Endothelial binding sites for heparin

Specificity and role in heparin neutralization

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The specificity of endothelial binding sites for heparin was investigated with heparin fractions and fragments differing in their M_r , charge density and affinity for antithrombin III, as well as with heparinoids and other anionic polyelectrolytes (polystyrene sulphonates). The affinity for endothelial cells was estimated by determining I_{50} values in competition experiments with ^{125}I -heparin. We found that affinity for endothelial cells increases as a function of M_r and charge density (degree of sulphation). Binding sites are not specific receptors for heparin. Other anionic polyelectrolytes, such as pentosan polysulphates and polystyrene sulphonates, competed with heparin for binding to endothelial cells. Fractions of standard heparin with high affinity for antithrombin III also had greater affinity for endothelium. However, these two properties of heparin (affinity for antithrombin III and affinity for endothelial cells) could be dissociated. Oversulphated heparins and oversulphated low- M_r heparin fragments had lower anticoagulant activity and higher affinity for endothelial cells than did their parent compounds. Synthetic pentasaccharides, bearing the minimal sequence for binding to antithrombin III, did not bind to endothelial cells. Binding to endothelial cells involved partial neutralization of heparin. Bound heparin exhibited only 5% and 7% of antifactor IIa and antifactor Xa specific activity, respectively. In the presence of 200 nM-antithrombin III, and in the absence of free heparin, a limited fraction (approx. 30%) of bound heparin was displaced from endothelial cells during a 1 h incubation period. These data suggested that a fraction of surface-bound heparin could represent a pool of anticoagulant.

INTRODUCTION

Heparins are anticoagulant drugs which act by antithrombin III-mediated inhibition of serine proteinases involved in blood coagulation. However, the antithrombotic properties of heparin fractions and fragments *in vivo* are not always quantitatively correlated with their anticoagulant potency *in vitro* (Jaques, 1980, 1982) nor with their affinity for antithrombin III (AT III) (Barrowcliffe *et al.*, 1984; Merton *et al.*, 1984; Thomas, 1984).

Binding of heparin to endothelial cells has been proven by experiments *in vivo* and *in vitro* (Hiebert & Jaques, 1976a,b; Mahadoo *et al.*, 1978; Hiebert, 1981; Glimelius *et al.*, 1978; Bauer *et al.*, 1983; Bârzú *et al.*, 1984; Gajdusec, 1984). Binding has been implicated as a possible indirect mechanism of antithrombotic action (Jaques, 1982; Vairel *et al.*, 1983; Barrowcliffe *et al.*, 1984; Fareed, 1985) despite a lack of data allowing a correlation of the antithrombotic effects of different heparins with their affinity for endothelial cells.

In a previous study we described the kinetics of binding of standard heparin and low- M_r heparin fragments to human endothelial cells in culture (Bârzú *et al.*, 1985). Much lower affinity for endothelial cells was found with low- M_r fragments than with standard heparin. Our results also suggested that a fraction of standard heparin is internalized by endothelial cells, as was shown with other cell types (Bleiberg *et al.*, 1983;

Reyser *et al.*, 1983; Castellot *et al.*, 1985). The aim of the present work was to elucidate whether endothelial binding sites for heparin are specific receptors, able to elicit a specific response, or are nonspecific binding sites, involved in heparin neutralization and/or degradation. The specificity of binding sites was determined with various heparin fractions or fragments differing in their M_r , charge density, and AT III affinity, as well as with various analogues and polyanionic electrolytes. We also tried to determine whether binding and/or endocytosis contribute to the inactivation of heparin and to what extent these events might potentiate the antithrombogenic properties of the endothelial cells. We therefore examined the availability of endothelial bound heparin for interaction with AT III as well as the AT III-dependent antiproteinase activity of endothelial bound heparin. Our results show that endothelial binding sites are not specific receptors for heparins. These binding sites are involved in partial neutralization of the anticoagulant activity of heparins.

