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Heparan sulphate with no affinity for antithrombin III and the control of haemostasis

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Heparan sulphate with no affinity for antithrombin III (ATIII) was observed to cause acceleration of the factor Xa:ATIII interaction by 1100-fold (k_2, 7×10^7 $M^{-1} \cdot min^{-1}$) and the prothrombinase:ATIII interaction by 2900-fold (k_2, 2.5×10^7 $M^{-1} \cdot min^{-1}$). Although high-affinity heparan sulphate catalyzed higher acceleration and at lower concentration, in natural mixtures of the two forms the activity of the no affinity form predominated. Heparan sulphate had no significant effect on the thrombin:ATIII interaction but inhibited its potentiation by heparin (K_d 0.3 μM). From the estimated concentration of heparan sulphate on the endothelial cell surface it is proposed that the non-thrombogenic property of blood vessels is due to the acceleration of the factor Xa or prothrombinase:ATIII interaction by the greater mass of surface-bound heparan sulphate rather than by the much smaller proportion of heparin-like molecules (with high affinity for antithrombin III) which may be present.

Heparan sulfate; Dermatan sulfate; Antithrombin III; Hemostasis

1. INTRODUCTION

It is well established that the serine protease inhibitor, antithrombin III (ATIII) is important in the control of haemostasis and familial deficiency of ATIII (blood levels less than about 50% of normal) is associated with recurrent thromboembolic disease [1,2]. However, the rate of interaction between the inhibitor, at physiological concentrations, and the activated coagulation factors is substantially less than that which is characteristic for proteinase inhibitors [3]. The rate is accelerated, catalytically, by heparin [4] and it has been proposed that heparin-like molecules (i.e. with the property of tight binding to ATIII) located on the vascular endothelium are responsible for the ATIII-dependent, non-thrombogenic properties of blood vessels [5]. Heparan sulphate proteoglycans with this property have been isolated from aortic tissue [6] and from endothelial cell cultures [7]. In relationship to the total mass of sulphated glycosaminoglycans (GAGS) in such material the heparin-like material is present at very low concentrations (about 0.2% of the total) [8]. Although heparin is particularly effective in accelerating the protease:ATIII interaction, other GAGS (e.g. dermatan sulphate) present within the vasculature have been found to possess this property but to a lesser degree [9,10]. These latter studies compared the potency of these GAGS to heparin and did not evaluate their potential to be involved in the non-thrombogenic property of blood vessels. In this present work, we have evaluated the potency of these GAGS, using a kinetic approach and propose that the ATIII-dependent, non-thrombogenic property of the vascular endothelium may arise from the mass of surface-bound heparan sulphate rather than to the small proportion of heparan sulphate molecules with a high affinity for ATIII.

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2. MATERIALS AND METHODS

2.1. *Materials*

Human factor Xa, prothrombin and ATIII were prepared as described previously [11–13]. Human α-thrombin was kindly supplied by Dr J. Fenton, New York Dept of Health, Albany, USA. Pure heparan sulphate (SO_4/COOH ratio, 1.68; anticoagulant activity, 5 U/mg) and dermatan sulphate (SO_4/COOH, 1.19; anticoagulant activity, 8 U/mg) prepared from porcine intestinal mucosa were obtained from Mediolanum Farmaceutici, Milan, Italy and gave single bands upon electrophoresis. Chondroitin sulphate A (whale cartilage) and chondroitin sulphate C (shark cartilage) were obtained from Sigma, Poole, England. Standard heparin (average molecular mass 15 kDa) and low molecular mass heparin (Fraxiparine®, 4.2 kDa) were obtained from Leo Laboratories, England and Choay Laboratories, France, respectively. Washed platelets were prepared as previously described [14]. Fractionation of heparan sulphate and heparin over ATIII immobilized on concanavalin A (conA) Sepharose were made as described previously [15].

2.2. *Determination of the rate of inhibition of factor Xa, platelet-bound factor Xa (prothrombinase) and thrombin by ATIII in the presence of various GAGS*

This was made under pseudo first-order conditions as described previously [14,16].

2.3. *Measurement of antiheparin activity*

The inhibition, by increasing the concentration of GAGS, of the rate of interaction between ATIII and thrombin in the presence of heparin was measured in mixtures containing 25 nM ATIII, 1.5 nM thrombin, 3.3 nM heparin (with high affinity for ATIII) and 0.2 mM chromogenic substrate S2238 (KabiVitrum, England) in 0.1 M triethanolamine/HCl (pH 7.8)/0.1 M NaCl containing 0.1% (w/v) poly(ethylene glycol) 6000 at 37°C. The change in absorbance was followed at timed intervals in an Abbott bichromatic analyzer using 380/450 nm filter and first-order rate constants calculated from semi-logarithmic plots of absorbance against time, as described previously [16].

