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THE ANTICOAGULANT PROPERTIES OF MAST CELL PRODUCT, CHONDROITIN SULPHATE E

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Summary The anticoagulant potency in vitro of chondroitin sulphate E has been found to be similar to that of the heparinoids. In purified systems chondroitin sulphate E was shown to be principally an activator of heparin cofactor II. Maximum acceleration of heparin cofactor II:thrombin interaction was 185-fold (9.3×10^7 M^{-1} min^{-1}), antithrombin III:thrombin interaction was 11-fold (4.16×10^6 M^{-1} min^{-1}) and antithrombin III:factor Xa was 146-fold (3.86×10^6 M^{-1} min^{-1}). Chondroitin sulphate E was observed to prolong the thrombin clotting time of fibrinogen in the absence of antithrombin III and heparin cofactor II. The effect appeared to be related to interference in thrombin:fibrinogen interaction rather than in fibrin monomer polymerization.

INTRODUCTION: Chondroitin sulphate E is a form of oversulphated galactosaminoglycan with repeating disaccharide units of Glu-UA-Gal-NAc-4-6-di SO_4. It was first isolated from squid cranial cartilage[1,2]. More recently it has been identified in and purified from mast cells of bone marrow, blood, mucosal tissues, foetal liver and lymph node (reviewed in 3). Interestingly, it would appear to be present as an alternative to heparin in mast cells and may indicate a heterogeneity of these secretory cells[4]. An anticoagulant property has been reported for chondroitin sulphate E previously[5] but in this paper we have carried out a detailed investigation of its properties. These findings show chondroitin sulphate E to have anticoagulant properties similar to heparinoids and dependent principally on activation of heparin cofactor II and interference in fibrinogen:thrombin interaction.

MATERIALS AND METHODS: Human α thrombin was obtained from Dr. J. Fenton, Albany, U.S.A. Human factor Xa and human antithrombin III were prepared as described previously[6,7]. Human heparin cofactor II was prepared according

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to the method of Yamagishi et al[8]. Chondroitin sulphate E was prepared as described previously[9]. Chondroitin sulphate 4 (whale cartilage) dermatan sulphate (porcine skin) and chondroitin sulphate 6 (shark cartilage) were obtained from Sigma Chemical Co., Poole, U.K. Pentosan polysulphate was obtained from Clin Midi, Paris, France, and heparin from Leo Pharmaceuticals, U.K. Fibrinogen (Kabi, Grade L) and chromogenic substrates, S2238 and S2222, were obtained from Flow Laboratories, Rickmansworth, U.K. Pooled plasma was obtained from blood taken into 9 volumes of 3.8% sodium citrate. Blood was taken from ten donors, centrifuged at 3000xg for 20 minutes at 4°C before pooling of plasma and storage at -20°C.

Kinetics of reaction between thrombin or factor Xa and heparin cofactor II or antithrombin III in the presence of chondroitin sulphate E were measured as described previously[10,11].

Clotting time measurements These were carried out in an automated coagulometer at 37°C (Schnigter und Gross). Kaolin cephalin clotting time was measured using a cephalin preparation obtained from Dr. L. Poller (UK Reference Laboratory for Anticoagulant Reagents, Manchester, UK). 0.1ml of plasma was prewarmed and 0.1ml of cephalin added followed by 0.1ml of Kaolin suspension (0.25g/100ml of Owrens buffer). After 10 minutes clotting was initiated by addition of 0.1ml of 25mM $CaCl_2$.

Thrombin time was measured by addition of 200µl of human ∝ thrombin (3.5 units/ml distilled water) to 100µl of prewarmed plasma. The anti-factor Xa activity in plasma was measured using a commercial kit (Heptest®, Invermed. Ltd., Fort William, U.K.). To 0.1ml of plasma was added 0.1ml of factor Xa, after 120 seconds 0.1ml of a calcium/cephalin mixture was added and clotting time measured.

Fibrin polymerization studies were made using the method of Laudano & Doolittle[12].

RESULTS: Influence of chondroitin sulphate E on clotting time Chondroitin sulphate E caused a prolongation of the KCCT but this effect reached a plateau at concentrations greater than 15µgs/ml (fig.1(a)). Double log plots of the data obtained with heparin and chondroitin sulphate E were not parallel but the slopes gave values for clotting time increase per microgram of glycosaminoglycan (GAG) of 150 seconds for heparin and 1.36 seconds for

