Unfractionated heparin (UFH), a glycosaminoglycan (GAG), in commercial preparations varies in size (3,000-30,000 Da) and charge (1, 2). Partial depolymerization of UFH produces low molecular weight heparin (LMWH) that has smaller chain lengths (1,800-12,000 Da) (3, 4). Heparin is used as an anticoagulant for prophylaxis and/or treatment of thrombotic events such as venous thrombosis, pulmonary embolism, and in patients with unstable angina (5).

In vivo, heparin exerts its anticoagulant effects by accelerating the interaction of antithrombin (AT) with various coagulant serine proteases such as thrombin, factor Xa (FXa), and factor IXa (FIXa) (6, 7). AT binds noncovalently to a specific pentasaccharide sequence on UFH causing an allosteric change in AT to a more active conformation (8). UFH also binds to thrombin, bridging the enzyme and AT, to form the thrombin-AT inhibitor complex, which then releases the heparin chain to catalyze further thrombin/AT reactions (9). In contrast, FXa neutralization by heparin/AT complex is achieved by binding of the enzyme directly to AT without formation of the ternary complex (1, 7). Rapid catalysis of thrombin inhibition by AT is a critical step in heparin’s action in vivo since thrombin is the pivotal enzyme in the coagulation cascade.

Binding of heparin to plasma proteins and endothelial surfaces is inhibited by covalent linkage to antithrombin

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Summary
Unfractionated heparin (UFH) and low molecular weight heparin (LMWH) are used for prophylaxis and treatment of thrombosis. However, UFH has a short plasma half-life and variable anticoagulant response in vivo due to plasma or vessel wall protein binding and LMWH has a decreased ability to inactivate thrombin, the pivotal enzyme in the coagulation cascade. Covalent linkage of antithrombin to heparin gave a complex (ATH) with superior anticoagulant activity compared to UFH and LMWH, and longer intravenous half-life compared to UFH. We found that plasma proteins bound more to UFH than ATH, and least to LMWH. Also, UFH bound significantly more to endothelial cells than ATH, with 100% of UFH and 94% of ATH binding being on the cell surface and the remainder was endocytosed. Competition studies with UFH confirmed that ATH binding was likely through its heparin moiety. These findings suggest that differences in plasma protein and endothelial cell binding may be due to available heparin chain length. Although ATH is polydisperse, the covalently-linked antithrombin may shield a portion of the heparin chain from association with plasma or endothelial cell surface proteins. This model is consistent with ATH’s better bioavailability and more predictable dose response.

Keywords
Anticoagulant, vessel wall, protein binding, heparinoid, endocytosis
Despite widespread use as an anticoagulant, heparin has pharmacokinetic and biophysical limitations. First, only one-third of UFH and a smaller fraction of LMWH have the unique AT-binding pentasaccharide sequence (10, 11), thus, a large proportion of pharmaceutical heparin is inactive against thrombin. Second, heparin’s anticoagulant activity and clearance from the circulatory system are influenced by the length of its polysaccharide chains, where larger molecular weight species have higher anticoagulant activity but are also cleared more rapidly than lower molecular weight species (12). The advent of LMWH has alleviated some of the complications/limitations of UFH with increased ease of administration, longer plasma half-life and a more predictable dose-response (5). However, although active against FXa, LMWH has a reduced ability to catalyze thrombin inhibition due to its short chain length (chains with <18 monosaccharides cannot simultaneously bind thrombin and AT (13)).

A major mechanism for loss of plasma heparin activity is the binding to other plasma proteins including platelet factor 4 (14), histidine-rich glycoprotein (14), vitronectin (15), fibronectin (16), and lipoproteins (17). Binding of heparin to these plasma proteins is nonspecific and chain-length dependent (18-20). It has been shown that the capacity of infants and children to generate thrombin is delayed and decreased compared to adults (21, 22) and that thrombin generation is decreased 30% more in heparinized (0.1-0.2 anti-FXa U/ml) plasma from newborns (23) or children (21) compared to similarly heparinized plasma from adults. The enhanced effect of heparin in the young occurs even though plasma AT is decreased (24-26) and may result from decreased concentrations of nonspecific heparin binding plasma proteins compared to that in adult plasma. Another factor affecting the bioavailability of heparin is its clearance rate. Heparin is cleared by a combination of saturable and nonsaturable (renal) mechanisms (27), the latter being effective at clearing therapeutic heparin doses and involves the binding of heparin to sites on the surfaces of endothelial or reticuloendothelial cells (28, 29). Binding of heparin to endothelial cells, like plasma protein binding, is chain-length dependent, where lower molecular weight heparins have lower affinity for endothelial cells (30). Thus, factors that reduce heparin binding to endothelial cells could slow heparin clearance giving improved heparin recovery in patients.

