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J Clin Invest. 1985;75(4):1169-1173. <https://doi.org/10.1172/JCI111812>.

Research Article

Clinical grade heparin is a very heterogeneous mucopolysaccharide, containing molecules with Mr ranging from 6,000 to 30,000 that have either a high affinity or a low affinity for antithrombin III (AT). In this study, the antithrombotic properties of intact high-affinity heparin (Mr = 15,000) and of two heparin fragments (h16, a 16-monosaccharide fragment, with Mr = 4,300, and h12, a 12-monosaccharide fragment, with Mr = 3,200) and of their functional covalent stoichiometric complexes with human AT were compared in a venous thrombosis stasis model in rabbits. Thrombosis was induced by injection of glass-activated human plasma and measured in a segment of the jugular vein that was isolated between two vascular clamps for 10 min. Injections of 55 micrograms/kg resulted in a clear antithrombotic effect for intact heparin, but not for the two fragments. Equivalent amounts (carbohydrate moiety) of covalent complexes of heparin or of both heparin fragments with human AT resulted in an antithrombotic effect lasting for 45-60 min. Injection of 110 micrograms/kg of heparin and of the heparin fragments yielded an antithrombotic effect, lasting 45-60 min; the corresponding amounts of covalent complexes caused an anti-thrombotic effect for 60-120 min. The free and conjugated fragments produced equal antithrombotic effects at equal plasma levels of anti-Factor Xa activity, but the specific antithrombotic activities of free and complexed intact heparin, on a [...]

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Antithrombotic Properties in Rabbits of Heparin and Heparin Fragments Covalently Coupled to Human Antithrombin III

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Abstract

Clinical grade heparin is a very heterogeneous mucopolysaccharide, containing molecules with M_r ranging from 6,000 to 30,000 that have either a high affinity or a low affinity for antithrombin III (AT). In this study, the antithrombotic properties of intact high-affinity heparin ($M_r = 15,000$) and of two heparin fragments (h_{16} , a 16-monosaccharide fragment, with $M_r = 4,300$, and h_{12} , a 12-monosaccharide fragment, with $M_r = 3,200$) and of their functional covalent stoichiometric complexes with human AT were compared in a venous thrombosis stasis model in rabbits. Thrombosis was induced by injection of glass-activated human plasma and measured in a segment of the jugular vein that was isolated between two vascular clamps for 10 min.

Injections of 55 $\mu\text{g}/\text{kg}$ resulted in a clear antithrombotic effect for intact heparin, but not for the two fragments. Equivalent amounts (carbohydrate moiety) of covalent complexes of heparin or of both heparin fragments with human AT resulted in an antithrombotic effect lasting for 45–60 min. Injection of 110 $\mu\text{g}/\text{kg}$ of heparin and of the heparin fragments yielded an antithrombotic effect, lasting 45–60 min; the corresponding amounts of covalent complexes caused an antithrombotic effect for 60–120 min. The free and conjugated fragments produced equal antithrombotic effects at equal plasma levels of anti-Factor Xa activity, but the specific antithrombotic activities of free and complexed intact heparin, on a molar basis, were 10–20-fold greater than those of the free and complexed heparin fragments. The plasma half-life of the covalent complexes of the heparin fragments with AT is, however, 10 times longer than that of the complex between intact heparin and AT and 30 times longer than that of free intact heparin. Covalent complexes between AT and heparin fragments could, therefore, be useful to maintain more stable levels of antithrombotic activity in plasma.

Introduction

The anticoagulant activity of heparin is due to catalysis of the inhibition of several activated coagulation factors by the plasma protease inhibitor antithrombin III (AT)¹ (1, 2). Active heparin

molecules contain a unique pentasaccharide sequence that binds with high affinity to AT (3); in clinical grade heparin preparations, this sequence is only present in about one-third of the molecules (4). In addition, clinical grade heparin is very heterogeneous with respect to M_r , ranging from 6,000 to 30,000 with an average of 10,000–15,000 (5).

