Carbohydrate Polymers **18** (1992) 9–16

Inhibition of angiogenesis by heparin fragments in the presence of hydrocortisone

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(Received 26 February 1991; revised version received 9 May 1991; accepted 18 June 1991)

Heparin in combination with hydrocortisone has been reported to be a potent inhibitor of angiogenesis in the chick chorioallantoic membrane (CAM). Since very little is known about which structural features in heparin are essential for this effect, fragments and chemically modified fragments of heparin were prepared and assayed for anti-angiogenic activity in a CAM assay. The bleeding induced by heparin limited the subcutaneous dose that could be administered together with cortisone to tumour-bearing mice and a further objective of this study was, therefore, to find heparin derived substances with a considerably reduced effect on bleeding time. Both a partially N-desulfated, N-acetylated heparin fragment (molecular weight, M_r 5000) with very little effect on the bleeding time in rats (prolongation $1{\cdot}1 \pm 0{\cdot}8$ min at 100 mg/kg i.v.) and a heparin fragment with M_r 3800, prepared by periodate oxidation of heparin (bleeding time prolongation $3{\cdot}6 \pm 2{\cdot}1$ min at 32 mg/kg i.v.) showed an anti-angiogenic activity in the CAM assay similar to that of heparin. The results also indicate that if heparin fragments are to show good anti-angiogenic activity in the CAM assay in the presence of hydrocortisone, they should retain some of the N-sulfated glucosamines which are a characteristic structural element of heparin and which are also found in heparan sulfate.

INTRODUCTION

Heparin is a linear, highly sulfated polysaccharide consisting of alternating uronic acid (either L-iduronic or D-glucuronic) and D-glucosamine residues. Variations in the size of the polysaccharide chains and in the degree and distribution of sulfate groups contribute to a high degree of heterogeneity, which makes it very difficult to assign a precise structure. The clinical use of heparin has been associated with its anti-coagulant and anti-thrombotic properties (for a review see Jorpes, 1946). The risk of hemorrhage, which is considered to be related to its anti-coagulant properties (Palm *et al.*, 1990), is the main limitation in its clinical use. More recently, heparin's biological role has been expanded to include a variety of functions associated with vascular growth control. An example of this is the finding by Folkman *et al.* (1983) that a combination of heparin and cortisone inhibited angiogenesis and caused inhibition of growth and regression of experimental tumours in mice. This finding was later confirmed by several investigators whilst others were not able to reproduce the data (for a review see Folkman and Ingber (1989) and Kyo-Lee *et al.* (1990)). Since then very little progress has been made on the elucidation of which structural features of heparin that contributed to this anti-angiogenic effect in the presence of cortisone. The complexity of the heparin structure and its bleeding-promoting effect have contributed to this slow progress. On the other hand, further research into the steroid component of this angiostatic mixture has yielded more potent and specific steroids (Crum *et al.*, 1985). Heparin fragments obtained by partial depolymerization have been shown to be less hemorrhagic in animals then heparin itself (Palm *et al.*, 1990), while still retaining their anti-thrombotic properties. The strategy for this study was, therefore, to prepare heparin fragments and examine them for their inhibitory effect on angiogenesis and coagulation as well as for their effect on bleeding time. By selective chemical modifications of heparin fragments, the anti-coagulant and bleeding effects could be further reduced and the influence of these structural changes on the inhibition of angiogenesis could be examined. In order to examine the anti-angiogenic activity of these heparin-derived substances they were

Carbohydrate Polymers 0144-8617/92/$05.00

assayed in the presence of hydrocortisone in the chick embryo chorioallantoic membrane (CAM) assay. Results of these studies show that heparin can be depolymerized to give heparin fragments of M_r 3800–5100 without significantly losing its anti-angiogenic activity. By the chemical alterations introduced in the heparin fragments a further reduction of the anti-coagulant activity and bleeding time was obtained but only a partial exchange of N-sulfate groups for N-acetyl groups was compatible with substantially retained angiostatic activity.

MATERIALS AND METHODS

Chick chorioallantoic membrane (CAM) assay

Inhibition of angiogenesis was assayed on the chick chorioallantoic membrane essentially as described by Auerbach *et al.* (1974). Fertilized eggs from Shaver Starcross (Linköpings Kontrollhönseri, Linköping, Sweden) were incubated in a standard egg incubator (Andersson & Bonde, Ängelholm, Sweden) for three days at 37°C. The eggs were then cracked and the content was placed in Falcon Petri dishes, size 20 mm × 100 mm (Becton & Dicinson, Plymouth, UK) and incubated for another three days in a CO_2-incubator (Flow laboratories), CO_2 concentration 2·5% and relative humidity 60–80%.