In order to overcome the limitations of UFH and LMWH, we produced a covalent complex of AT and heparin (ATH) which reacts rapidly with thrombin (31, 32) and has high catalytic activity due to increased pentasaccharide content (32, 33). By stabilizing the association of AT and heparin, binding of heparin to other fluid phase or surface bound proteins may be reduced through steric hindrance from the conjugated protein. Several features of ATH give support for this hypothesis. ATH’s plasma half-life is prolonged compared to UFH (31, 34), as would be expected if the heparin in ATH has decreased involvement with receptor-mediated or other elimination pathways. Thus, it is pertinent to determine if the alteration of heparin’s pharmacokinetics and pharmacodynamics in vivo, resulting from conjugation to AT, resides in a decreased affinity for other macromolecules. Here, we report that maintenance of the heparin complex with AT indeed obviates much of the unwanted interactions with plasma and cell-surface proteins.

Materials and methods

ATH and Low Molecular Weight ATH (LMWATH) covalent complexes

ATH production has been previously described (31). Briefly, UFH (Sigma, Mississauga, Canada) and AT (Affinity Biologics, Ancaster, Canada) were mixed in PBS buffer and incubated at 40°C for 14 days. The long incubation at high temperature allows for the production of ATH via Schiff base formation between the AT lysyl ε-amino and heparin aldose aldehyde groups, followed by Amadori rearrangement to produce a stable keto-amine (35). ATH was then purified by a two-step process involving butyl-agarose (Amersham Pharmacia, Uppsala, Sweden) hydrophobic chromatography to remove any excess heparin, followed by DEAE Sepharose (Amersham Pharmacia) anion exchange chromatography to remove unbound AT.

LMWATH was produced by gel filtration of ATH on a Sephacryl S-200 high-resolution (Amersham Pharmacia) chromatography column in 2 M NaCl. The fractions from the column chromatography were collected and a low molecular weight portion was pooled and confirmed by SDS-polyacrylamide gel electrophoresis followed by Alcian blue/silver staining of the gel. Binding of heparinoids to plasma proteins have been shown to be nonspecific and chain-length dependent (18-20). Thus, the use of LMWATH would give us insight into the chain-length dependence of ATH binding to plasma proteins.

Preparation of pooled plasmas

Normal adult plasmas were obtained from healthy volunteers by venipuncture. Cord plasmas were obtained from the umbilical cord vein of term neonates that have undergone uneventful deliveries. Whole blood (9 volumes) was drawn into a syringe containing 3.2% sodium citrate (1 volume) and then centrifuged for 20 minutes at 3,000 × g at 4°C to obtain plasma supernatant. The resultant plasmas were re-centrifuged. The individual plasmas from adults or cords were then pooled to make adult normal pooled plasma (NPP) or cord pooled plasma (CPP), respectively, aliquoted and kept frozen at ~70°C until assayed. Any haemolyzed plasmas were excluded from the pool. All blood samples were obtained in accordance with McMaster University, Henderson Research Centre, and St. Joseph’s Healthcare ethical guidelines.
Non-specific plasma protein binding studies

Heparin, when bound to plasma proteins, exhibit reduced activity (20), but the activity of heparin can be recovered when it is displaced from these basic heparin binding proteins by polyanions (19). To determine the effect of plasma protein binding to UFH (10,000 IU/ml, Leo Pharma Inc., Thornhill, Canada, approx. MW = 15000 D), ATH (753 units/mg of heparin, approx. MW of heparin chains = 18000 D), LMWH (953 IU/ml; Tinzaparin; Leo Pharma, approx. MW = 6500 D), or LMWATH (4500 units/mg of heparin, approx. MW of heparin chains = 5000 D), each heparinoid was added to NPP or CPP in the presence or absence of dextran sulfate (Sigma) and assayed for anti-FXa activity. The anti-FXa assay was used to determine the plasma protein binding to heparin instead of an anti-thrombin assay because anti-FXa assays are used clinically for assessing heparin activity in patients. Also, since inhibition of both FXa and thrombin activity in vivo involves antithrombin binding to the heparin, plasma protein binding would be globally assessed using just the anti-FXa assay.

Appropriate pooled plasma was used to dilute the heparinized samples into the range of the standard curves. Stock heparinoids were initially diluted in 0.15 M NaCl followed by a final dilution of 1/100 in plasma before being assayed. The target range of the heparinized adult and cord plasmas, with background (plasma only) subtracted, was 0.05-0.15 U/ml for UFH and ATH and 0.20-0.30 U/ml for LMWH and LMWATH. These ranges were purposely set at the lower end of levels required for therapeutic prophylaxis so that increased activity from displacement by dextran sulfate would still be within the analytical range of the assay.