High-affinity heparin fractions of low M_r retain the ability to catalyze the inhibition of some coagulation factors, among them Factor Xa, while having a markedly reduced effect on the neutralization of other coagulation enzymes, among them thrombin. Such low- M_r heparins have high anti-Factor Xa activity but do not significantly prolong the activated partial thromboplastin time (6–8).

It has been suggested that heparin fractions of low M_r and with a high anti-Factor Xa/activated partial thromboplastin time activity ratio still show antithrombotic activity but are associated with a reduced incidence of bleeding (9, 10). This was recently demonstrated in rabbits with experimental venous thrombosis, using heparin fragments obtained by controlled nitrous acid degradation (11, 12). In addition, the antithrombotic activity of different heparin fragments not only depends on their specific anticoagulant activity, but it also seems to be a function of the chain length of the fragment (11). Furthermore, it has been shown that low-affinity heparin potentiates the antithrombotic effect of high-affinity heparin fragments (13).

Complex formation between heparin and AT is an essential step in the mechanism of inhibition of activated coagulation factors (2, 7, 14). Recently, complexes between heparin and AT have been stabilized by covalently coupling AT to heparin or to heparin fragments (15, 16). These covalent complexes inhibit Factor Xa and thrombin with second order rate constants (k) very similar to those found for the corresponding reversible complexes (15–17).

In this study, the antithrombotic properties of covalent complexes between AT and heparin or heparin fragments were compared with those of free high-affinity heparin or high-affinity heparin fragments in an experimental venous thrombosis model in rabbits.

Methods

Materials. Human AT was purified from plasma by affinity chromatography on heparin Sepharose (18). Protein concentration was determined by absorbance measurements using an extinction coefficient, $A_{1\text{cm}}^{1\%}$ (280 nm) = 6.2, and an M_r of 56,000 (18).

High-affinity heparin of average $M_r = 15,000$ (H) was purified from clinical grade heparin (Kabi AB, Stockholm, Sweden) by affinity chromatography on matrix-bound AT (4). High-affinity heparin fragments of $M_r = 4,300$, containing an average of 16 monosaccharides (h_{16}) and of 3,200, containing an average of 12 monosaccharide units (h_{12}) were prepared by partial deaminative cleavage of heparin with nitrous acid (19, 20) and subsequent affinity chromatography on AT Sepharose. In this study only high-affinity heparin fragments purified on AT Sepharose were used.

The M_r of the fragments was determined by gel filtration on

Received for publication 24 August 1984 and in revised form 18 December 1984.

1. Abbreviations used in this paper: AT, antithrombin III; H, high-affinity heparin of average $M_r = 15,000$; h_{12} , high-affinity heparin fragment of $M_r = 3,200$, containing an average of 12 monosaccharide units; h_{16} , high-affinity heparin fragments of $M_r = 4,300$, containing an average of 16 monosaccharides; k , second order rate constant(s).

J. Clin. Invest.

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0021-9738/85/04/1169/05 \$1.00

Volume 75, April 1985, 1169–1173

Sephadex G75 (Pharmacia, Uppsala, Sweden) (11). The characteristics and homogeneity of the fragments were very similar to those described by Thomas et al. (21): h_{16} contained polysaccharides ranging from 14 to 18 monosaccharide units, and h_{12} consisted of material with 10 to 14 monosaccharide units. Heparin concentrations were measured using the carbazole- H_2SO_4 method (22), and heparin activity was determined by Factor Xa inhibition using the chromogenic substrate *N*-benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-pNA (S-2222; Kabi AB) (6).