Sample application

In order to obtain a simple slow release preparation, aliquots of the test samples were added to a 0·45% solution of methyl cellulose (4000 Centipoise, Fisher Scientific Co., Fair Lawn, NJ) Transparent methyl cellulose disks, containing the test compound in a concentration range of 5–50 μg/disk and hydrocortisone (50 μg/disk), were prepared by pipetting 10 μl of the test mixture on to the top of a Teflon rod (diameter 3·2 mm). When the disk was sufficiently dry (approximately 30 min) it was lifted from the Teflon rod with fine forceps and placed on the outer edge of the CAM on a six-day-old embryo, where the capillaries were still growing. After a final two days' incubation in a CO_2-atmosphere the embryos were observed under a stereoscope at ×10 to ×15 for the presence or absence of capillaries beneath and around the disk.

The evaluation of the results were graded as follows: (−) normal capillaries beneath and around the disk; (+/−) the area beneath the disk was avascular but normal capillaries were found outside; (+) area of avascularity extends beyond the disk. All the samples were coded in such a way that the person responsible for the scoring did not know the nature or dose of the test compound. In an average week, about 80% of the embryos survived the removal from their shell and about 70% (approximately 100 eggs) were alive on day 8 for examination of avascular zones. In order to check the reproducibility of the assay a standard sodium heparin from porcine intestinal mucosa (Abbott batch AK 19029) with a known activity, was always added to 10–15 eggs in every experiment. The results are presented as per cent of embryos showing an inhibition of angiogenesis (i.e. the sum of (+/−) and (+) divided by the total number of eggs). Each component was tested on a minimum of 20 eggs at four dose levels and in at least two different experiments at 1–2-week intervals.

Bleeding time assay

The template bleeding time test according to Dejana *et al.* (1979) was performed in Sprague-Dawley rats weighing 200–250 g, anesthetized with Mebumal/Stesolid (Dumex A/S, Copenhagen). The template device (Simplate, General Diagnostics, Durham, NC) was applied longitudinally to the dorsal part of the tail, taking care to avoid large veins. Blood from the wound was then carefully removed every 30 s with blotting paper. A minimum of six rats was used for each compound and dose. Bleeding times were measured from the moment the tail was incised until the first arrest of bleeding. The bleeding time was recorded with an accuracy of 30 s, bleeding times longer than 20 min being noted as >20. Two bleeding times were always determined in each rat, viz. 10 min before and 10 min after drug administration and the results are expressed as the prolongation of bleeding time.

Preparation, characterization and chemical modification of heparin fragments

Heparin fragment 1

Heparin fragment 1 (see Fig. 4 for a schematic presentation of the relation between heparin and the heparin fragments of the investigation) was prepared from heparin from porcine intestinal mucosa (Hepar Ind.) by controlled partial depolymerization by nitrous acid, essentially as described by Thunberg *et al.* (1980) followed by reduction by excess sodium borohydride (Horton & Philips, 1973).

Heparin fragment 2

Heparin fragment 2 was prepared by periodate oxidation (Fransson, 1978) of heparin (Hepar Ind.) (1·00 g) which was dissolved in 0·05 M phosphate buffer, 0·2 M NaCl, pH 7·0 (50 ml) and heated to 39°C in a dark flask. Sodium periodate (1·07 g) was added and the reaction mixture was stirred at 39°C for 19 h. Ethylene glycol (0·5 ml) was added and the solution ultrafiltrated. The retentate was washed with water before being dissolved in 0·02 M NaOH (100 ml). After 35 min at 21°C the product was reduced by $NaBH_4$ (0·20 g) over 2 h. Excess $NaBH_4$ was decomposed by HOAc (1·2 ml)

after which the solution was neutralized by NaOH, desalted by ultrafiltration and lyophilized to yield 0·56 g. This product was subjected to ion exchange chromatography on DEAE-Sepharose. The column was washed with 0·3 M NaCl and then eluted with 2 M NaCl. The latter fraction was precipitated with EtOH to yield 0·39 g heparin fragment 2 after drying at 60°C for 3 h.