To evaluate heparinoid recovery, anti-FXa activity of each plasma sample was performed before and after the addition of 100 µg/ml final concentration of dextran sulfate, which had relatively low baseline activity (specific anti-FXa activity of dextran sulfate alone was <0.2 U/ml). Dextran sulfate is a negatively charged, sulfonated polysaccharide used to displace heparinoids from the plasma proteins. Although more highly charged than heparin, dextran sulfate does not contain the AT-specific pentasaccharide binding sequence and has been demonstrated to bind nonspecifically to plasma proteins (19). Dextran sulfate was very consistent at releasing heparinoid activity from plasma proteins. Anti-FXa activities were measured using the Stachrom heparin kit (Diagnostica Stago, Parsippany, NJ, USA). This assay, adapted for use on an automated coagulation analyzer ACL 300+ (Instrumentation Laboratories, Milan, Italy), measures the ability of UFH to potentiate the inhibition of FXa by AT using a chromogenic substrate. The assay was performed in the presence of excess AT in the reaction mixture. The increase in anti-FXa activity of the heparinized plasmas after dextran sulfate addition (anti-FXa activity of [dextran sulfate + plasma] is subtracted as background) represents the proportion of active ATH, UFH, or LMWH displaced from the plasma proteins.

The amount of heparinoid bound to plasma proteins was calculated by taking the difference in anti-FXa activity between dextran sulfate displacement of heparinoid in plasma ([heparinoid + plasma + dextran sulfate] – [plasma + dextran sulfate]) and heparinoid alone ([heparinoid + plasma] – [plasma]). This difference was expressed as % increase in anti-FXa activity of the heparinoid in the presence of dextran sulfate. All values are expressed as mean ± SEM (n=5).

Radiolabelling of ATH, AT, UFH and Bovine Serum Albumin (BSA)

125I-ATH and 125I-AT were prepared by reacting either ATH or AT (Affinity Biologicals) with Na125I (Perkin Elmer Life Sciences, Markham, Canada) and Iodobeads (Pierce Biotechnology, Brockville, Canada) according to manufacturer’s instructions. Briefly, the Iodobeads were washed in PBS buffer (12 mM sodium phosphate, 145 mM NaCl, pH 7.4), pre-incubated with PBS buffer containing 0.5 mCi of Na125I, then mixed with 1.8 mg/ml of ATH or AT. After 5 minutes, the entire reaction mixture was transferred onto a PD-10 column (Amersham Pharmacia) followed by PBS wash for fractionation of 125I-ATH or 125I-AT from unreacted Na125I. The specific activity of unlabelled ATH was 506 units/mg and radiolabelled product was 0.70-1.99 µCi/µg 125I-ATH. The specific activity of radiolabelled AT was 0.49 µCi/µg 125I-AT.

UFH (Sigma) was labelled by mixing 1 mCi of 3H-NaBH4 (Perkin Elmer) in 20 µL of NaOH with 1 mg of UFH in 980 µL of 1 M Tris-HCl buffer, pH 8.0, in a 1.5 ml eppendorf tube and incubating at 23°C for 20 hours. The reaction mixture was then dialyzed against 0.15 M NaCl to remove any unreacted 3H-NaBH4. The specific activity of 3H-UFH was 7.15 × 10^4 µCi/µg.

Radiolabelling of BSA with Na131I followed a method similar to that for Na125I-labelling of ATH and AT. Briefly, a PD-10 gel filtration column was pre-equilibrated the day before iodination with 20X column volume of PBS buffer, pH 7.4. The Iodobead (Pierce) was washed with 0.5 M phosphate buffer, pH 7.0, then pre-incubated with PBS buffer containing 0.5 mCi of Na131I. BSA was added to the mixture (1.8 mg/ml of BSA in the final incubation solution) and after 5 minutes, the entire reaction mixture was transferred onto the pre-equilibrated PD-10 column (Amersham Pharmacia), followed by PBS wash for fractionation of 131I-BSA from unreacted Na131I.