MATERIALS AND METHODS

Chemicals

All chemicals for culture medium and fetal calf serum were purchased from Flow Laboratories (Irvine, Scotland, U.K.). Ultrosor G (a serum substitute containing growth and adhesion factors, mineral trace elements, hormones,

binding proteins, vitamins, but a low concentration of total proteins) was a product of IBF, France. Collagenase was obtained from Boehringer-Mannheim. Proteinase type XIV (Pronase), bovine serum albumin and Polybrene were products of Sigma. Chromogenic substrates (CBS 31.39 and 34.47) and purified human factor Xa was obtained from Diagnostica Stago, France. AT III purified from human plasma (Thaler & Schmer, 1975) and human thrombin generated from purified prothrombin by using the prothrombinase complex (Pletcher & Nelsestuen, 1982) were gifts from Dr. Lindhout (University of Limburg, the Netherlands). The concentration of AT III was calculated from $A_{280}^{1\% \cdot 1\text{ cm}} = 5.7$. The concentration of thrombin (specific activity 3000 NIH units/mg) was determined by active-site titration (Chase & Shaw, 1969) with *p*-nitrophenyl-*p*'-guanidinobenzoate hydrochloride.

The following heparins and low- M_r heparins were obtained from Institut Choay, Paris: porcine mucosal standard heparin (160 USP units/mg and 155 anti-Xa units/mg); partially desulphated and Bolton-Hunter derivatized heparin (8.9 nmol of aromatic residues/mg, 130 USP units/mg, 130 anti-Xa units/mg) as described by Dawes & Pepper (1979); CY 216 (2500–8000 Da) and CY 222 (1500–8000 Da) were obtained by chemical depolymerization with HNO_3 and gel filtration (patent application in progress). Other heparin fractions (noted as A, B, C and D) were obtained by ion-exchange chromatography (Sache *et al.*, 1982) and were kindly supplied by Dr Sache. High-affinity and low-affinity fractions were separated by chromatography on AT III-Sepharose (Hopwood *et al.*, 1976). Pentosan polysulphate (Hemoclar), its partially desulphated derivative (SR 24776) and nonsulphated pentosans were kindly supplied by Dr. Mafrand (Sanofi Recherche, Toulouse, France). Polystyrene sulphonates were purchased from Chrompack (Orsay-Les-Usis, France).

Gel filtration of heparin and heparin fractions

In order to compare the profiles of labelled and nonlabelled heparins, we used a column of Sephadex G-100 (fine grade) (145 cm $\times$ 2 cm) eluted with 0.2 M-NaCl. Homogeneous oligosaccharides (hexa-, octa- and dodeca-saccharides) as well as the 8000- M_r fraction were obtained from CY 222 by gel filtration on Sephadex G-50 (superfine grade) (300 cm $\times$ 2.5 cm) eluted with 0.2 M-NaCl. The degree of polymerization of the compound was deduced from analysis of the chromatogram and confirmed by ^{13}C -n.m.r. analysis for hexa- and octa-saccharide. The compounds were desalted by gel filtration on Sephadex G-25 with water as eluent.

M_r determination

The M_r of heparin preparations was obtained by h.p.l.c. with TSK SW 2000 (Toyo Soda, Japan) or Lichrospher Si 100 (Merck, Germany) columns. The columns were calibrated using standards of known M_r , previously determined by ultracentrifugation (Barlow, 1983). M_r values for oligosaccharides (hexa-, octa- and dodeca-saccharides) were deduced from their degree of polymerization assuming an M_r of 316 for the average monosaccharide. M_r values of polystyrene sulphonates, pentosan polysulphate and related compounds were provided by the manufacturers.

Degree of sulphation

Degree of sulphation was determined by conductometry according to Casu & Gennaro (1975). The degree of sulphation of pentosan polysulphonates was provided by the manufacturer.

Labelling procedure

Standard, derivatized heparin (see under 'Chemicals'), was labelled with stoichiometric amounts of Na^{125}I according to Greenwood *et al.* (1963) by the chloramine T method, as described previously (Bâzu *et al.*, 1984). Antithrombin activity and the gel filtration pattern on Sephadex G-100 of labelled heparin were compared with the same parameters of derivatized standard heparin and found to be practically identical. The specific radioactivity of labelled heparin was 15–17 mCi/mg. At 1 week after labelling, the radioactive ligand was dialysed for 24 h against phosphate-buffered saline at 4 °C, using a membrane with a cut-off of 3500 Da, to avoid any trace of free ^{125}I resulting from radiolysis.

