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 activity for endothelial cells was estimated by determining \(K_{50}\) values in competition experiments with \(^{125}\text{I}\)-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; Gilmeius et al., 1978; Bauer et al., 1983; Bărzu 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 (Bărzu 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 antiprotease 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.). Ulotroser 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 $A_{1}^{1}1.0\, cm = 5.7$. 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 desulfated 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 desulfated derivative (SR 24776) and nonsulfated pentosans were kindly supplied by Dr. Mafrand (Sanofi Recherche, Toulouse, France). Polystyrene sulphonates were purchased from Chrompack (Orsay-Les-Ulis, 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 x 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 x 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$^{131}$I according to Greenwood et al. (1963) by the chloramine T method, as described previously (Bärzu 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 $^{131}$I resulting from radioisly.

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% 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 immuno-fluorescent 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% Ultroser G. This medium was again changed just before the experiment. As previously shown (Bärzu et al., 1985) medium containing Ultroser 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 $^{131}$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 Ultrosen 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 μl of 0.5 μM) was added and preincubated for 5 min at 37 °C. The reaction was started by the addition of either 25 μl of thrombin (0.1 μM) or 25 μl of factor Xa (0.1 μM). At different time intervals aliquots (40 μl) 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 μM), or CBS 31.39 (188 μM), 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 (min⁻¹·μg⁻¹), were determined on the basis of the amount of cell-bound or cell-released radioactivity, in experiments conducted with trace amounts of 125I-heparin, diluted in 0.2 μM 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 Mr, 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 125I-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 I₅₀, 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 Mr, 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 Mr, 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 I₅₀ values of high- and low-affinity fractions, separated by chromatography on AT III-Sepharose (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 hexasaccharide, 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-Mr, heparin fractions, such as CY 216 or fraction A, have much lower affinity for endothelium than does standard heparin. Low-Mr, heparin fragments such as CY 222 competed with no more than 30% binding of standard heparin at 100 μM (Bärzu et al., 1985). These results suggested that only the higher-Mr, fragments from the mixture of CY 222 could bind to the endothelial cells. Therefore, narrow Mr-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 125I-heparin binding to endothelial cells by heparin fractions obtained by DEAE-Sepharose fractionation

<table>
<thead>
<tr>
<th>Compound</th>
<th>Average Mr*</th>
<th>Degree of sulphation (OSO₄/COO)*</th>
<th>High-affinity fraction for AT III (%)*</th>
<th>Affinity for endothelium [I₅₀ (μM)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard heparin</td>
<td>15000</td>
<td>2.16</td>
<td>35</td>
<td>0.17</td>
</tr>
<tr>
<td>Fractions</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>7300</td>
<td>1.31</td>
<td>22</td>
<td>2.0</td>
</tr>
<tr>
<td>B</td>
<td>13200</td>
<td>2.22</td>
<td>43</td>
<td>0.13</td>
</tr>
<tr>
<td>C</td>
<td>17300</td>
<td>2.46</td>
<td>71</td>
<td>0.04</td>
</tr>
<tr>
<td>D</td>
<td>20300</td>
<td>2.48</td>
<td>100</td>
<td>0.03</td>
</tr>
</tbody>
</table>

* 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

<table>
<thead>
<tr>
<th>Compound</th>
<th>APTT</th>
<th>Anti-Xa*</th>
<th>Affinity for endothelium [I$_{50}$ (μM)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard heparin fractions</td>
<td>144</td>
<td>165</td>
<td>0.17</td>
</tr>
<tr>
<td>High affinity heparin</td>
<td>236</td>
<td>168</td>
<td>0.07</td>
</tr>
<tr>
<td>Low affinity heparin</td>
<td>106</td>
<td>61</td>
<td>0.25</td>
</tr>
<tr>
<td>Synthetic pentasaccharide</td>
<td>0</td>
<td>4000</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>Synthetic tetrascarhide</td>
<td>0</td>
<td>0</td>
<td>&gt; 100</td>
</tr>
</tbody>
</table>