Endothelial cell binding studies

The following experiments were performed to compare the binding of ATH to endothelial cells with UFH. Human umbilical vein endothelial cells (HUVECs), pooled from several donors, were obtained from BioWhittaker (Walkersville, MD, USA). HUVECs were cultured according manufacturer’s guidelines using their supplemented endothelial cell growth media (EGM-2 MV, no heparin). Cells were initially cultured in...
100 mm Falcon Primaria (BD Biosciences, Mississauga, Canada) tissue culture dishes then transferred into Falcon Primaria 24-well tissue culture plates. HUVECs were allowed to grow at 37°C in a humidified atmosphere containing 5% CO₂ until confluence before being assayed. Once the cells were confluent, the plates were moved into a warm room (37°C). The culture media from each well was removed and rinsed with 1 ml of pre-warmed Minimum Essential Media (MEM; Gibco BRL/Invitrogen Canada Inc., Burlington, Canada). After rinsing, the cells were incubated for 30 minutes with various radiolabelled proteins in MEM.

To examine ATH binding, the wells containing HUVECs were incubated with 17 pM ¹²⁵I-ATH with or without 50- and 1000-fold molar excess of unlabelled ATH. Similarly, 17 pM ¹²⁵I-AT + 17 pM UFH with or without 50- and 1000-fold molar excess of unlabelled [AT + UFH] were placed in the wells to examine AT binding. Some wells were incubated with 68 pM AT + 67 pM ³H-UFH with or without 13- and 250-fold molar excess of [AT + UFH] to examine UFH binding. Finally, 17 pM ¹²⁵I-ATH was incubated with the endothelial cells in the presence or absence of 13- and 254-fold molar excess of unlabelled UFH to assess the involvement of ATH's heparin moiety in endothelial cell binding. The difference in binding of labelled material between experiments with or without 250- to 1000-fold molar excess non-labelled ligand was taken as high affinity specific binding.

After incubation at 37°C for 30 minutes with various radiolabelled compounds ± unlabelled material, a 100 µL aliquot of the bathing MEM solution from each well, representing unbound (free) radiolabelled material, was kept for radioactive counting. The plates were then moved into the cold room (4°C), which time the rabbits were euthanized. Five minutes prior to the time of death, 1 ml of cold MEM containing 1 M NaCl was placed in each well to displace any surface-bound material and the entire supernatant was removed for radioactive counting. Finally, to determine the amount of material endocytosed by the endothelial cells, the cells were dissolved in a total of 1 ml of 0.1% SDS solution and the samples kept for radioactive counting. Total binding of the heparinoid is the amount of radioactive heparinoid to the endothelium cells on the vena cava. Rabbits were injected (time = 0 s) with low-dose (3 µg/kg) or high-dose (3 mg/kg) radiolabelled (¹²⁵I) ATH (~ 10⁶ CPM/kg) via the catheter in the ear artery. The amount of bound radioactive heparinoid was multiplied by Avagadro’s number (6.023 x 10²³ molecules/mole) and then divided by the number of cells (2.87 x 10⁵ cells; counted by trypsinization of the cells and subsequent counting on a haemocytometer) to obtain the number of molecules bound/cell (high affinity binding sites). All values are expressed as mean ± SEM (n=3).

**In vivo ATH binding to endothelium (vena cava)**

Male New Zealand white rabbits (~ 3 kg) were used in these experiments to determine the binding of ATH to the endothelial cells on the vena cava. Rabbits were injected (time = 0 s) with low-dose (3 µg/kg) or high-dose (3 mg/kg) radiolabelled (¹²⁵I) ATH (~ 10⁶ CPM/kg) via the catheter in the ear artery. Rabbits were divided into 3 experimental groups post-injection: Group 1 = 20 minutes; Group 2 = 4 hours; Group 3 = 24 hours, after which time the rabbits were euthanized. Five minutes prior to euthanization, rabbits were injected with a plasma marker (~ 5 x 10⁴ CPM of ¹³¹I-BSA per animal) to account for trapped blood in the sampled tissue. Three minutes after the injection of ¹³¹I-BSA, a 3 ml blood sample was taken into a heparinized syringe to determine the amount of radiolabelled ATH and BSA in the blood (samples counted on a γ-counter) so that this amount could be taken into account (as a ratio of background ATH/BSA trapped in the blood) when the tissue was examined for ATH binding. After the appropriate time had lapsed for each experimental group, rabbits were euthanized and a sample length of the vena cava was harvested and weighed.
Approximately 1 g of sample was placed in a test tube and filled to a total volume of 2 ml with water and the sample was counted. Results are expressed as the percentage of the dose administered per gram of wet tissue (with radiolabel in the trapped blood subtracted) (n≥5 per group).

**Statistical analysis**

Multiple data sets were analyzed by ANOVA. Upon finding significance, pairs of data sets were analyzed by using Student’s \( t \)-test. Differences were considered significant at a value of \( p<0.05 \).