Cell culture

Primary cultures of human umbilical vein endothelial cells were prepared according to Jaffe *et al.* (1973) and were allowed to grow in 35 mm Petri dishes in medium M 199 supplemented with 20% (v/v) fetal calf serum, 20 μmol of glutamine/ml, 50 units of penicillin/ml and 50 units of streptomycin/ml at 37 °C in a humid atmosphere containing 5% CO_2 . Monolayers were confluent by days 5–6 [(6–8) $\times 10^5$ cells/dish]. The identity of endothelial cells was confirmed by immunofluorescent demonstration of the presence of factor VIII-related antigen as described by Booyse *et al.* (1981).

Binding experiments

At 24 h before each experiment the medium containing fetal calf serum was removed and cultures were washed and changed to medium containing 2% Ultrosor G. This medium was again changed just before the experiment. As previously shown (Bâzu *et al.*, 1985) medium containing Ultrosor G did not interfere with the binding of heparin to endothelial cells, but prevented cell detachment during repetitive manipulation. Binding was performed during a 5 h incubation with 10 pmol of ^{125}I -heparin/dish in the presence of different unlabelled compounds. After incubation, cells were gently washed three times with 1.5 ml of phosphate-buffered saline, then detached and solubilized by incubation for 30 min at 37 °C with 0.5 ml of phosphate-buffered saline containing 1 mg of Pronase/ml and 0.1% Triton X-100. The radioactivity of cell lysates and of the incubation medium was measured with a Beckman 7000 gamma counter. The affinity of the endothelium for different heparin fractions, fragments or analogues was indirectly determined from competition experiments, using a low concentration of labelled ligand (5 nM). Under these conditions the I_{50} value for unlabelled compounds could reflect the K_d (Hollenberger & Cuatrecasas, 1979; Naqui, 1983) and consequently the affinity of the compound for the endothelial cells. Each I_{50} value represents the mean of three or four experiments conducted in duplicate, with different batches of labelled heparins.

Determination of anti-IIa and anti-Xa activity of heparin bound to endothelial cells

Confluent cells in 35 mm culture dishes were incubated for 5 h with medium M 199 and Ultrosor G, in the absence or the presence of different concentrations of heparin, then washed five times with 1.5 ml of modified Hanks' balanced salt solution containing 1 mg of bovine serum albumin/ml and 15 mM-Hepes (pH 7.4). The reaction was carried out in 0.45 ml of washing buffer. AT III (25 μ l of 0.5 μ M) was added and preincubated for 5 min at 37 °C. The reaction was started by the addition of either 25 μ l of thrombin (0.1 μ M) or 25 μ l of factor Xa (0.1 μ M). At different time intervals aliquots (40 μ l) were taken to determine residual amidolytic activity. Aliquots were placed in spectrophotometric cuvettes containing 1 ml of 50 mM-Tris/HCl (pH 8), 175 mM-NaCl, 0.5 mg of ovalbumin/ml and 0.4 mg of Polybrene/ml. Amidolytic activity was determined by following the absorption change at 405 nm after addition of either CBS 34.47 (94 μ M), or CBS 31.39 (188 μ M), which are specific chromogenic substrates for thrombin and factor Xa, respectively.

The pseudo-first order rate constant was estimated by plotting log(residual amidolytic activity) versus time. This rate constant was linearly related to the concentration of heparin in the measuring system, allowing the calculation of the anti-IIa and anti-Xa activities present in the cell culture dish or released in the medium. The anti-IIa and anti-Xa specific activities, k ($\text{min}^{-1} \cdot \mu\text{g}^{-1}$), were determined on the basis of the amount of cell-bound or cell-released radioactivity, in experiments conducted with trace amounts of ^{125}I -heparin, diluted in 0.2 μ M of unlabelled heparin.

