation of endogenous heparin cofactor II (Parish et al., 1999; Khachigian and Parish, 2004).

In the present paper the activities of these heparinomimetics were evaluated using biochemical and biological assays and compared to those of UFH. Also, the binding of such compounds to endothelial and smooth muscle cells in culture was investigated by confocal microscopy and ELISA-like assay.

2. Results and discussion

2.1. Biochemical characteristics of the heparinomimetics

The electrophoretic behavior of the different heparinomimetics in agarose gel is depicted in Fig. 1. The compounds show different electrophoretic mobilities reflecting the interaction with the diamine in the PDA buffer (Fig. 1A). This binding is not related to net charge, but to the conformation of the sugar residues and the distribution of charges in the molecules (Dietrich et al., 1977; Nader et al., 2004). In the discontinuous barium/PDA buffer (Fig. 1B), all heparinomimetics showed a single component, contrasting with UFH, that is divided into three bands named fast, intermediate and slow according to differences in molecular masses and charge distribution (Dietrich et al., 1977; Medeiros et al., 2000). All compounds were detected in the gel, including the pentasaccharide. PI-88 displayed the strongest interaction with diamine and barium ions.

Glycosaminoglycan lyases act upon the polysaccharide chain cleaving the glycosidic bond by a beta elimination mechanism leaving an unsaturated uronic acid (C4–C5 double bond) at the non-reducing end. Among the lyases that degrade heparin and heparan sulfate, heparitinase I and heparinase show selective substrate specificity requirements for their activities. Heparinase acts upon α-D-glucosaminido-iduronate linkages. The sulfation at the C-2 position of the iduronate residue is obligatory for activity, and *N*-acetylation of the glucosamine moiety impedes enzyme action (Nader et al., 1999). On the other hand, heparitinase I acts only upon α-D-glucosaminido-glucuronate, and sulfation at the C-6 position of the glucosamine impedes enzyme activity. In contrast, heparitinase II has broad specificity, acting upon heparin and heparan sulfate chains. Nevertheless, this enzyme shows no action upon glucosaminido-glucuronate linkages when the glucosamine is *N*-acetylated or in clusters of trisulfated disaccharides (Nader et al., 1990; Nader et al., 1999; Sampaio et al., 2006). UFH as well as heparinomimetics were subjected to degradation using specific enzymes (Fig. 1C) and the products analyzed both by agarose gel electrophoresis and chromatography. The lower molecular mass products were not precipitated in the gel with quaternary ammonium salts after enzymatic degradation, yet they were separated and quantified by chromatography. UFH was totally degraded by the combined action of heparinase and heparitinase II, which also occurred with enoxaparin. On the other hand, C3 was shown to be a poor substrate for heparitinase II, being mainly degraded by heparinase (Fig. 1C). Upon the action of heparinase and heparitinase II, UFH, enoxaparin and C3 yielded the typical heparin disaccharides in different proportions (Fig. 1D) (Nader et al., 1990). Glucosamine N,6-disulfate is located at the non-reducing end of the compounds and its relative amount increases as the molecular weight decreases (Dietrich et al., 1998). As expected, the lyases did not degrade PI-88. Although sulfated at the various positions required for the recognition by the heparin lyases, this compound does not possess a heparin/heparan backbone rendering it resistant to the enzymatic depolymerization. The pentasaccharide incubated under the same conditions was not degraded by either heparinase or heparitinase II, indicating that even though the compound retains several of the heparin structural characteristics the methylation at the reducing end of the chain makes the enzymatic action difficult. Nevertheless, incubation of the pentasaccharide in the presence of 100 times more enzyme for several days yields the production of disaccharide and trisaccharide to a certain extent (data not shown) as previously described (Yu et al., 2000). Also, heparitinase I showed no action on any of the compounds (data not shown).

2.2. Anticoagulant, antithrombotic and hemorrhagic activities

All compounds showed anticoagulant activity in the APTT test (Fig. 2A). Nevertheless, the activity for UFH is around 10 times higher when compared to the other compounds. A dose–response curve of the heparinomimetics in a venous thrombosis model in rats is shown in Fig. 2B. All compounds except C3 showed similar activity to UFH. On the other hand, in the thrombosis model the pentasaccharide showed much lower antithrombotic activity, about 10 times less than that observed for UFH, LMWH as well as C3. C3 is composed mainly of highly sulfated hexasaccharides, in which, according to the degradation by heparinase, the internal uronic acid is α-L-iduronic acid 2-O-sulfate. Thus the difference in activity between C3 and the pentasaccharide is a