**Results**

**Nonspecific plasma protein binding in NPP**

To determine the effect of plasma protein binding to UFH, ATIII, LMWH, and LMWATH, each heparinoid was added to NPP or CPP and anti-FXa activities were measured before and after the addition of dextran sulfate (Table 1). Because dextran sulfate displaces the bound heparinoid from plasma proteins, the increase in anti-FXa activity after dextran sulfate addition represents the total activity of heparinoid bound to non-AT protein. UFH bound significantly more to plasma proteins in NPP than ATIII or LMWH. There was significantly higher % increase in anti-FXa activity, after dextran sulfate addition, of plasmas containing UFH (175.0 ± 16.4) compared to ATIII (107.4 ± 8.9; \( p=0.0067 \)) or LMWH (25.0 ± 10.1; \( p<0.0001 \)). Also, ATIII had significantly higher % increase in anti-FXa activity compared to LMWH (10.2 ± 9.8). A trend towards a higher % increase in anti-FXa activity for UFH in cord plasma in the presence of dextran sulfate was observed compared to ATIII (UFH had a 1.5-fold higher % increase in anti-FXa activity than ATIII).

Table 1: Anti-Factor Xa activities of heparinoids in normal adult or cord pooled plasmas in the absence or presence of dextran sulfate.

<table>
<thead>
<tr>
<th>Pooled Plasma</th>
<th>Heparinoid</th>
<th>Anti-Xa Activity (u/ml)</th>
<th>% Activity Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(no DS)</td>
<td>(+ DS)</td>
<td></td>
</tr>
<tr>
<td>NPP</td>
<td>UFH</td>
<td>0.102 ± 0.007</td>
<td>0.276 ± 0.008</td>
</tr>
<tr>
<td></td>
<td>ATIII</td>
<td>0.098 ± 0.007</td>
<td>0.202 ± 0.013</td>
</tr>
<tr>
<td></td>
<td>LMWH</td>
<td>0.244 ± 0.009</td>
<td>0.302 ± 0.016</td>
</tr>
<tr>
<td></td>
<td>LMWATH</td>
<td>0.151 ± 0.023</td>
<td>0.141 ± 0.012</td>
</tr>
<tr>
<td>CPP</td>
<td>UFH</td>
<td>0.126 ± 0.010</td>
<td>0.264 ± 0.010</td>
</tr>
<tr>
<td></td>
<td>ATIII</td>
<td>0.128 ± 0.004</td>
<td>0.224 ± 0.006</td>
</tr>
<tr>
<td></td>
<td>LMWH</td>
<td>0.262 ± 0.006</td>
<td>0.290 ± 0.031</td>
</tr>
<tr>
<td></td>
<td>LMWATH</td>
<td>0.133 ± 0.024</td>
<td>0.122 ± 0.016</td>
</tr>
</tbody>
</table>

NPP, adult normal pooled plasma; CPP, normal cord pooled plasma; UFH, unfractionated heparin; ATIII, covalent anthithrombin-heparin complex; LMWH, low molecular weight heparin (Tinzaparin); LMWATH, low molecular weight ATIII; DS, dextran sulfate. For each heparinoid, anti-Factor Xa activity assay was performed before and after the addition of 100 µg/ml final concentration of DS (used to displace heparinoids from the plasma proteins). The increase in anti-Factor Xa activity after DS addition represents the proportion of active UFH, ATIII, or LMWATH displaced from the plasma proteins. All values are expressed as mean ± SEM.

**Nonspecific plasma protein binding in CPP**

Both UFH and ATIII bound significantly more to proteins in cord plasma than LMWH (\( p=0.0035 \) and \( p=0.0003 \), respectively). The % increase in anti-FXa activity of UFH (116.6 ± 24.2) and ATIII (75.4 ± 4.9) was 11.4- and 7.4-fold higher, respectively, than LMWH (10.2 ± 9.8). A trend towards a higher % increase in anti-FXa activity for UFH in cord plasma in the presence of dextran sulfate was observed compared to ATIII (UFH had a 1.5-fold higher % increase in anti-FXa activity than ATIII).

There was no significant difference between NPP and CPP in the amount of UFH and LMWH bound to (displaced from) plasma proteins, but there was significantly more plasma proteins bound to ATIII in NPP than CPP (\( p=0.0133 \)) (Fig. 1). The results showed that plasma proteins in both NPP and CPP bound more to UFH than ATIII, and least to LMWH. There was a trend toward higher plasma protein binding to these heparinoids in NPP compared to CPP but only ATIII showed a significant difference.