Heparin fragment 3
The pyridinium salt of heparin fragment 1 was prepared essentially as described by Inoue *et al.* (1976). This salt (10 g) dissolved in Me_2SO containing water (5%) (500 ml) was heated at 35°C for 1 h. The solution was then added to a 10% solution of NaOAc in MeOH (2 liters). The precipitate was isolated by centrifugation, washed with EtOH and dried in air. A small amount (0·1 g) was dissolved in 2 M NaCl, ultrafiltrated and the retentate washed with water and lyophilized to give partially N-desulfated heparin fragment 3 (41 mg). A ^{1}H-NMR spectrum in 0·1 M NaOD/D_2O showed that 70% of the N-sulfate groups were desulfated.

Heparin fragment 4
The air-dried precipitate fragment 3 was N-acetylated according to the method of Danishefsky *et al.* (1960) to give partially N-desulfated, N-acetylated heparin fragment 4 (7·4 g).

Heparin fragment 5
Totally N-desulfated heparin fragment 5 was prepared in the same way as partially N-desulfated fragment 3 but heated in Me_2SO containing water (5%) at 50°C for 1·5 h.

Heparin fragment 6
Heparin fragment 6 was prepared by re-N-sulfating fragment 5 (0·20 g) according to Lloyd *et al.* (1971). Yield 0·19 g.

Heparin fragment 7
This was obtained by N-acetylating fragment 5 according to Danishefsky *et al.* (1960).

Heparin fragment 8
Heparin fragment 8 was prepared by O-oversulfating fragment 7 essentially according to Ogamo *et al.* (1985) and Nagasawa *et al.* (1986). The tri-*n*-butylammonium salt of fragment 7 (2·29 g) was dissolved in dry *N,N*-dimethylformamide (23 ml), cooled to 4°C, after which sulfur trioxide–pyridine complex (2·29 g in 15 ml *N, N*-dimethylformamide) was added. After 21 h at 4°C, cold water (80 ml) and 2 M NaOH (11·5 ml) were added, and the mixture was left in the cold for 1 h, poured into 3% NaOAc in EtOH (500 ml). The precipitate was centrifuged, washed with EtOH, dried, dissolved in 2 M NaCl and ultrafiltrated. The retentate was washed with water and lyophilized yielding O-oversulfated N-acetylated heparin fragment 8 (1·86 g).

The molecular weights (M_r) were determined by analytical gel filtration on Sephadex G-75 (Pharmacia, Uppsala, Sweden) using reference samples with a known degree of polymerization, obtained by nitrous acid depolymerization of heparin (Thunberg *et al.*, 1980). The anti-coagulant activity of heparin and heparin fragments was determined by an APTT (Activated Partial Thromboplastin Time) assay (Andersson *et al.*, 1976) and by an anti-FXa assay using the chromogenic substrate S-2222 (Kabi Diagnostica, Sweden) (Bergkvist *et al.*, 1983). The APTT activity and the anti-FXa activity of standard heparin was measured against a house standard which was calibrated against the 4th International Standard preparation of heparin. The anti-FXa activity of heparin fragments and chemically derived heparin fragments was measured against a house standard calibrated against the 1st International Standard of LMW-heparin (85/600). Elemental analyses (S) were performed by Mikrokemi, Uppsala, Sweden, on samples dried for 3 h at 60°C in vacuum. NMR spectra were recorded in D_2O or D_2O/NaOD in a JEOL GX-400 instrument (400 MHz ^{1}H, 100 MHz ^{13}C) at 40°C. The sulfate to carboxyl molar ratio (SO_3/COO) was determined by conductimetric titrations according to Casu and Gennaro (1975) using 50 mg samples of lyophilized material. Results are expressed as the average of duplicate titrations. Heparin batch AK 19029 was obtained from Abbott.

RESULTS

Heparin fragments and chemically modified heparin fragments; chemistry, anticoagulant activities and effects on bleeding