RESULTS

Affinity for endothelium of the fractions of standard heparin separated by ion-exchange chromatography

Standard heparin is a heterogenous mixture of polysaccharide chains differing in M_r , degree of sulphation, and affinity for the specific receptor, AT III. In order to find out which of these components have a greater affinity for endothelium, four heparin fractions (A–D) separated by ion-exchange chromatography were tested for their ability to compete with ^{125}I -heparin in binding to endothelial cells (Table 1). These fractions were studied by Sache *et al.* (1982) for their anticoagulant activity *in vitro* and their affinity for AT III. It first appeared that their affinity for endothelial cells, as

estimated by I_{50} , increased as a function of charge density or degree of sulphation. However, it is important to point out that separation by ion-exchange chromatography, like other techniques of heparin fractionation, results in more than one structural variable at the same time (Hurst *et al.*, 1983). The fractions with higher charge density also had a higher M_r and greater proportion of chains with high affinity for AT III. Therefore we could not exclude the possibility that affinity for endothelium might also depend on M_r and/or affinity for AT III.

Affinity for endothelium as compared with affinity for AT III

To determine whether binding to endothelial cells requires the same structural factors as binding to AT III, we measured I_{50} values of high- and low-affinity fractions, separated by chromatography on AT III–Sephacrose (Table 2). High-affinity fractions exhibited approximately three times higher affinity for endothelial cells than did low-affinity fractions. To compare further the structural requirements, two synthetic oligosaccharides were also tested: a tetrasaccharide, which is completely devoid of affinity for AT III, and a pentasaccharide which contains the minimal sequence necessary for binding to AT III (Choay *et al.*, 1983). Neither the penta- nor the tetra-saccharide was able to compete with heparin binding to endothelial cells.

Role of chain length and charge density in binding to endothelium

To establish the influence of the chain length on binding to endothelial cells, the affinity of different fractions or fragments of heparin was measured (Table 3). Low- M_r heparin fractions, such as CY 216 or fraction A, have much lower affinity for endothelium than does standard heparin. Low- M_r heparin fragments such as CY 222 competed with no more than 30% binding of standard heparin at 100 μ M (Bârzu *et al.*, 1985). These results suggested that only the higher- M_r fragments from the mixture of CY 222 could bind to the endothelial cells. Therefore, narrow M_r -range fragments such as the 8000 Da fraction (containing approx. 25 oligosaccharides), or the hexadeca-, dodeca-, and octa-saccharide, were separated by gel filtration. Only the 8000 Da fraction was able to compete with standard heparin binding. The other fragments, at concentration as high as 0.1 mM, did not bind to endothelium.

These results suggest that binding to endothelium requires either a high degree of polymerization or a large number of negatively charged groups. Standard heparin

Table 1. Competition of ^{125}I -heparin binding to endothelial cells by heparin fractions obtained by DEAE-Sephacel fractionation

Compound	Average M_r *	Degree of sulphation (OSO ₃ /COO)*	High-affinity fraction for AT III (%)*	Affinity for endothelium [I_{50} (μ M)]
Standard heparin	15000	2.16	35	0.17
Fractions				
A	7300	1.31	22	2.0
B	13200	2.22	43	0.13
C	17300	2.46	71	0.04
D	20300	2.48	100	0.03

* As determined by Sache *et al.* (1982).

Table 2. Competition of ^{125}I -heparin binding to endothelium as a function of affinity for AT III

Compound	Anticoagulant activity <i>in vitro</i> (units/mg)		Affinity for endothelium [I_{50} (μM)]
	APTT	Anti-Xa*	
Standard heparin	144	165	0.17
High affinity heparin fractions	236	168	0.07
Low affinity heparin fractions	106	61	0.25
Synthetic pentasaccharide	0	4000	> 100
Synthetic tetrasaccharide	0	0	> 100

* Determined by the method of Yin *et al.* (1973).

and the above mentioned fractions and fragments were additionally sulphated (M. Petitou, unpublished work). Their affinity for endothelial cells and their anticoagulant activity *in vitro* were then compared with those of the parent compounds. Increased sulphation strongly enhanced the affinity of heparin, fraction A, CY 216 and CY 222 fragments. Even the lower- M_r fragments, such as dodeca- or octa-saccharides, showed increased affinity for the endothelium. It should be mentioned however, that for an equal degree of sulphation, the I_{50} was still dependent on M_r . It is also shown (Table 3) that the variation in affinity for endothelial cells was not correlated with the AT III-dependent anticoagulant activity of these compounds. In general, additional sulphation was accompanied by a significant decrease of the anticoagulant potency *in vitro* of the tested compounds (Table 3).