**Endothelial cell binding studies**

To investigate the influence of endothelial surfaces on the removal of ATIII from the fluid phase, we examined ATIII binding to endothelial cells *in vitro* and compared it to ATIII + UFH.
HUVECs were incubated for 30 minutes at 37°C with $^{125}$I-ATH, AT + $^3$H-UFH, or $^{125}$I-AT + UFH in the presence or absence of corresponding unlabelled AT or AT + UFH (up to 1000-fold molar excess), to determine the high affinity specific binding to the cells. Results showed that $^3$H-UFH had the greatest total binding to endothelial cells, significantly more than $^{125}$I-ATH (p=0.0217) or $^{125}$I-AT (p=0.0077) (Table 2). $^{125}$I-ATH also showed significantly greater total binding to endothelial cells than $^{125}$I-AT (p=0.0012). Upon further analysis, $^3$H-UFH had significantly greater binding to the surface of endothelial cells compared to $^{125}$I-ATH (p=0.0201) or $^{125}$I-AT (p=0.0077). $^{125}$I-ATH cell surface-associated binding (displaced by 1 M NaCl) was also significantly greater than $^{125}$I-AT binding (p=0.0012). Analysis of the bound material revealed that all of UFH (104% or $2.44 	imes 10^6 \pm 4.81 	imes 10^5$ molecules/cell), 94% of ATH (6.21 $\times 10^5 \pm 6.49 	imes 10^4$ molecules/cell) and 96% of AT (4.63 $\times 10^4 \pm 2.46 	imes 10^4$ molecules/cell) was cell-surface associated (Fig. 2). Only 6% of bound ATH (3.72 $\times 10^4 \pm 9.20 \times 10^3$ molecules/cell), compared to 4% for AT (1.76 $\times 10^3 \pm 3.10 \times 10^3$ molecules/cell), was endocytosed (cell-associated material remaining after displacement of cell surface-bound ligand) over the 30 minute incubation period (Fig. 3).

**Table 2:** Total number of molecules bound per endothelial cell.

<table>
<thead>
<tr>
<th>Heparinoid</th>
<th>Total # molecules bound/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>UFH</td>
<td>$2.35 \times 10^5 \pm 6.34 \times 10^5$</td>
</tr>
<tr>
<td>ATH</td>
<td>$6.58 \times 10^5 \pm 7.13 \times 10^4$</td>
</tr>
<tr>
<td>AT</td>
<td>$4.81 \times 10^4 \pm 2.32 \times 10^4$</td>
</tr>
</tbody>
</table>

UFH, unfractionated heparin; ATH, covalent antithrombin-heparin complex; AT, antithrombin. Binding studies were done on confluent human umbilical vein endothelial cells (HUVECs; BioWhittaker). HUVECs were incubated for 30 minutes with various radiolabelled ligands ($^3$H-UFH, $^{125}$I-ATH, $^{125}$I-AT), with or without excess unlabelled material (see Materials and Methods). The difference in binding of labelled material between experiments with or without non-labelled ligand was taken as specific binding. All values are expressed as mean $\pm$ SEM (n=3).
These results show that ATH, like UFH, binds to endothelial cell surfaces, but to a lesser degree (3.6-fold less) than UFH. Upon binding to the endothelial cells, a small percentage of ATH is subsequently endocytosed after 30 minutes of incubation at 37°C with the endothelial cells.

**Competitive binding of 125I-ATH vs. unlabelled UFH**

To test which moiety of ATH is responsible for endothelial cell surface binding, an experiment was carried out whereby 125I-ATH was incubated with HUVECs in the absence or presence of excess unlabelled UFH. The results showed that excess unlabelled UFH interfered with 125I-ATH binding (Fig. 4). There was a significant decrease in 125I-ATH binding to endothelial cell surfaces in the presence of increasing amounts of excess unlabelled UFH (p=0.0004).

**In vivo ATH binding to endothelium (vena cava)**

There was very low binding of ATH to the vena cava (Fig. 5). However, at a low-dose injection of ATH (3 µg/kg), we observed measurable binding to vessel wall, especially 20 minutes after injection, and this binding was significantly decreased over time (p=0.0001). At high-dose injection of ATH (3 mg/kg), there was minimal binding of ATH to the vena cava, and over a 24-hour period, the amount of binding did not change significantly (p=0.4745).

**Discussion**

Our previous studies have shown that ATH exhibits superior anticoagulant properties and has a longer intravenous half-life compared to UFH (31, 32, 36), and could potentially be suitable for use as a safe and effective anticoagulant. Experiments in our laboratory have shown that soluble PF4, as well as protamine, bind to ATH and caused full neutralization of its anti-FXa activity. We have now extended our findings by demonstrating the differences in ATH, UFH, and LMWH with respect to non-specific plasma protein binding and endothelial cell-surface binding.