Partial depolymerization of heparin from porcine intestinal mucosa by nitrous acid, resulted in heparin fragment 1 with an M_r of 5100. The APTT activity of fragment 1 was reduced to 59 IU/mg (Table 1) and the bleeding time was 50% shorter than the standard heparin from which it was prepared (Table 2). When the same heparin was depolymerized by periodate oxidation fragment 2 with M_r 3800 was obtained. Fragment 2 showed a more pronounced decrease in anti-coagulant activity than fragment 1 (Table 1). The prolongation of the bleeding time of fragment 2 at 32 mg/kg (3·6 ± 2·1 min) corresponded to approximately 2 mg/kg of fragment 1 or approximately 1 mg/kg of heparin (Table 2). Partial N-desulfation of fragment 1, where 70% of the original N-sulfate groups were removed, gave fragment 3 which was almost devoid of anti-coagulant activity (Table 1). N-acetylation of fragment 3 resulted in the partially N-acetylated heparin fragment 4 (Table 1), in which the anti-

coagulant effect remained very low, whilst the M_r increased to the value of the heparin fragment from which the chemical modifications started (Table 1). The bleeding time was further reduced compared to fragment 1 and even at 100 mg/kg fragment 4 showed a prolongation of only 1·1 ± 0·8 min compared to saline (Table 2). On N-resulfation of the completely non-anticoagulant N-desulfated heparin fragment 5, to give fragment 6 the anti-FXa activity as well as the APTT value returned to the same value found before desulfation. Although sulfur analysis indicated a slight drop in sulfur content (from 11·9% to 11·3%) no loss of O-sulfate was seen by ^{1}H-NMR and ^{13}C-NMR (not shown). When the completely N-desulfated heparin fragment 5 was N-acetylated to give fragment 7, the anti-coagulant activity remained very low (Table 1). When the totally N-acetylated fragment 7 was oversulfated, to give fragment 8, the sulfur content as well as the M_r increased. The APTT of the oversulfated heparin fragment 8 increased (Table 1).

Effect of heparin fragments and chemically modified heparin fragments on inhibition of angiogenesis

Heparin batch AK 19029, which was included as a positive control in all experiments, gave in the presence of hydrocortisone an average inhibition of 56 ± 11% (n = 616) at a concentration of 25 μg/10 μl. The very few experiments in which the control heparin was outside the range of 45–67% (mean ±1 SD) were not included in the results. From Fig. 1 it is seen that the weak effect by heparin itself on the inhibition of angiogenesis in the CAM assay is enhanced by hydrocortisone. This effect is most pronounced at the two highest concentrations (25 and 50 μg/disk). Pooled data from these two doses reveals that inhibition of angiogenesis is significantly enhanced if heparin is assayed in the presence of hydrocortisone (50 μg/disk).

Hydrocortisone by itself showed an inhibition of 6%, while the empty methyl cellulose disk by itself did not show any inhibition at all. When fragments 1 and 2,

Table 1. Chemical and biochemical properties of heparin, heparin fragments and chemically modified heparin fragments

	Anti-FXa (IU/mg)	APTT (IU/mg)	M_r	Per cent sulfur	$\frac{SO_3^a}{COO}$
Heparin	165	179	14000	12·0	2·19
Heparin fragment (1)	130	59	5100	11·9	2·29
Heparin fragment (2)	1	13	3800	12·8	2·35
Partially N-desulfated (3)	2	nd	4500	10·6	nd
Partially N-desulfated, N-acetylated (4)	2	3	5000	9·2	1·58
Totally N-desulfated (5)	<1	1	4600	9·7	nd
Totally N-desulfated, re-N-sulfated (6)	120	60	5200	11·3	nd
Totally N-acetylated (7)	<1	2	5200	8·7	1·57
O-oversulfated, totally N-acetylated (8)	2	78	7200	13·9	3·31

nd, Not determined.
[a]Sulfate to carboxyl molar ratio (see experimental section).

Table 2. Bleeding time of heparin and heparin fragments

Compound	Dose			Bleeding time (min)	BT > 20 min %
	mg/kg	Anti-FXa (IU/kg)	APTT (IU/kg)		
Heparin	1	165	179	4·9 ± 1·5	0
	2	330	358	16·5 ± 4·1	90
Heparin fragment (1)	1	130	59	1·5 ± 0·9	0
	2	260	118	3·6 ± 3·2	0
	4	520	236	8·6 ± 6·6	33
Heparin fragment (2)	4	4	52	0·9 ± 0·9	0
	8	8	104	0·7 ± 0·7	0
	32	32	416	3·6 ± 2·1	0
Partially N-desulfated, N-acetylated heparin fragment (4)	50	100	150	1·3 ± 0·8	0
	100	200	300	1·1 ± 0·8	0

The bleeding time (BT) (min) is expressed as the difference in bleeding time 10 min after and 10 min before drug administration. The frequence (%) of a bleeding time longer than 20 min is given in the last column.

