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Effects of glycosaminoglycans on platelet and leucocyte function: role of N-sulfation

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Abstract—The effect of glycosaminoglycans (GAGs) such as sulodexide, low molecular mass dermatan sulfate, heparin and some derivatives with different degrees and types of sulfation was studied on cathepsin G- or thrombin-stimulated platelets and *n*-formyl-methionyl-leucyl-phenylalanine (fMLP)-stimulated polymorphonuclear leucocytes (PMNs). All GAGs (0.01–20 μg/mL) inhibited both platelet aggregation induced by cathepsin G and its catalytic activity. Thrombin-induced platelet aggregation in contrast was only prevented by heparin, sulodexide and dermatan (2–100 μg/mL). All GAGs, except 2-O,N-desulfated heparin, inhibited β-glucuronidase and lysozyme release, as well as β-glucuronidase activity and PMN superoxide production by the peptide fMLP. The efficacy of GAGs was clearly dependent on the degree and type of sulfation since dermatan and N-desulfated heparins were comparatively less effective. The observation that heparin and other GAGs inhibit platelet activation induced by the PMN protease cathepsin G may help determine whether mechanisms of action other than anticoagulation are critical in the antithrombotic activity of heparin and related compounds.

Heparin is a major anticoagulant drug widely used in the prevention and treatment of thrombosis. The main action of heparin on blood clotting is the potentiation of thrombin inhibition by antithrombin III. However, it has been questioned whether anticoagulation is fully responsible for the antithrombotic activity of heparin [1]. It has been reported for instance that various heparins prevent platelet activation, induced either by stimulated polymorphonuclear leucocytes (PMNs*) or by purified cathepsin G, a neutral serine protease contained, together with elastase, in the azurophilic granules of PMNs [2–5]. The mechanism suggested for this inhibition is ionic interaction between the negatively charged heparin and a cationic molecule, such as cathepsin G; a similar mechanism was proposed for the inhibition by heparin of another PMN protease, elastase [6–8].

In order to understand the mechanism of action of some heparins, with different types of sulfate bonds and degrees of sulfation, and some glycosaminoglycans (GAGs), we investigated their action on cathepsin G- or thrombin-stimulated platelets, and on *n*-formyl-methionyl-leucyl-phenylalanine (fMLP)-stimulated PMNs.

Materials and Methods

Unfractionated heparin sodium salt, total N-desulfated, 2-O-desulfated and 2-O,N-desulfated heparin, low molecular mass dermatan sulfate ( )esmin 370) [9] and sulodexide from Alfa Wassermann S.p.A. (Bologna, Italy) were dissolved in isotonic saline just before use. Sulodexide, extracted from pig intestinal mucosa, contained two principal components: 80% fast-moving heparin and 20% dermatan sulfate [10]. Table 1 reports the chemico-physical characteristics of the tested compounds. Preparation of compounds and determination of molecular mass have been reported elsewhere [10, 11].

Human washed platelets (10^8 cells/mL) and PMNs (0.5×10^7 cells/mL), prepared as described [12], were resuspended in the presence of 1 mM Ca^{2+}. Purified human fibrinogen (0.38 mg/mL; Kabi Diagnostica, Stockholm, Sweden) (except when thrombin was used as a stimulus) and cytochalasin B (2.5 μg/mL; Sigma Chemical Co., St Louis, MO, U.S.A.) were added to platelet and PMN suspensions, respectively. Samples were incubated in an Elvi 840 aggregometer (Elvilogos, Milano, Italy) for 2 min at 37° without stirring, followed by 1 min under constant stirring at 1000 rpm, with or without GAGs. Different preparations of heparin, sulodexide or dermatan were added to the cell suspension at appropriate concentrations in a volume of 10 μL/mL. The cell suspension was then stimulated by cathepsin G (0.2 μM; purified from human PMNs, Calbiochem, San Diego, CA, U.S.A.) or thrombin (0.1–0.3 U/mL; purified from human plasma, 2000 NIH U/mg protein, Sigma). Platelet aggregation was expressed as per cent inhibition of maximal light transmission at 3 min. Three minutes after stimulation with fMLP (1 μM; Sigma), PMNs were cooled to 0° and centrifuged for 1.5 min (14,000 rpm); supernatants were stored at −20° for measurement of β-glucuronidase and lysozyme release [12]. The effect of GAGs on enzyme activity was evaluated by the addition of different concentrations of compounds to the cell-free enzyme obtained from PMNs lysed by 0.1% Triton X-100.