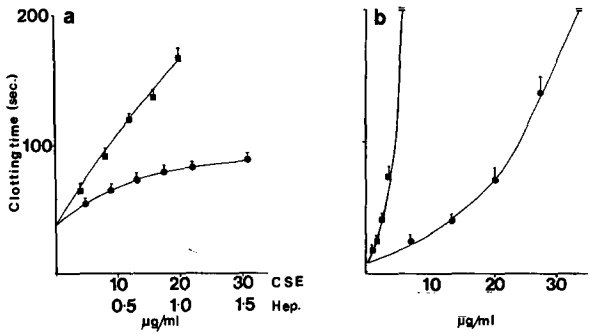

Figure 1. Influence of increasing concentrations of chondroitin sulphate E (CSE) and heparin on a) APTT and b) thrombin time of plasma ●—● CSE, ■—■ heparin. Shown as mean (±SE) of three determinations.

chondroitin sulphate E indicating a potency in this method of 1.4iu/mg. At low concentrations of chondroitin sulphate E there was a shallow response in the thrombin time increasing rapidly at higher concentrations as we have noted previously with the heparinoid, pentosan polysulphate , fig. 1(b)[13]. Calculated potency from parallel plots was 25 units/mg. When measured in a clotting factor Xa assay a potency of 1.6iu/mg was calculated (not shown).

Influence of chondroitin sulphate E on interaction between protease inhibitor and thrombin or factor Xa When increasing concentrations of chondroitin sulphate E were incubated with mixtures of protease inhibitor, heparin cofactor II or antithrombin III and enzyme, thrombin or factor Xa, a corresponding increase in rate of interaction was observed. When the reciprocal of the observed first order rate constant was plotted against inverse of concentration of chondroitin sulphate E, a straight line was obtained. The results permitted calculation of maximum increase in rate and the apparent dissociation constants of chondroitin sulphate E, as described previously in study of pentosan polysulphate[10,11], Table 1.

Influence of chondroitin sulphate E on thrombin:fibrinogen interaction Chondroitin sulphate E was observed to increase the thrombin clotting time of fibrinogen solutions in the absence of added antithrombin III or heparin cofactor II, fig.2. Chondroitin sulphate 4 and 6 and dermatan sulphate at concentrations up to 100µgs/ml had no effect on the clotting time under

TABLE 1

Effect of chondroitin sulphate E (CSE) on the interaction between protease inhibitors and coagulation proteinases

Proteinase inhibitor	Proteinase	$k'' M^{-} min^{-1}$ -CSE	$k'' M^{-} min^{-1}$ +CSE	Apparent Kd µgs/ml
Heparin cofactor II	Human α thrombin	$5x10^5$(14)	$9.3x10^7$	80
Antithrombin III	Human α thrombin	$3.78x10^5$(15)	$4.16x10^6$	8
Antithrombin III	Factor Xa	$2.64x10^4$(6)	$3.86x10^6$	80

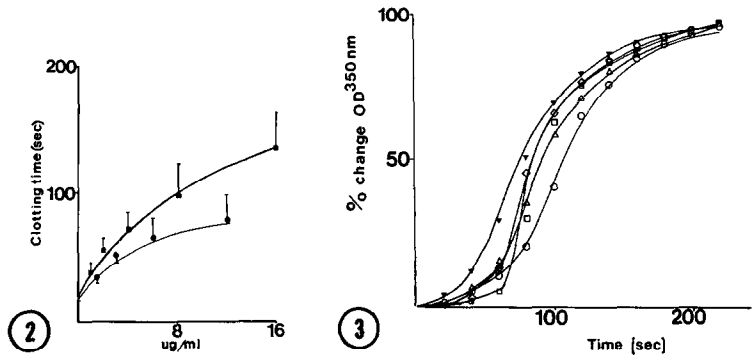

Figure 2. Influence of chondroitin sulphate E and heparin on clotting time of fibrinogen. 0.3ml of fibrinogen (0.2% w/v in water) was prewarmed at 37°C and 0.1ml of thrombin (4 NIH units/ml) was added. μ=0.036. ●–●, CSE, ■–■, heparin. Shown as mean (±SE) of three determinations.

Figure 3. Influence of chondroitin sulphate E and heparin on clotting of fibrinogen measured by increase in absorbance at 350nm. 1.0ml of fibrinogen (0.2% w/v in 0.02M Tris-HCl, 0.15M NaCl pH7.4, μ=0.12) was prewarmed and 0.1ml of thrombin (5 NIH units/ml) added at zero time ▼–▼control, ○–○CSE 30µgs/ml Δ–Δ, CSE, 15µgs/ml, ◇–◇heparin 60µgs/ml, □–□ heparin, 240µgs.ml Results are shown as percentage of the absorbance at 350nm after five minutes incubation and mean of two to three determinations.

these conditions. Pentosan polysulphate at a concentration of 12.5µgs/ml tended to shorten the clotting time as we have reported previously[13].

Chondroitin sulphate E at concentrations up to 30ugs/ml had no effect on the rate of fibrin monomer polymerization as measured in the system of Laudano & Doolittle[12] (not shown). Heparin was observed to cause some increase in the rate of fibrin monomer polymerization when tested at 10ugs/ml.