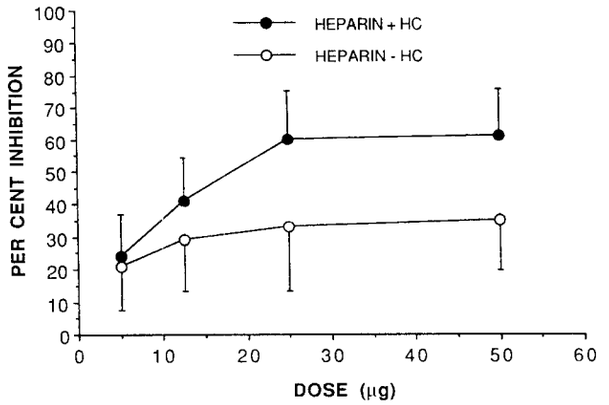

Fig. 1. Inhibition of angiogenesis in the CAM assay by heparin in the presence (+HC) and absence (−HC) of hydrocortisone. CAM assay procedure is described in the Materials and Methods section. The error bars have been calculated as follows: the proportion of positive and negative eggs are $p = a/n$ and $1 - p = q = b/n$, respectively. The confidence interval for p is obtained from $p \pm \lambda \cdot \text{std}(p)$ and the standard deviation for p; $\text{std}(p) = \sqrt{p \cdot q/(n-1)}$, where n is the total number of observations. $\lambda \approx 2$ at a 5% significance level.

which were prepared from the same batch of heparin but by different methods, were compared in the CAM assay with the heparin from which they were made, they all showed a high degree of inhibition (Fig. 2). Partial N-desulfation of fragment 1 resulted in a fragment (3) of virtually no anti-angiogenic activity. The activity was, however, restored in fragment 4, which was obtained after N-acetylation of the free amino groups of fragment 3 (Fig. 3(a)). Total N-desulfation of fragment 1 to give fragment 5 resulted in a loss of all inhibitory activity. Most of the original inhibitory activity of 1 was, however, restored after N-resulfation of 5 to give fragment 6 but inhibitory activity after N-acetylation (to give fragment 7) was only seen at the highest concentration of 50 μg (Fig. 3(b)). O-oversulfation of the totally N-acetylated heparin fragment 7, caused a slight increase in inhibitory activity in the low dose range (5–25 μg fragment 8) but did not reach the level of activity of fragment 1 in this dose range (Fig. 3(c)).

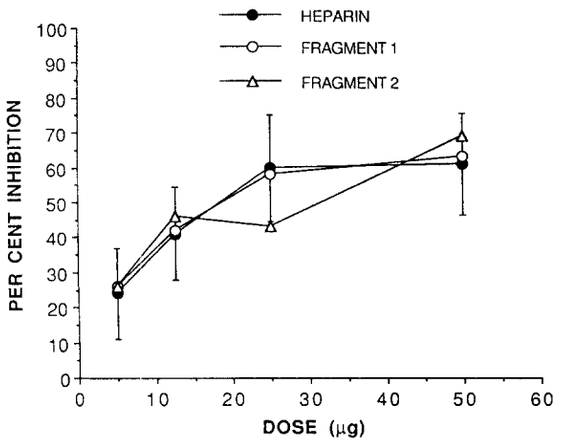

Fig. 2. Inhibition of angiogenesis in the CAM assay by two heparin fragments and the heparin they were prepared from in the presence of hydrocortisone. Heparin fragment 1 was prepared by nitrous acid depolymerization and heparin fragment 2 by periodate oxidation. Preparations of fragments and the CAM procedure are described in the Materials and Methods section. For simplicity, error bars are only indicated for heparin. Fragments 1 and 2 show a similar variation (i.e. ±10–15%).