Various plasma proteins that bind to UFH have been identified, thereby, decreasing the amount of heparin molecules available to interact with AT (14, 15, 37), limiting its anticoagulant activity in vivo. Plasma levels of heparin-binding proteins of patients with thrombotic disease are considerably diverse (38) providing a plausible explanation for the unpredictable anticoagulant response of UFH (18, 20, 39). Conversely, because of its short chain length, LMWH have decreased non-specific plasma protein binding, increased bioavailability, and hence, a more predictable intravenous half-life (40). Many studies have cited differences in the composition of the haemostatic system between adults and children, such as lower plasma AT concentrations in children compared to adults (24-26). It has

**Figure 3**: Endocytosis of bound heparinoid by endothelial cells. UFH, unfractionated heparin; ATH, covalent antithrombin-heparin complex; AT, antithrombin. Binding studies were done on confluent human umbilical vein endothelial cells (HUVECs; BioWhittaker). HUVECs were incubated for 30 minutes with various radiolabelled proteins (3H-UFH, 125I-ATH, 125I-AT), with or without excess of unlabelled material (see *Materials and Methods*). The amount of bound heparinoid endocytosed by the endothelial cells was determined by displacing the HUVECs in a total of 1 ml of 0.1% SDS solution (after displacement of surface-bound material by washing with 1 M NaCl in Minimum Essential Media). The difference in binding of labelled material between experiments with or without non-labelled ligand was taken as specific binding. All values are expressed as mean ± SEM (n=3).

**Figure 4**: Binding of ATH to the surface of endothelial cells in the presence of excess UFH. ATH, covalent antithrombin-heparin complex; UFH, unfractionated heparin. Binding studies were done on confluent human umbilical vein endothelial cells (HUVECs; BioWhittaker). HUVECs were incubated at 37°C for 30 minutes with 125I-ATH in the presence or absence of unlabelled UFH (see *Materials and Methods*). ATH surface binding was determined by displacing any surface-bound material with cold (4°C) Minimum Essential Media containing 1 M NaCl after incubation. The difference in binding of labelled material between experiments with or without non-labelled ATH ligand was taken as specific binding. All values are expressed as mean ± SEM (n=3).
been shown that plasma levels of some heparin-binding proteins in children, such as vitronectin (41) and fibronectin (42), are lower than adults. Thus, increased sensitivity to heparin in the young may be due to decreased concentrations of nonspecific heparin-binding plasma proteins.

In this study, significant differences were observed in the amount of nonspecific plasma proteins bound to heparin covalently linked to AT in comparison to either UFH or LMWH (Table 1). In normal adult plasma, UFH had the greatest increase in anti-FXa activity after displacement from plasma proteins by dextran sulfate, followed by ATH, then LMWH. Further studies showed that when AT was conjugated to low molecular weight heparin chains (LMWATH), essentially no binding to NPP proteins could be observed (Table 1). In cord plasma, UFH and ATH had significantly higher plasma protein binding than LMWH. Again, no significant plasma protein binding could be observed by LMWATH in CPP. Although, there was no statistically significant difference between adult and cord plasma in the amount of UFH and LMWH bound to (displaced from) plasma proteins, significantly more plasma proteins bound to ATH in adult than cord plasma (Fig. 1). These findings suggest that the difference in plasma protein binding may be due to available heparin chain length. Although ATH is polydisperse, the covalently linked AT in ATH may shield a portion of the heparin chain, thus, plasma proteins would bind less to ATH than UFH. Thus, it might be expected that ATH molecules containing shorter heparin chains would have an even further reduction in plasma protein binding, regardless of the plasma tested. Data from the experiments with LMWATH confirmed this hypothesis. These results are consistent with ATH’s increased intravenous half-life, better bioavailability, and a more predictable dose response in vivo. LMWH had the lowest plasma protein binding coincident with the shortest chain length. Binding of LMWH to plasma proteins was 86% and 91% less than UFH in adult and cord plasma, respectively. This result is comparable to those found by Young et al (18) where they observed that various preparations of LMWH bound 70-90% less plasma proteins than UFH in adult plasma and that the variability of the degree of nonspecific plasma protein binding to these LMWHs was most likely due to their molecular weight distribution. More studies are needed to elucidate what type of proteins in adult and cord plasmas are bound by these heparinoids.