Covalent complexes between AT and heparin or heparin fragments were synthesized as previously described (15, 16). Primary amino groups were introduced in the mucopolysaccharide through reaction with hexamethylenediamine, and the fraction of the substituted heparin having a high affinity for AT was linked to AT with the bifunctional reagent tolylene-2,4-diisothiocyanate. The covalent complexes were purified by chromatography on DEAE-Sephacel (Pharmacia). The AT-H complex was further purified by gel filtration on Ultrogel AcA 44 (LKB Produkter, Bromma, Sweden) in 0.1 M Tris HCl buffer, pH 7.6. The purified complexes were quantified by the carbazole- H_2SO_4 method (heparin moiety) and by radial immunodiffusion using antibodies against human AT in the gel. Both determinations agreed within 10%. Carbazole measurements were not influenced by the presence of AT. The AT concentration in the complexes, as determined by titration against thrombin, was >85% of that estimated by the method of Lowry et al. (23). The disappearance of anticoagulant activity from the plasma in rabbits *in vivo* was followed by measuring Factor Xa inhibition in a plasma medium (11, 24, 25).

The antithrombotic properties of the heparin or heparin fragments and of their corresponding covalent complexes with AT were evaluated in a rabbit thrombus model originally described by Wessler et al. (26) and previously applied to compare the antithrombotic effect of intact heparin and heparin fragments (11). The model involves activation of the rabbit's coagulation system by intravenous injection of glass-activated human plasma. New Zealand white rabbits weighing 2.5–3.5 kg were premedicated with atropine (0.1 mg/kg) and Hypnorm® (0.1 ml/kg) (Leo AB, Helsingborg, Sweden) and then anesthetized with 2% pentobarbitone. After injection of 1.5 ml/kg of glass-activated human plasma, a 2-cm-long segment of the jugular vein (containing 0.2–0.3 ml blood) was isolated between two vessel clamps. The isolated vein segment was removed after 10 min of stasis, its contents were emptied into a petri dish containing 0.9% NaCl, and both the size and appearance of the formed clot(s) were scored on a scale graded from 0 to 4 (26). A score of 4 was given for a single large thrombus forming a cast of the isolated segment, 3 for two or more large thrombi, 2 for several small thrombi, 1 for a few macroscopic strands of fibrin, and 0 for fluent blood. The antithrombotic effect was tested 15, 30, and 45 min after injection of the test compounds. The covalent complexes between AT and h_{16} or h_{12} with a longer duration of the antithrombotic effect were also tested after 60, 90, and 120 min.

A minimum of four separate determinations was made for each time point and test compound; when results were too variable another two to three experiments were performed. About every fifth rabbit served as a control injected with saline only. The *in vivo* applications and the scoring of thrombi were carried out by an investigator unaware of the test compound that was injected.

For each test compound, a mean antithrombotic score was calculated by averaging the individual data for each time point. A score below 2 was considered to represent a significant antithrombotic effect; a score between 2 and 3, a moderate effect; and scores above 3, no antithrombotic activity. Differences in anticoagulant activity were statistically analyzed using *t* test.

Immediately before the injection of the thrombogenic plasma, 1.8-ml blood samples were collected from a cannulated ear artery and mixed with 200 μ l of 10% sodium citrate for measurement of anti-Factor Xa activity.

Anti-Factor Xa activity was measured in a plasma environment using the chromogenic substrate S-2222. Details of the assay and its calibration are reported elsewhere (11).

Results

Table I summarizes the specific anticoagulant activities of the free heparin moieties and of their corresponding AT-heparin complexes, as determined by the anti-Factor Xa assay. The specific activities of the complexes are very similar to those of the corresponding free fragments both for AT-H and for AT- h_{12} . By repeated assays, the specific activity of AT- h_{16} appears to be somewhat higher than that of the free fragment. This difference is larger than can be explained by experimental error, because the anti-Factor Xa assay has a coefficient of variation of 10–15%.

The antithrombotic effect of the free and complexed heparins is illustrated in Fig. 1. 15 min after an intravenous injection of 55 μ g/kg, the mean thrombosis scores for H and AT-H were 1.7 and 0.7, respectively ($n = 6$). Corresponding values after 30 min were 3.4 and 2.3 (Fig. 1 A). The results suggest a somewhat higher antithrombotic effect or a prolonged duration of the effect for the complex as compared with the free heparin. Intravenous injection of 55 μ g/kg of h_{16} or h_{12} did not produce an antithrombotic effect, whereas equivalent amounts of their covalent complexes were active (Fig. 1, C and E).