When fibrinogen was clotted by thrombin and the rate of formation of fibrin followed by absorbance at 350nm it was observed that chondroitin sulphate E caused a significant lag before the rapid phase of fibrin monomer polymerization, fig.3. Heparin also caused such a lag but this was more pronounced and followed by a rather accelerated increase in polymer formation. It is probable, therefore, that the effect of chrondroitin

sulphate E on clotting is related to direct interference with thrombin: fibrinogen interaction.

DISCUSSION: The physiological role of mast cell proteoglycans has yet to be determined but may be related to packaging and release of cationic amine mediators and also intracellular inhibition of the large amounts of potent degradative enzymes and pH and osmoregulation in the granule. Both heparin and chondroitin sulphate E inhibit activation of the alternate complement pathway[16,17], and can initiate the Hageman factor dependent coagulation pathway of contact activation *in vitro*[18]. Heparin is also a uniquely potent anticoagulant. An anticoagulant property for chondroitin sulphate E has been reported previously[5], but in this paper we have carried out a detailed investigation and reach a conclusion as to the mechanisms by which chondroitin sulphate E may act as a weak anticoagulant.

Testing of chondroitin sulphate E in plasma showed its weak anticoagulant properties with potencies of 1.4 APTT units/mg and 1.6 units of antifactor Xa activity. The APTT potency is comparable to that of dermatan sulphate and heparan sulphate[19,20], and the antifactor Xa activity is comparable to that of dermatan sulphate[19]. Chondroitin sulphate 4 and 6 show no anti-coagulant activity in these systems at concentrations up to 100ug/ml[19]. Chondroitin sulphate E showed high potency in the thrombin clotting time probably related to mechanisms discussed below. In this respect it is 10-fold more potent than the synthetic heparinoid, pentosan polysulphate, which has much higher potency (38iu/mg) in APTT units[13].

The mechanisms involved in its anticoagulant properties would appear to arise mainly from its ability to activate the protease inhibitors, antithrombin III and heparin cofactor II and to interfere in thrombin/fibrinogen interaction. Maximum acceleration of the heparin cofactor II:thrombin interaction was calculated as 185-fold (9.24×10^7 M^{-1} min^{-1}) and this can be compared to 900-fold, 1500-fold and 500-fold for catalysis of this rate by heparin, dermatan sulphate and pentosan polysulphate respectively[11,14,21]. A 185-fold increase over basal rate would produce a

$t_{\frac{1}{2}}$ for thrombin in plasma of 600 msecs approaching the rate of 100 msecs thought to be necessary for physiological control[22]. Chondroitin sulphate E causes a 11-fold (4.16×10^6 M^{-1} min^{-1}) increase in rate of antithrombin III:thrombin interaction compared to 10,000-fold by heparin[23], and 40-fold by pentosan polysulphate[10]. Acceleration of antithrombin III:factor Xa interaction was 146-fold (3.86×10^6 M^{-1} min^{-1}) compared to 4500- and 260-fold for these reactions catalyzed by heparin[24] and pentosan polysulphate[24], respectively. In these purified systems the results show chondroitin sulphate E to have properties similar to the heparinoid, pentosan polysulphate, and to the heparan sulphate containing heparinoid, Endoglycan (Mediolanum Pharmaceuticals, Milan, Italy.)[25] in acceleration of protease inhibition:coagulation factor interaction. This acceleration is probably not related to any specific interaction between inhibitor and the glycosaminoglycan (as is the case for antithrombin III:heparin interaction) but to approximation of the inhibitor and coagulation factor by non-specific binding to the same carbohydrate molecule[26].

Chondroitin sulphate E also has the property to interfere directly with fibrinogen:thrombin interaction. Experiments show it to have no significant effect on fibrin monomer polymerization (unlike heparin which causes a slight increase in rate). Since chondroitin sulphate E had no effect on the kinetics of interaction with chromogenic substrate S2238 the inhibition is probably related to binding to a cationic site on thrombin which has been shown to be important for thrombin:fibrinogen interaction[27]. In that work it was demonstrated that heparin was a potent interactant with the site and dermatan sulphate and heparan sulphate to a lesser extent.

Chondroitin sulphate E is a weak anticoagulant as measured in vitro. These properties do not necessarily correlate with its antithrombotic properties since heparinoids with weaker anticoagulant properties than heparin <u>in vitro</u> have been found to be effective therapeutically in experimental and clinical situations[28-30]. These properties are somewhat

similar to heparan sulphates and heparinoids and there is an additional ability to interfere with thrombin:fibrinogen interaction.

The role of mast cells as a source of anticoagulant in the primary haemostatic balance has yet to be elucidated[31]. Heparin in plasma is present[32] but not in a freely available form[33]. Whether chondroitin sulphate E may play some role in this respect is now being investigated.

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