In addition to plasma protein binding, heparin binding to endothelial cells affects its bioavailability by altering its clearance rate, contributing to the variable anticoagulant response in vivo. UFH not only binds to endothelial cells, but also is internalized and degraded (30, 43). Heparin is cleared by nonsaturable and saturable mechanisms, the latter involving the binding of heparin to the endothelium (27). Factors influencing heparin binding to endothelial cells could increase the heparin clearance rate resulting in diminished heparin recovery in patients.

In order to investigate the influence of endothelial cell surfaces on ATH clearance, we examined ATH binding to endothelial cells in vitro and compared it to UFH and AT binding. Our findings indicate that UFH significantly bound to endothelial cells 3.6-fold and 44.9-fold more than ATH or AT, respectively (Table 2). The total binding capacity of the endothelial cells was highest for UFH at 2.44 × 10^6 ± 4.81 × 10^4 molecules/cell, which is in agreement with Young et al (2.5 × 10^6 sites/cell) (44), followed by ATH and AT. The majority of the binding was cell-surface associated (104% of UFH, 94% of AT, 96% AT) (Fig. 2) and the remainder was endocytosed (Fig. 3). Other studies (28, 45) employing much longer incubation times found that 25-30% of heparin molecules were not dissociable from endothelial cells, suggesting that a fraction of bound heparin is internalized by the vascular endothelium. However, the 30-minute
incubation time used in the present study is unlikely to have more than one endocytic turnover (46).

It is not surprising that ATH bound to endothelial cells to a lesser degree than UFH since the affinity of heparin for endothelial cells is directly related to its molecular weight, charge density, and relative affinity for AT (47, 48). Although ATH is produced using UFH, free AT selects for the polysaccharide chains with specific AT pentasaccharide binding sequences. Also, the AT in the covalent ATH complex may shield a portion of the heparin chain, thereby decreasing the heparin chain length available for interaction with the endothelial surface. Binding of ATH to cells was most likely through its heparin moiety, since the binding of AT alone was much reduced and competitive surface binding experiments showed that excess UFH was able to outcompete ATH for interaction with the cell surface (Fig. 4). Inhibition of ATH endothelial cell binding by UFH was a result of competition by the free heparin for ATH’s binding site on the endothelium and not binding of the endothelium for ATH’s AT moiety, given that previous work has shown that ATH has no affinity for heparin immobilized on Sepharose beads (33). In vivo data showed that ATH, when injected at a low dose (3 µg/kg), binds to the endothelial cells of the rabbit vena cava and this binding is decreased over time (Fig. 5). However, when 1000-fold greater amounts of ATH were injected into the rabbits, there was very little binding to the vena cava, suggesting that the system was saturated and that the clearance of ATH may be of a different route such as elimination through the kidneys. The half-life in humans of intravenously injected heparin ranges from 0.4-2.5 hours (49), whereas the half-life of AT is 2.7 days (50). ATH more closely resembled AT plasma clearance than heparin in the rabbit model, where the intravascular clearance of ATH was found have an α-phase of 2.6 hours, followed by a β-phase of 13 hours (31).

Thus, the longer plasma half-life and better bioavailability of ATH, compared to heparin, may be a result of reduced binding to endothelial cells. ATH could potentially be used as an anticoagulant with better efficacy (31, 51) and similar bleeding risk compared to UFH (34). Covalent linkage of AT to heparin eliminates the rate-limiting step of AT binding to heparin (52) and AT in the conjugate is permanently activated since AT cannot dissociate from heparin in the ATH complex (32). Formation of ATH favours heparin with the AT-specific pentasaccharide sequence (thus a larger proportion of the ATH pool would be active against thrombin and FXa) (31, 32) and ATH has the ability to inhibit fibrin-bound thrombin (36). In this study, ATH has been shown to have decreased plasma protein and cell surface binding compared to UFH, most likely due to the partial concealment of heparin by the complexed AT. Reduction of heparin’s non-AT protein binding, by stabilization of the AT-heparin complex, would facilitate both in vivo activity and bioavailability.

Our present study confirms that loss of heparin function due to fluid and surface phase binding can be minimized by the presence of AT in a complex possessing high activity (both non-catalytic and catalytic) against thrombin. It would be valuable to conduct studies to determine the class of plasma or cell surface heparin binding proteins that have reduced affinity for ATH. Also, studies should be performed to assess if binding of ATH to plasma or cell surface proteins is dependent on the molecular weight of the conjugate. Regardless of the mechanisms involved, covalent linkage of UFH to AT results in a reduction in plasma protein and cell surface binding approaching that of LMWHs. In a sense, ATH combines many positive attributes exhibited by either UFH or LMWH, without a number of the deficiencies.

References

14. Lane DA, Pijler G, Flynn AM. Neutralization of heparin related saccharides by histidine-