At a dose of 110 μ g/kg a significantly higher antithrombotic effect was obtained as evidenced by lower mean thrombosis scores. A prolonged duration of the antithrombotic effect is observed for all heparin substances tested. 15 min after administration, both for H and AT-H, zero scores were observed, corresponding to full protection, while scores after 30 min averaged 1.2 and 1.0, both for H and AT-H, respectively (Fig. 1 B). For the free fragments h_{16} and h_{12} , injection of 110 μ g/kg yielded a significant antithrombotic effect (Fig. 1, D and F). After 45 min, however, the protection by h_{16} and h_{12} disappeared, while, on the contrary, for AT- h_{16} and AT- h_{12} it took 120 min and 90 min, respectively (Fig. 1, D and F), before the antithrombotic protection disappeared.

The disappearance rate of the anti-Factor Xa activity from the plasma of the rabbits after injection of 110 μ g/kg of the free or complexed heparin moieties is shown in Fig. 2. Because the different heparin substances have different specific activities (Table I) and because an equal dose of 110 μ g/kg was given in all cases, different initial anti-Factor Xa activities were obtained in the blood. Initially, the anticoagulant activity disappeared rapidly, reaching 50% after 9 min for H, 18 min

Table I. Specific Anti-Factor Xa Activity of the High-Affinity Heparins and of Their Covalent Complexes with AT

Heparin substance	Heparin moiety IU/mg
H	300
AT-H	270
h_{16}	540
AT- h_{16}	850
h_{12}	860
AT- h_{12}	760

AT-H, AT- h_{16} , and AT- h_{12} , covalent complexes between AT and H, h_{16} , or h_{12} .

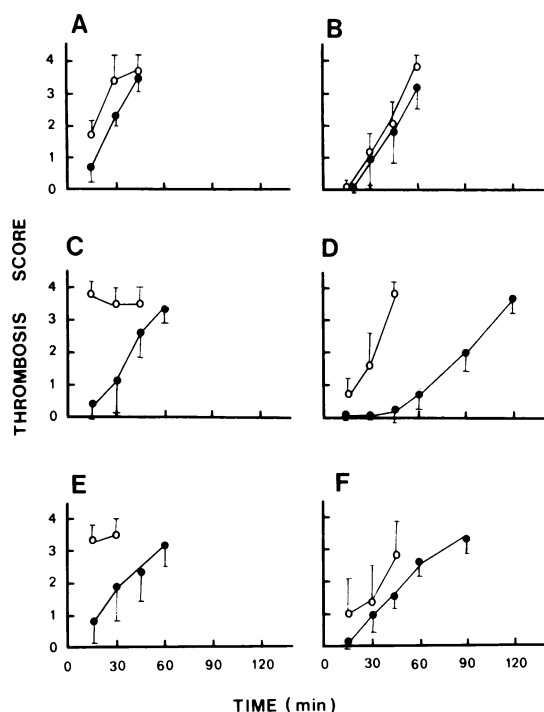


Figure 1. Antithrombotic effect of intact H (A, B) and of h₁₆ (C, D) and h₁₂ (E, F) in a venous thrombosis model in rabbits. ○, free heparin moieties; ●, covalent complexes between the heparin moieties and AT. A, C, and E, 55 µg/kg dose; B, D, and F, 110 µg/kg dose. The data represent mean±SD of at least four experiments.

for AT-H, 53 min for h₁₆, 80 min for AT-h₁₆, 50 min for h₁₂, and 85 min for AT-h₁₂.