Affinity of other sulphated polysaccharides (heparinoids) or organic polyanions for endothelial cells

In order to find out if endothelial binding sites exhibit any specificity for the polysaccharide chain of heparins, sulphated pentosans and polystyrene sulphonate were

tested in competition experiments with standard heparin. Pentosan polysulphate (Hemoclar) had a higher affinity for endothelial cells than did standard heparin (Table 4), despite its lower average M_r and its lower affinity for AT III (Fischer *et al.*, 1982). The affinity of pentosan polysulphate for endothelial cells was diminished by a partial desulphation (compound SR 24776). The non-sulphated parent pentosan was completely devoid of affinity for endothelium. Other sulphated polymers such as polystyrene sulphonates were also tested. As shown in Table 4, they competed with heparin binding and their affinity was dependent on their M_r and their degree of sulphation.

Competition of ^{125}I -heparin binding to endothelial cells by AT III

In our previous work (Bârzu *et al.*, 1985), we showed that binding of radiolabelled heparin to endothelial cells in the presence of 20% fetal calf serum was five times lower than in the absence of serum. Many serum components might be responsible for this competition, including AT III, platelet factor 4, histidine-rich glycoproteins, and low density lipoproteins (Marciniak, 1974; Handin & Cohen, 1976; Lijnen *et al.*, 1983;

Table 3. Competition of ^{125}I -heparin binding to endothelial cells by low- M_r heparin fractions and their additionally sulphated derivatives

Compound	M_r	Degree of sulphation (OSO_3/COO)	Anticoagulant activity <i>in vitro</i> (units/mg)		Affinity for endothelium [I_{50} (μM)]
			APTT	Anti-Xa	
Standard heparin	15000	2.16	144	165	0.17
Fraction A*	(6000–25000)	3.01	60	35	0.025
	7300	1.31			2.0
		1.90			0.23
CY 216	4500	2.1	26	224	15
CY 222	(1800–8000)	2.5	22	30	0.15
	2500	2.1	12	250	> 100
	(1500–8000)				
Fraction 8000	8000	2.1	12	250	0.52
Hexadecasaccharide	5000	2.04	10	274	> 100
		3.35	16	23	0.32
		2.26	6	200	> 100
Dodecasaccharide	3800	2.91	5	30	8.0
		2.41	3	143	> 100
		2.95	4	39	20
Octasaccharide	2500				

* See Table 1.

Table 4. Competition of ^{125}I -heparin binding to endothelium by polysulphated pentosans and other polyanions

Compound	Average M_r	Degree of sulphation (% S)*	Affinity for endothelium [I_{50} (μM)]
Standard heparin	15000	11	0.17
Pentosan polysulphate	4700	17.5–18.5	0.13
SR 24.776	3400	14	8.2
Non-sulphated pentosans	2000	0	> 100
Polystyrene	31000	10.2†	0.52
sulphonates	4000	16.0†	6.0

* As reported by manufacturer.

† Determined by conductometry.

Kecskes *et al.*, 1983). We studied the effect of purified human AT III on the binding of ^{125}I -heparin, in the range of concentrations for high affinity binding. Concentrations of AT III higher than 10 nM inhibited binding, with an I_{50} value of 27 nM (Fig. 1). This result strengthened the data of Table 1, which showed that the fractions of standard heparin having a high proportion of high-affinity chains for AT III might be involved in binding to endothelium.