3. RESULTS

3.1. *Fractionation of heparan sulphate upon immobilized ATIII*

Heparan sulphate was chromatographed over ATIII immobilized upon ConA-Sepharose [15] and fractions pooled separately and concentrated to represent no affinity (NA), low-affinity (LA) and high-affinity (HA) fractions, respectively. The yield of each was measured and found to be, as a percentage of the total, 97.4% for NA, 2.0% for LA and 0.6% for HA. Pooling of fractions after chromatography of a similar amount of heparin gave the following percentages: 45% (NA), 33% (LA) and 23% (HA). Very little heparan sulphate was bound, therefore, to a column capable of binding substantial amounts of low- and high-affinity heparin.

3.2. *Acceleration of the rate of inhibition of factor Xa and prothrombinase by ATIII in the presence of various GAGS*

Neither chondroitin sulphate A nor chondroitin sulphate C showed any significant effect on the rate of inhibition of free factor Xa or prothrombinase-bound factor Xa by ATIII. Both heparan sulphate with no affinity and dermatan sulphate were able to accelerate these reactions (fig.1). With free factor Xa the maximum observed rate at 140 μM heparan sulphate was equal to a second-order rate constant of $7 \times 10^7\ M^{-1} \cdot min^{-1}$ or 1100-fold increase over the basal rate of $6.3 \times 10^4\ M^{-1} \cdot min^{-1}$ in the absence of GAGS [14]. The maximum rate of inhibition of factor Xa in the prothrombinase complex was equal to a second-order rate constant of $2.5 \times 10^7\ M^{-1} \cdot min^{-1}$ or 2900-fold increase over a basal rate of $0.85 \times 10^4\ M^{-1} \cdot min^{-1}$ [13]. High-affinity heparan sulphate was a potent activator of the factor Xa:ATIII interaction (6500-fold, $4.1 \times 10^8\ M^{-1} \cdot min^{-1}$) but in the natural mixture of forms, the activity of heparan sulphate with no affinity tended to predominate and an acceleration of 1000-fold was observed.

Dermatan sulphate was somewhat less potent but reached a maximum rate with free factor Xa equal to a 580-fold acceleration ($3.6 \times 10^7\ M^{-1} \cdot min^{-1}$) and 850-fold of the prothrombinase:ATIII interaction ($7.5 \times 10^6\ M^{-1} \cdot min^{-1}$). The maximal fold acceleration by a low molecular mass heparin (high affinity for ATIII, M_r 4.2 kDa) was 660-fold ($4 \times 10^7\ M^{-1} \cdot min^{-1}$) with free factor Xa and 1930-fold ($1.7 \times 10^7\ M^{-1} \cdot min^{-1}$) with prothrombinase.

3.3. *Influence of GAGS on the thrombin:ATIII interaction*

None of the GAGS, apart from the HA heparan sulphate, caused significant acceleration of the thrombin:ATIII interaction (<30-fold maximum acceleration) as we have previously observed for non-heparin GAGS [16]. However, all caused inhibition of the rate of interaction between thrombin and ATIII occurring in the presence of heparin (fig.2). This anti-heparin activity has been iden-