Superoxide anion production was determined by measuring the reduction of cytochrome *c* (Sigma) [12]. Cathepsin G and thrombin catalytic activities were measured with the specific chromogenic substrates *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Sigma) and chromozym TH (Tos-Gly-Pro-Arg-*p*-nitroanilide, Boehringer Mannheim, Mannheim, Germany), respectively [13]. GAGs were added 30 sec before the addition of cathepsin G or thrombin. For other methodological details not mentioned in this section, see Refs 4 and 12.

Results and Discussion

As summarized in Table 1, all GAGs tested inhibited both the catalytic activity of cathepsin G and platelet aggregation induced by this enzyme in a concentration-dependent manner and with a comparable range of potency. This inhibition was also dependent on the type and degree of molecule sulfation. The less active compounds were 2-O,N-desulfated heparin and low molecular mass dermatan sulfate. The *N*-sulfate group appeared to be more important than the *O*-sulfate group for the anti-cathepsin G action of GAGs. In fact, both N-desulfated heparin and dermatan, which lack *N*-sulfate groups, were less potent than sulodexide, heparin and its 2-O-desulfated derivative. A correlation between the inhibitory efficacy of heparin-derived oligosaccharides with a different molecular mass and degree of sulfation has also been reported against the other PMN-derived protease, elastase. However, in the latter case the *O*-sulfate group of GAGs was more

* Abbreviations: GAGs, glycosaminoglycans; PMN, polymorphonuclear leucocyte; fMLP, *n*-formyl-methionyl-leucyl-phenylalanine.

Table 1. Chemico-physical characteristics and inhibitory concentrations (IC_{50}, μg/mL) of glycosaminoglycans on platelet aggregation induced by cathepsin G or thrombin and on the catalytic activity of cathepsin G and thrombin

	Molecular mass (Da)	SO_3^-/COO^-	Cathepsin G		Thrombin	
			Aggregation	Catalytic activity*	Aggregation	Catalytic activity
Sulodexide	7000	1.7	0.16 ± 0.02	0.30 ± 0.04	33.40 ± 1.80	0.77 ± 0.07
Dermatan	4400	1.1	2.63 ± 0.32	2.88 ± 0.53	>100	45.65 ± 6.70
Heparin	12,000	2.1	0.12 ± 0.01	0.37 ± 0.05	24.85 ± 4.55	0.19 ± 0.07
2-O-desulfated heparin	10,700	1.4	0.31 ± 0.03	0.48 ± 0.03	>100	>100
N-desulfated heparin	10,600	1.3	1.08 ± 0.03	0.62 ± 0.15	>100	>100
2-O,N-desulfated heparin	9300	0.6	24.30 ± 0.77	5.68 ± 0.44	>100	>100

IC_{50} values were calculated from mean inhibition curves of at least five different experiments using the Allfit program [4].

* Since inhibition of cathepsin G catalytic activity never exceeded 60% inhibitory concentrations, for this parameter IC_{30} was calculated.

important than the *N*-sulfate group for inhibitory activity [8]. In contrast with platelet aggregation, which could be fully suppressed, maximal inhibition of the protease activity never exceeded 60%. The postulated cause of this incomplete inhibition was the use of a small substrate, such as *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide, for cathepsin G catalytic activity measurements: the latter, at variance with the receptor bound to the platelet membrane, could maintain some residual access to the catalytic site of cathepsin G [2, 8]. Heparin might also exert a direct effect on platelets, in view of heparin-binding sites on their surface [14, 15]. Polyanions, such as heparin and dextran sulfate, reportedly blocked the effect of cathepsin G on endothelial [16] and epithelial [17] cells, through the charge or charged site(s) on the enzyme, while proteolytic activity did not seem essential. At variance, the effect of cathepsin G on platelets was dependent on its intact proteolytic activity [18].