Effect of AT III on the release of endothelial bound heparin

Endothelial cells were prelabelled with ^{125}I -heparin. The displacement of the bound fraction from endothelium by AT III was then investigated (Fig. 2). Only high concentrations of AT III (approx. 100 times higher than

bound heparin) were able to displace significantly more heparin than was spontaneously released (Fig. 2). No displacement was found at the concentrations and at the short time of incubation (10 min) used in the kinetic study of anti-IIa or anti-Xa activities.

Anti-IIa and anti-Xa activity of bound heparin

The AT III-dependent antiprotease activity of endothelium bound heparin as well as of the released fraction was measured. This was done in order to examine the role of binding in the neutralization of heparin and in the antithrombogenic properties of endothelium. Adequate conditions for pseudo-first-order kinetics of factor IIa and factor Xa inactivation reactions were selected. The rate of inactivation of the two proteinases was measured using albumin-coated dishes and dishes containing cell monolayer with or without previously bound heparin. No difference was

Fig. 1. Competition of ^{125}I -heparin binding to endothelial cells by AT III

Endothelial cell monolayers were incubated at 37 °C for 5 h with 5 pmol of ^{125}I -heparin/dish in the absence or in the presence of different concentrations of purified AT III, and cell bound radioactivity was measured. The results represent a typical experiment conducted in triplicate. Similar results were obtained in the other two experiments. The broken line represents the level of nonspecific binding in the presence of 10 μM unlabelled heparin.

Fig. 2. Effect of AT III on the release of endothelial bound heparin

Endothelial cells in culture were incubated at 37 °C for 5 h with 0.2 μM standard heparin containing trace amounts (10 pmol/dish) of ^{125}I -heparin. Cells were then washed five times in Hanks' buffer + albumin (1 mg/ml) and incubated for different time intervals in 0.5 ml of buffer, in the absence (○) or in the presence of 25 nM- (●) or 200 nM- (▲) AT III. The released and cell-bound radioactivity was determined in triplicate dishes. Results are expressed as percentage of initially bound heparin.

Fig. 3. Inactivation of thrombin (a) and factor Xa (b) by AT III in the presence of endothelial bound heparin

The rate of inactivation of factor IIa and factor Xa was measured in triplicate dishes using: albumin-coated plastic dishes (●); dishes containing endothelial cells (○); endothelial cells previously incubated for 5 h with $0.2 \mu\text{M}$ standard heparin ('heparinized' cells) (■) and washed five times in incubation buffer to determine the activity of total cell-bound heparin; supernatant of 'heparinized' cells after 10 min incubation in Hanks' buffer albumin (1 mg/ml) (□) to determine the activity of released heparin fraction. Reaction mixture: 0.45 ml of Hank's/albumin buffer, $25 \mu\text{l}$ of $0.5 \mu\text{M}$ -AT III and $25 \mu\text{l}$ of $0.1 \mu\text{M}$ -thrombin or factor Xa.

found in the kinetics of IIa or Xa inactivation between albumin-coated dishes and cell monolayers (Fig. 3). An anti-IIa and anti-Xa activity was found in dishes containing cells with previously bound heparin. These activities were dependent on the amount of heparin bound (results not shown). When cell supernatants were used for kinetic assays the activity was partially recovered, showing that the activity found in culture dishes is partially due to the release of a fraction of heparin from endothelial cells during incubation (Fig. 3).

Simultaneously, on the same primary cultures, we measured the amount of bound and released heparin, using ^{125}I -heparin as tracer. The total and specific antiproteinase activity of bound and released heparin was compared with the activity of heparin in solution (in the absence of cells). The activity of the bound fraction was very low: 5% and 7% for anti-IIa and anti-Xa activity respectively, as compared with standard heparin in solution (Table 5). Moreover, the released heparin exhibited lower specific activity and a higher ratio of anti-Xa/anti-IIa activity as compared with applied material.

DISCUSSION

Binding of heparin to vascular endothelium has been described after intravenous injection into various animal species (Mahadoo *et al.*, 1978; Hiebert, 1981) and was confirmed by incubating vessel segments (Hiebert & Jaques, 1976a,b) or cell cultures (Glimelius *et al.*, 1978; Bauer *et al.*, 1983; Bârză *et al.*, 1984; Gajdusec, 1984) with heparin-containing medium. Binding was considered as evidence for an additional vascular mechanism of action of heparins (Jaques, 1982; Fareed, 1985; Barrowcliffe *et al.*, 1984). However, there is no experimental support to show that binding to endothelium has a significant effect on either the pharmacokinetics or action of heparin. In the present paper we studied the effect of binding of heparin to endothelial cells on its activity in the AT III-dependent inhibition of factors IIa and Xa.