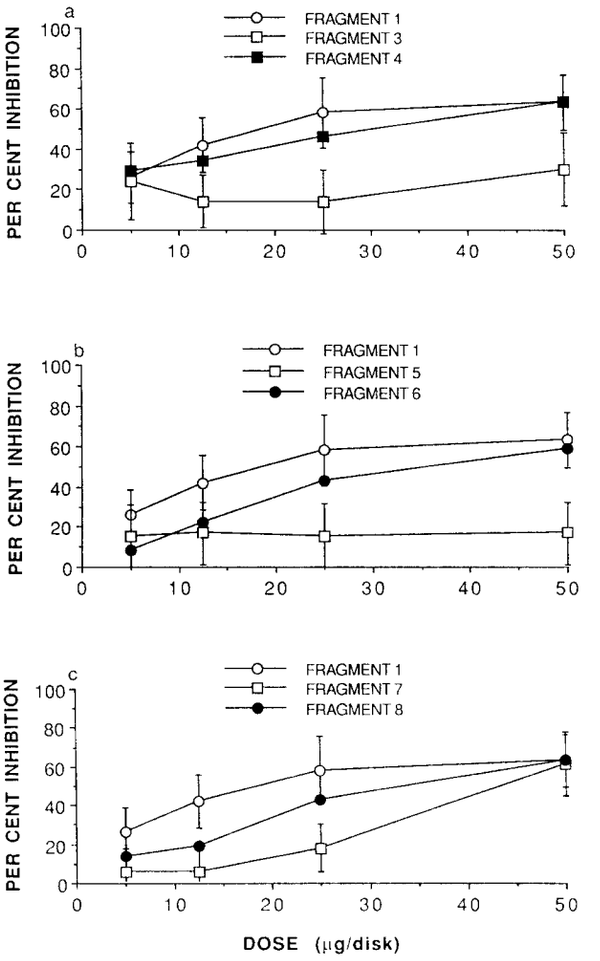

Fig. 3. Inhibition of angiogenesis in the CAM assay in the presence of hydrocortisone by chemically modified heparin fragments compared to the nitrous acid depolymerized heparin fragment 1 from which they were prepared. The CAM assay and the preparation of the fragments are described in the Materials and Methods section. Fragment 3 is a partially N-desulfated heparin fragment. Fragment 4 is a partially N-desulfated, N-acetylated heparin fragment. Fragment 5 is a totally N-desulfated heparin fragment. Fragment 6 is a totally N-desulfated, N-resulfated heparin fragment. Fragment 7 is a totally N-desulfated, N-acetylated heparin fragment. Fragment 8 is a totally N-desulfated, N-acetylated O-oversulfated heparin fragment. Error bars are only indicated for the two extreme components. Remaining fragments show a variation in the range of 10–18%.

DISCUSSION

When heparin was, in combination with cortisone or hydrocortisone, applied to the chick embryo chorioallantoic membrane (CAM) during its rapid growth phase (6–8 days), avascular zones were developed within 48 h of implantation (Folkman *et al.*, 1983). The authors used this assay to investigate the effect of the changes introduced in the heparin structure by selective chemical modifications. The purpose of chemically changing the heparin structure was to study the relationship between different structural components and their influence on the anti-angiogenic activity of the heparin–hydrocortisone mixture. Heparin's bleeding effect limited the subcutaneous dose that could be administered together with cortisone to tumour-bearing mice (Folkman *et al.*, 1983). A further goal was, therefore, to reduce the bleeding side-effect of heparin, which has often been ascribed to its anti-coagulant activity (Palm *et al.*, 1990).

It has been reported that a non-anti-coagulant hexasaccharide, prepared by enzymatic depolymerization of heparin (Folkman *et al.*, 1983), and a synthetic non-anti-coagulant heparin pentasaccharide (Crum *et al.*, 1985) were, on a weight basis, each as inhibitory in the presence of steroids in the CAM assay as whole heparin. These small saccharides are not easily accessible on a larger scale for pharmacological and toxicological evaluation. Since the authors initially found that a heparin fragment (1) of M_r 5100 had the same anti-angiogenic activity as the heparin from which it was prepared, the chemical modifications were performed on this heparin fragment (Fig. 4). Heparin fragments with M_r of about 5000 also have the advantage over heparin of having a high bioavailability after subcutaneous administration (Bratt *et al.*, 1986). Heparin fragment 1 as well as the other fragments prepared in this study (2–8) are easily prepared on a large scale.

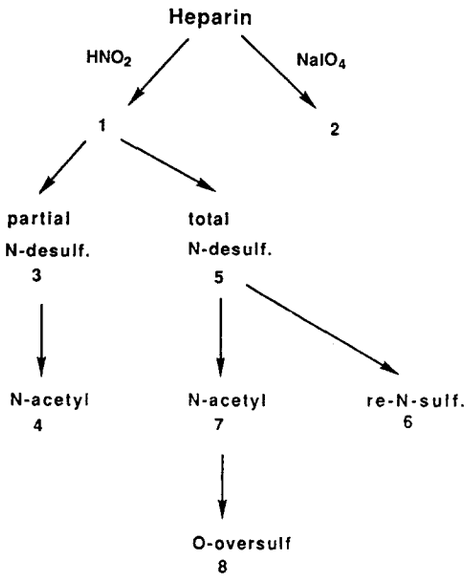

Fig. 4. Schematic presentation of the relation between heparin and the heparin fragments of the investigation.