Sulodexide and heparin (but not its desulfated derivatives), with equivalent potency, inhibited thrombin-induced platelet aggregation in a range of concentrations from 5 to 100 μg/mL, while the effect of dermatan was much less pronounced. Even at the highest concentration tested (100 μg/mL), dermatan did not exceed 40% inhibition of platelet aggregation, in agreement with previously reported data [19]. However, GAGs seem to be much more effective in preventing thrombin-induced platelet aggregation in platelet-rich plasma, where antithrombin III and heparin cofactor II complexes to thrombin may play a role [20]. Direct binding of sulfated polysaccharides to the platelet may also affect thrombin-induced aggregation.

Heparin, sulodexide and dermatan inhibited the hydrolysis of the thrombin-specific substrate in a concentration-dependent manner; dermatan and, in particular, desulfated heparin derivatives were significantly less active and did not completely prevent the hydrolysis of the specific substrate even at the highest concentration used. Since catalytic function is essential for thrombin aggregating activity, the discrepancy between the concentrations of GAGs inhibiting thrombin aggregating effect and thrombin catalytic activity remains to be elucidated.

All GAGs tested, except the 2-O,N-desulfated heparin derivative, inhibited the release of β-glucuronidase and lysozyme in a concentration-dependent manner (Fig. 1). Besides their number, a relation seems to exist between the position of the sulfate groups in the GAGs and their interaction with lysosomal enzyme release, as N-desulfated

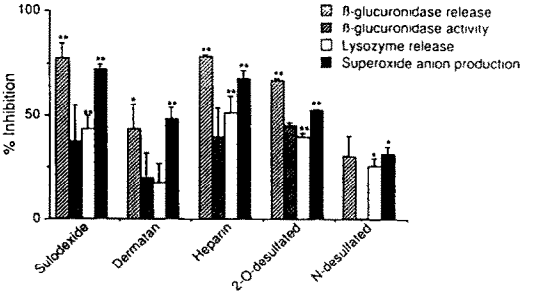

Fig. 1. Effect of different GAGs (100 μg/mL) on β-glucuronidase and lysozyme release by fMLP-stimulated PMNs, on the activity of β-glucuronidase from lysed PMNs and on superoxide anion production by fMLP-stimulated PMNs. Data (means ± SEM; N = 4) are expressed as per cent inhibition of controls. Control values for β-glucuronidase and lysozyme release were 34.5% ± 1.6 and 58.9% ± 4.8 of total enzyme content, respectively. Control values for superoxide anion production were 8.4 ± 1.7 nmol cytochrome *c* reduced/10^6 PMNs/40 min. *$P < 0.05$, **$P < 0.01$ significantly different from control group by ANOVA and Dunnett's test.

heparin derivatives and dermatan were less active than the O-desulfated derivative. The reduction of β-glucuronidase activity release was twice as high as compared to that of lysozyme. GAGs also exerted a direct inhibitory action on β-glucuronidase activity, but did not affect the activity of lysozyme. The compounds tested also impaired superoxide production by fMLP-stimulated PMNs and in this case also the 2-O-desulfated derivative of heparin was completely inactive, while the N-desulfated derivative was less effective than the others. These results provide further evidence for a role of GAGs as regulatory molecules of the intragranular digestive activity of PMNs [21, 22].

Platelet activation by cathepsin G, released from activated PMNs, could be one of the mechanisms by which these cells contribute to venous or arterial thrombosis [12, 23]. The observation that heparin and other GAGs inhibit platelet activation induced by cathepsin G may help to determine whether mechanisms of action other than

anticoagulation are critical in the antithrombotic activity of heparin and related compounds.

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