Table 5. Anti-IIa and anti-Xa activity of the heparin bound to endothelial cells

Experimental conditions are given in detail in the legend of Fig. 3. Calculation of rate constants, total and specific anti-IIa and anti-Xa activities is described under 'Material and methods'. The values represent the mean of three separate experiments conducted in triplicate. The amount of total cell bound heparin and of heparin released during 10 min of incubation was determined by measuring the radioactivity after incubation of triplicate dishes of the same primary culture with $6 \mu\text{g}$ of heparin/dish ($0.2 \mu\text{M}$), containing trace amounts of ^{125}I -labelled heparin. Percentage of activity recovered was calculated for total cell-bound and released heparin, taking the anti-IIa and anti-Xa activity of $0.08 \mu\text{g}$ of heparin, in the absence of cells, as 100%.

Heparin sample	Anti-IIa activity		Anti-Xa activity	
	Total activity $k (\text{min}^{-1})$	Specific activity $k (\text{min}^{-1} \cdot \mu\text{g}^{-1})$	Total activity $k (\text{min}^{-1})$	Specific activity $k (\text{min}^{-1} \cdot \mu\text{g}^{-1})$
Total cell-bound heparin ($0.08 \mu\text{g}$)	0.233 (5%)	2.9	0.264 (7%)	3.3
Heparin released during 10 min incubation ($0.0071 \mu\text{g}$)	0.07 (1.5%)	9.9	0.155 (4.2%)	21.7
Standard heparin in solution ($0.08 \mu\text{g}$)	4.65	58.2	3.77	47.2

Since standard heparin is a heterogenous mixture of polysaccharide chains differing in M_r , charge density and affinity for AT III, it was necessary to establish the relative affinities of endothelial binding sites for the different components of the mixture. Our results show that higher- M_r fractions with high charge density bind more strongly to endothelial cells. The same fractions also contain a higher proportion of chains with high affinity for AT III (Table 1), which is able to inhibit heparin binding to endothelium (Fig. 1). It seems, however, that different structural factors are involved in binding to endothelial cells and in binding to AT III, since the synthetic pentasaccharide, bearing the minimal sequence involved in binding to AT III, did not compete with binding of heparin to endothelial cells. The correlation between binding to endothelial cells and affinity for AT III (Table 2, lines 2 and 3) could be secondary to a variation in chain length. As has been previously shown by Laurent *et al.* (1978), affinity for AT III increases with M_r of the polysaccharide chains.

Binding to endothelial cells was strongly dependent on M_r . Low- M_r heparin fractions or fragments (generated by depolymerization) had very low affinity for endothelial cells. Therefore, there is no reason to consider that binding to endothelial cells is the basis for a vascular mechanism of action, especially as concerns low- M_r heparin.

Oversulphation of different heparin fractions or fragments induced a higher affinity for endothelial cells. Nonetheless, affinity continued to be dependent on M_r (Table 3). The anticoagulant activity *in vitro* of these randomly oversulphated compounds was significantly lower compared with the activity of parent compounds. Since oversulphation yields compounds with high affinity for endothelium and low AT III-dependent anticoagulant activity, these two properties of heparins can be dissociated. Oversulphation of heparin fractions emphasizes the importance of electrostatic interactions in binding to endothelial cells.