In order to avoid differences in anti-angiogenic activity due to different heparins (Folkman *et al.*, 1983) the authors used only one batch of heparin as starting material for the depolymerization experiment. Also, all chemical modifications were carried out on the same batch of heparin fragment 1. Although the Hepar and Abbott heparins tested showed a similar anti-angiogenic activity, the authors have also found porcine mucosal heparins from other manufacturers with lower anti-angiogenic activity in the CAM assay (not shown), as have others (Folkman *et al.*, 1983).

When heparin was depolymerized by nitrous acid to give fragment 1, anti-coagulant activity, as measured in the anti-FXa assay, was largely retained and the APTT activity was reduced. This makes this fragment (1) a good anti-thrombotic heparin fragment (Holmer *et al.*, 1982) besides retaining the anti-angiogenic activity of the heparin from which it was prepared. In the periodate procedure employed for depolymerization, the heparin chains are cleaved at non-sulfated uronic acid (Fransson, 1978). This procedure presumably cleaves heparin chains at the non-sulfated glucuronic acid within the specific pentasaccharide sequence that is responsible for the binding of heparin to antithrombin III (Lindahl *et al.*, 1980), thus depriving fragment 2 of its anti-thrombin III mediated anti-coagulant activity (Table 1). Interestingly, fragment 2 still retained the anti-angiogenic activity of the original heparin used (Fig. 2), indicating that most of the non-sulfated uronic acid in heparin is dispensable with respect to its anti-angiogenic activity in the CAM assay in the presence of hydrocortisone.

The loss of anti-angiogenic activity by N-desulfation of fragment 1, partially (Fig. 3(a)) or totally (Fig. 3(b)), could be due to the formation of an amphoteric substance instead of the highly anionic heparin fragment. When the positive charge on the free amino groups of the desulfated heparin fragments was blocked by acetylation, the partially N-desulfated N-acetylated heparin fragment 4 regained anti-angiogenic activity (Fig. 3(a)). That the totally N-desulfated heparin fragment regained the original anti-coagulant (Table 1) and most of the anti-angiogenic activity of fragment 1 on N-resulfation (Fig. 3(b)) indicated that the N-desulfation as well as the N-resulfation was mild and selective. An increase over the normal heparin level of sulfate substitution of hydroxyl groups (O-oversulfation) could not compensate for the absence of N-sulfation in glucosamines (Fig. 3(c)). However, in β-cyclodextrin the anti-angiogenic activity increased with the number of sulfate substitutions when assayed in the CAM model in the presence of hydrocortisone

(Folkman *et al.*, 1989). In a sulfated polysaccharide-peptidoglycan complex from an Artherobacter species neither desulfation nor sulfation of the complex greatly affected the anti-angiogenic activity as tested in the presence of cortisone acetate on the CAM (Inoue *et al.*, 1988). It was also found that dextran sulfates and mannoglycan sulfates were lacking anti-angiogenic activity (Inoue *et al.*, 1988). Thus, it seems that for the sulfate substitution in carbohydrates to be advantageous for the inhibition of angiogenesis in the presence of hydrocortisone or cortisone, at least some of it should be in specific structures such as N-sulfate groups in the heparin fragments or in 2- and 6-sulfate groups in β-cyclodextrin tetradecasulfate.

In patients there does not seem to be a clear correlation between bleeding time measurements and bleeding (Rodgers & Levin, 1990). In clinical practice low molecular weight heparins such as fragment 1 have not yet convincingly shown less bleeding than heparin, although the bleeding time in rats was 50% shorter for fragment 1 than for heparin (Table 2). However, the very low effect on the bleeding time of fragments 2 and 4 (Table 2) and their retained anti-angiogenic activity on the CAM assay should make it possible to further evaluate these fragments in combination with steroids for anti-angiogenic effects in animals such as tumour-bearing mice, with reduced risk for bleeding being a limiting factor at s.c. or i.v. administration.