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

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

<table>
<thead>
<tr>
<th>Compound</th>
<th>$M_r$</th>
<th>Degree of sulphation (OSO$_2$/COO)</th>
<th>APTT</th>
<th>Anti-Xa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard heparin</td>
<td>15000</td>
<td>(6000-25000)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fraction A*</td>
<td>7300</td>
<td></td>
<td>1.16</td>
<td>144</td>
</tr>
<tr>
<td>CY 216</td>
<td>4500</td>
<td>(1800-8000)</td>
<td>2.1</td>
<td>26</td>
</tr>
<tr>
<td>CY 222</td>
<td>2500</td>
<td>(1500-8000)</td>
<td>2.1</td>
<td>12</td>
</tr>
<tr>
<td>Fraction 8000</td>
<td>8000</td>
<td></td>
<td>2.1</td>
<td>12</td>
</tr>
<tr>
<td>Hexadecasaccharide</td>
<td>5000</td>
<td></td>
<td>2.04</td>
<td>10</td>
</tr>
<tr>
<td>Dodecasaccharide</td>
<td>3800</td>
<td></td>
<td>2.26</td>
<td>6</td>
</tr>
<tr>
<td>Octasaccharide</td>
<td>2500</td>
<td></td>
<td>2.41</td>
<td>3</td>
</tr>
</tbody>
</table>

* See Table 1.
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 antiproteinase 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 reactions were selected. The rate of inactivation of the two proteinases was measured using albumin inactivation reactions were selected. The rate of inactivation of the two proteinases was measured using albumin.

**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 125I-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.**

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

<table>
<thead>
<tr>
<th>Compound</th>
<th>Average Mr</th>
<th>Degree of sulphation (% S)*</th>
<th>Affinity for endothelium [I50 (μM)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard heparin</td>
<td>15000</td>
<td>11</td>
<td>0.17</td>
</tr>
<tr>
<td>Pentosan polysulphate</td>
<td>4700</td>
<td>17.5–18.5</td>
<td>0.13</td>
</tr>
<tr>
<td>SR 24.776</td>
<td>3400</td>
<td>14</td>
<td>8.2</td>
</tr>
<tr>
<td>Non-sulphated pentosans</td>
<td>2000</td>
<td>0</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>Polystyrene sulphonates</td>
<td>31000</td>
<td>10.2†</td>
<td>0.52</td>
</tr>
<tr>
<td>Polystyrene</td>
<td>4000</td>
<td>16.0†</td>
<td>6.0</td>
</tr>
</tbody>
</table>

* As reported by manufacturer.
† Determined by conductometry.

Kecskes et al., 1983). We studied the effect of purified human AT III on the binding of 125I-heparin, in the range of concentrations for high affinity binding. Concentrations of AT III higher than 10 nM inhibited binding, with an I50 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 125I-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

![Fig. 1. Competition of 125I-heparin binding to endothelial cells by AT III](image)

![Fig. 2. Effect of AT III on the release of endothelial bound heparin](image)
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 18O-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 (Gilmielius et al., 1978; Bauer et al., 1983; Bárzu 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 μg of heparin/dish (0.2 μM), containing trace amounts of 18O-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 μg of heparin, in the absence of cells, as 100%.

<table>
<thead>
<tr>
<th>Heparin sample</th>
<th>Anti-IIa activity</th>
<th>Anti-Xa activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total activity</td>
<td>Specific activity</td>
</tr>
<tr>
<td></td>
<td>k (min⁻¹)</td>
<td>k (min⁻¹ μg⁻¹)</td>
</tr>
<tr>
<td>Total cell-bound heparin (0.08 μg)</td>
<td>0.233</td>
<td>2.9</td>
</tr>
<tr>
<td>(5%)</td>
<td>(5%)</td>
<td>(7%)</td>
</tr>
<tr>
<td>Heparin released during 10 min incubation (0.0071 μg)</td>
<td>0.07</td>
<td>9.9</td>
</tr>
<tr>
<td>(1.5%)</td>
<td>(1.5%)</td>
<td>(4.2%)</td>
</tr>
<tr>
<td>Standard heparin in solution (0.08 μg)</td>
<td>4.65</td>
<td>58.2</td>
</tr>
</tbody>
</table>

**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 μM 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 μl of 0.5 μM-AT III and 25 μl of 0.1 μM-thrombin or factor Xa.
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 fibrinogen (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 physiologiological 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 the surface bound heparin might represent a source of circulating anticoagulant.

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