Other highly sulphated polysaccharides (pentosan polysulphate) having low affinity for AT III (Fischer *et al.*, 1982) and sulphated organic polyanions (polystyrene sulphonates) also compete with heparin binding (Table 4). The affinity of these compounds similarly depended on their M_r and their degree of sulphation. The binding of pentosan polysulphate and polystyrene sulphonates to endothelial cells demonstrated once again that interactions are mostly electrostatic, as has been described for the binding of heparin to thrombin (Heuck *et al.*, 1985) to platelet factor 4 (Lane *et al.*, 1984) and to fibronectin (Ogamo *et al.*, 1985). It might be concluded that endothelial binding sites are not specific receptors for heparins, but rather nonspecific binding sites or so-called 'acceptor' sites for polyanionic electrolytes. The question then arises whether binding to these nonspecific binding sites has any significance for the activity of heparin as an anticoagulant, or for the antithrombogenicity of the endothelial cell lining.

One of the natural mechanisms of the antithrombogenicity of endothelium should be the occurrence on the endothelial cells of 'heparin-like molecules' (Rosenberg & Rosenberg, 1984). The presence of such a heparin-like activity was demonstrated in organ perfusions (Busch & Owen, 1982; Marcum *et al.*, 1984), using bovine aorta segments (Stern *et al.*, 1985) or by extraction from bovine

or rat microvascular endothelial cells in culture (Marcum & Rosenberg, 1984, 1985). In our experimental conditions (primary culture at confluence, chromogenic assay) the human umbilical vein endothelial cells did not exhibit a heparin-like activity. Our results are in agreement with data reported by Rodgers *et al.* (1983) working with bovine aorta endothelial cells in culture, in similar experimental conditions.

Here, the endothelial bound heparin slightly accelerated factor IIa and factor Xa inactivation by AT III. However, the bound fractions were less available to interaction with AT III (25 nM) and their activities represented respectively 5% and 7% of anti-IIa and anti-Xa activities of an equivalent amount of heparin in solution. These activities are partially provided by a release of a fraction of bound heparin into the incubation medium. It seems therefore unlikely that cell bound heparin could express full biological activity as an 'immobilized anticoagulant' (Larsson *et al.*, 1980; Hatton *et al.*, 1983) or mimic the action of the endogenous heparin-like compound reported by Marcum and coworkers (Marcum & Rosenberg, 1984, 1985; Marcum *et al.*, 1984). Instead, binding to endothelial cells was accompanied by a neutralization of heparin. The fraction of heparin released into the medium exhibited lower specific activity and a higher ratio of anti-Xa/anti-IIa potency than the applied heparin. These results suggest that lower- M_r chains are released from the endothelial cells. Further investigations are necessary to verify the possibility that heparin is degraded following binding and endocytosis by the endothelial cells. In the presence of an excess of AT III in the medium and in the absence of free heparin, surface-bound heparin fraction is partially dissociated and therefore may represent a source of anticoagulant.

The question arises whether endothelial binding, neutralization and release of heparin occur *in vivo*, in the presence of physiological concentrations of AT III and other plasma proteins with high affinity for heparin. Several points must be emphasized when trying to extrapolate data obtained *in vitro* to conditions *in vivo*.

The inhibitory effect on heparin binding to endothelium was found with concentrations of AT III higher than 10 nM. But the ratio between the number of endothelial binding sites for heparin and the concentration of AT III *in vivo* is different from that *in vitro*. This difference is due to a much higher endothelial surface/blood volume ratio *in vivo*, especially in the capillary bed (Busch *et al.*, 1982). It is therefore difficult to predict the relative distribution of heparin *in vivo* between AT III, other plasma proteins, and endothelial binding sites.

As mentioned above, uptake of heparin by endothelial cells has been found *in vivo* in animals after heparin administration (Mahadoo *et al.*, 1978; Hiebert, 1981). Moreover, the pharmacokinetics of standard heparin are consistent with the existence of a saturable (cellular) pool of compound (Caranobe *et al.*, 1985). This saturable pool was not found with low- M_r heparin fragments (CY 222) which have much lower affinity for endothelium.

In conclusion, data obtained with human endothelial cells in culture showed that these cells have nonspecific binding sites for anionic polyelectrolytes. The more charged high- M_r heparin fractions, which have also high affinity for AT III, are selectively bound by endothelial cells. Binding to endothelial cells involved neutralization

of most of the anticoagulant activity of heparin. Only a fraction of surface bound heparin might represent a source of circulating anticoagulant.

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