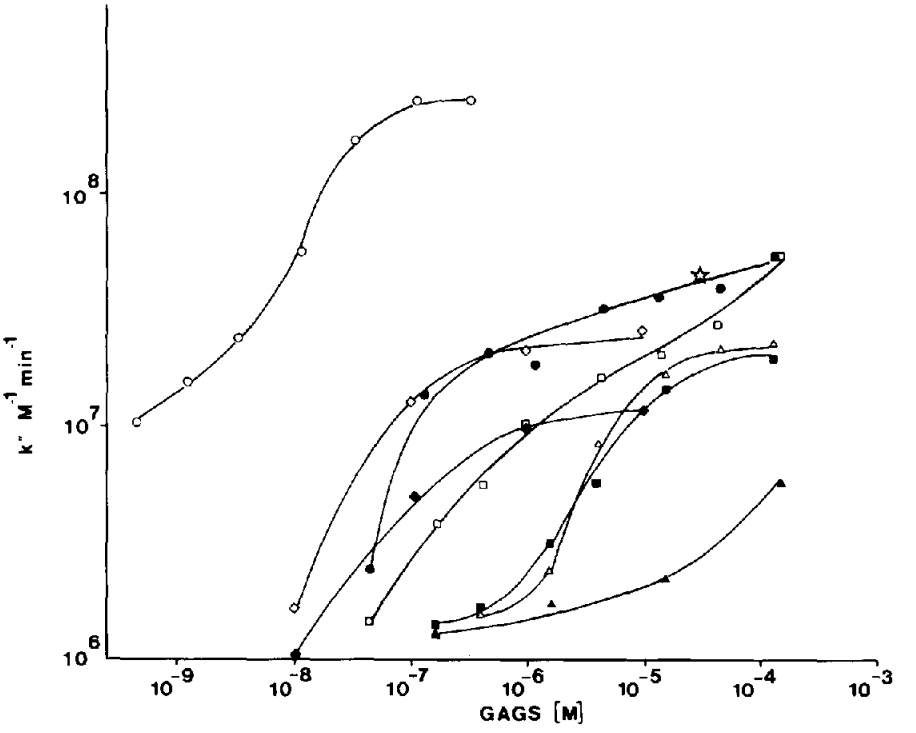

Fig.1. Effect of GAGS on the rate constant for the inhibition of free and platelet-bound factor Xa (prothrombinase) by ATIII. The second-order rate constant is plotted against molar concentration of GAGS on a double logarithmic scale. The second-order rate constant was calculated from the rate of inhibition of 3.6 nM factor Xa by 30 nM ATIII in 0.05 M Tris-HCl (pH 7.4)/0.1 M NaCl/5 mM $CaCl_2$/0.1% poly(ethylene glycol) 6000 at 37°C [14]. The molecular mass values for heparan sulphate and dermatan sulphate were taken as 25 kDa. (□) NA heparan sulphate, free factor Xa; (■) NA heparan sulphate, prothrombinase; (○) HA heparan sulphate, free factor Xa; (●) natural mixture of heparan sulphate, free factor Xa; (△) dermatan sulphate, free factor Xa; (▲) dermatan sulphate, prothrombinase; (◇) heparin (4.2 kDa), free factor Xa; (◆) heparin (4.2 kDa), prothrombinase; (☆) mixture of heparan sulphate, dermatan sulphate, CSA and CSC (see text).

tified by us previously as a property of sulphated polysaccharides and is due to their ability to bind thrombin [12]. The binding affinity of each GAGS for thrombin was estimated by plotting the inverse of the observed rate against their concentration [12] and these were calculated as 0.3 μM heparan sulphate, 4 μM dermatan sulphate, 14 μM chondroitin sulphate A, and 6 μM chondroitin sulphate C.

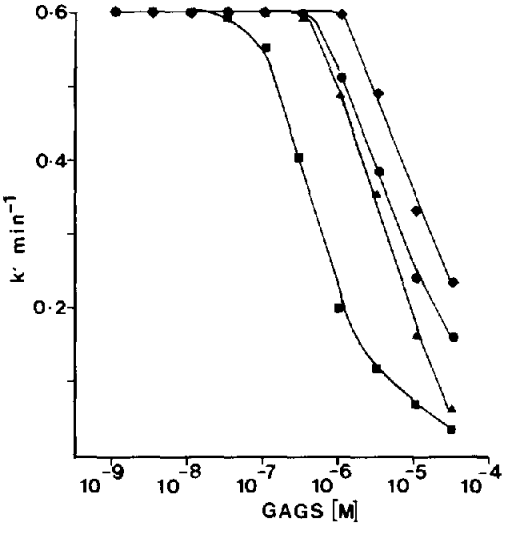

Fig.2. Antiheparin effect of GAGS. The effect was measured of increasing concentrations of GAGS upon the rate of the ATIII (25 nM): thrombin (1.5 nM) interaction in the presence of 3.3 nM HA heparin. (■) Heparan sulphate; (▲) dermatan sulphate; (◆) chondroitin sulphate A; (●) chondroitin sulphate C.

4. DISCUSSION

From the evidence presented here we propose that, although heparin-like molecules may play a part in the ATIII-dependent, non-thrombogenic properties of blood vessels, other GAGS, by virtue of their greater mass, may constitute the principal catalyst.

The concentration of proteoglycans on intact native endothelium has been estimated, by labelling with $^{35}SO_4$ and also by X-ray microanalysis to be about 10^{-12} mol polysaccharide chain per cm^2 or about 0.2 mM assuming the thickness of the glycocalyx to be 100 nm [17,18]. Of this concentration about half is heparan sulphate. Although heparan sulphate with no affinity for ATIII will be available for binding other plasma proteins, e.g. lipoprotein lipase, its concentration is 10–100 times higher than that which we observed here to cause maximal acceleration of the factor Xa (prothrombinase):ATIII interaction. This degree of acceleration was similar to the maximum achieved by a low molecular mass, high-affinity heparin with clinically proven antithrombotic properties [19]. Furthermore, when the reaction is accelerated by the natural mixture of heparan sulphate forms then the activity of heparan sulphate with no affinity for ATIII predominates. As also shown in fig.1, when a mixture containing 30 μM heparan sulphate (natural mixture) and 10 μM of each chondroitin sulphates A and C and dermatan sulphate, respectively, was tested, the rate observed was similar to that seen with heparan sulphate alone.

From our observations we also propose that catalysis of the thrombin:ATIII interaction by any heparin-like species on the endothelium will be inhibited by the anti-heparin properties of other GAGS. We have shown previously that the antiheparin property of other sulphated polysaccharides, is due to abortive binding to thrombin interfering with the mechanism of heparin catalysis [12] (which requires coincident binding of ATIII and thrombin to the same heparin molecule). Thrombin activity will be controlled in vivo presumably, by sequestering onto high-affinity sites on which it is not active, e.g. thrombomodulin or by clearance through the RE system. The control of factor Xa (prothrombinase) may, therefore, be a central feature in the ATIII-dependent modulation of coagulation, coinciding with the findings of Bauer et al. [20] who observed that in plasma samples from ATIII-deficient patients there was evidence of a reduction in the control of factor Xa activity rather than of thrombin activity.

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