It was noticed by Folkman *et al.* (1983) that too high a dose of heparin in combination with hydrocortisone or cortisone, caused the inhibitory effect of the mixture to decline when used to treat malignant tumours in mice. This was thought to be due to stimulation of tumour angiogenesis by heparin (Taylor & Folkman, 1982; Folkman *et al.*, 1983). In the presence of acidic fibroblast growth factor (aFGF) it was found that oligosaccharides, obtained by fractionation of heparin fragments to give fractions of high sulfur content, potentiated the growth of bovine adrenal capillary endothelial cells, whereas oligosaccharides of low sulfur content did not (Sudhalter *et al.*, 1989). Similar results were obtained by Barzu *et al.* (1989) using human umbilical vein endothelial cells. Having been assayed according to Sudhalter *et al.* (1989), heparin fragments 1, 2 and 8 were shown to enhance the endothelial cell growth to the same extent as heparin in the presence of aFGF, while heparin fragments 4 and 5 did not show any effect at all (data not shown).

Compared to heparin, fragment 2 is characterized mainly by having a lower M_r and by being essentially devoid of glucuronic acid (Fig. 5). Moreover its content of N-acetyl glucosamine units is reduced. These monosaccharides are a characteristic feature of heparin particularly from porcine intestinal mucosa though they occur in rather small amounts. In fragment 2 the fairly large amounts of the most frequently occurring disaccharide ((1 → 4)O-α-L-idopyranosyl-uronic acid 2-sulfate)-(1 → 4)-(2-deoxy-2-sulfamino-α-D-glucopyranosyl 6-sulfate) in heparin were preserved. The retained activity of fragment 2 points to an important role for this highly sulfated disaccharide for the anti-angiogenic activity of heparin in the presence of hydrocortisone. Fragment 4 having a larger amount of N-acetyl groups than N-sulfate groups and also a lower total sulfate content than heparin resembles heparan sulfate. While containing much less of the highly sulfated disaccharide of fragment 2 it might be expected to be less inhibitory. However, since this was not the case the fewer highly sulfated disaccharide units which remained might be sufficient for inhibition. The lack of stimulation as found on the endothelial cells might also contribute to the overall inhibitory effect as seen in the CAM assay.

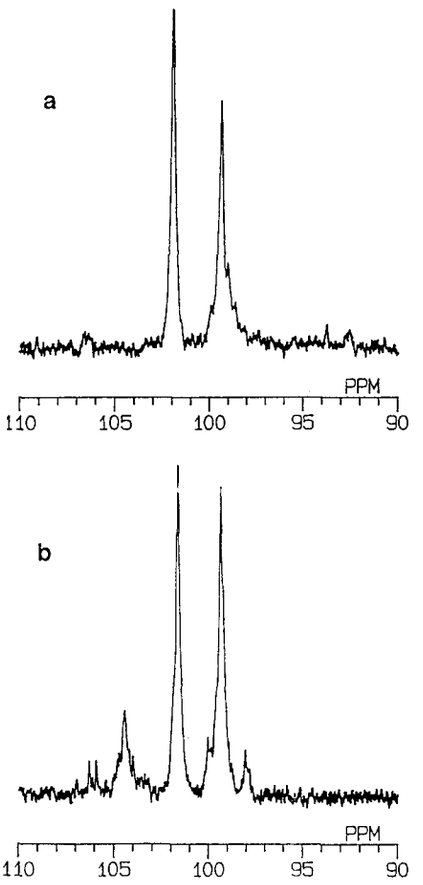

Fig. 5. ^{13}C NMR spectrum of the anomeric region (90–110 ppm) of fragment 2 (a) compared to the anomeric region of the heparin used as starting material (b). Methanol was used as an internal standard (51·6 ppm). The disappearance of the signal at 104·5 (C-1 of glucuronic acid) is clearly seen in (a), as well as changes in the region 97·5–100 ppm due to the decrease in the number of N-acetyl groups.

Heparin fragment 4 was also tested in a slightly different CAM assay (Jakobson *et al.*, 1989). When compared to heparin and several other natural and

chemically modified glycosamino-glycans, fragment 4 was found to be a very anti-angiogenic compound both in the presence and, to a lesser extent, in the absence of hydrocortisone (Hahnenberger & Jacobson, 1990).

Further studies on well defined heparin saccharides are needed in order to understand fully how heparin modulates angiogenesis in the presence as well as in the absence of steroids.

ACKNOWLEDGEMENTS

The authors thank Professor Judah Folkman for help in setting up the CAM assay and for assaying heparin fragment 1, showing very good inter-laboratory correlation between Kabi Pharmacia and the Surgical Research Laboratory, Harvard Medical School, Boston.

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