Table II represents the duration of the antithrombotic effect, measured as the time to reach an antithrombotic score of 2. This duration varied from 18 min (55 µg/kg H) to 90 min (110 µg/kg AT-h₁₆). The anti-Factor Xa levels in plasma measured at the time of the thrombosis score 2 are also represented in Table II. These data show that the anti-Factor Xa levels at score 2 were similar for free and conjugated

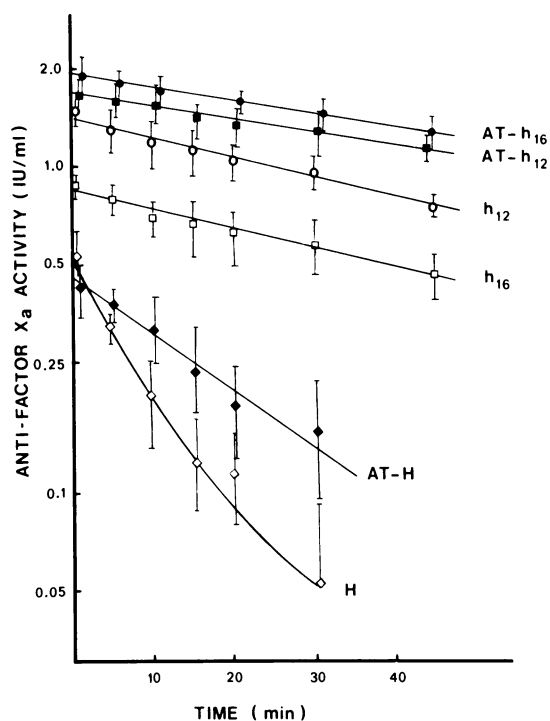


Figure 2. Initial disappearance rate of anti-Factor Xa activity after intravenous injection into rabbits of 110 µg/kg free or complexed heparin moieties.

heparin moieties but very different for H as compared with h₁₆ and h₁₂.

Discussion

The antithrombotic effect of intact H and of h₁₆ and h₁₂ was investigated in a venous thrombosis model in rabbits. Both the free heparin moieties and their covalent complexes with AT were studied. At doses of 55 µg/kg only free H but not free h₁₆ or h₁₂ had a significant antithrombotic effect, notwithstanding the fact that the anti-Factor Xa activity in plasma

Table II. Duration of the Antithrombotic Effect After Intravenous Injection of High-Affinity Heparin (Fragments) or AT-Heparin (Fragment) Complexes in Rabbits

Heparin compound	Time to reach score 2*		Anti-Factor Xa level at score 2‡		Concentration of heparin moiety at score 2 (×10 ⁻⁷ M)§
	55 µg/kg group	110 µg/kg group	55 µg/kg group	110 µg/kg group	
	min	min	IU/ml	IU/ml	Mean of both groups
H	18	38	0.06	—	0.13
AT-H	28	47	0.07	0.07	0.17
h ₁₆	—	33	—	0.6	2.6
AT-h ₁₆	39	90	0.7	0.9	2.2
h ₁₂	—	36	—	0.9	3.3
AT-h ₁₂	34	52	0.7	1.1	3.7

* Derived from Fig. 1. ‡ Derived from anti-Factor Xa assays on serial blood samples after injection of the heparin moieties (e.g., Fig. 2).

§ Obtained as the quotient of the anti-Factor Xa level at the time of score 2 and the specific anti-Factor Xa activity of the heparin moieties expressed in molar concentration.

after injection of the fragments was two- to fourfold greater than that obtained with H. In previous studies (11) it has been shown that heparin fragments with molecular weights of $\sim 4,000$ are equipotent to heparin with respect to antithrombotic effects. Barrowcliffe et al. (13), however, demonstrated that low-affinity forms of heparin potentiate the antithrombotic effect of high-affinity forms. At a dose of 110 $\mu\text{g}/\text{kg}$, h_{16} and h_{12} exerted an antithrombotic effect that lasted until the anti-Factor Xa level had decreased by $\sim 50\%$.

The covalent complex between AT and H had a slightly more pronounced antithrombotic effect than free H, a result that agrees with the complex's two- to threefold longer half-life (15) (Fig. 2). The covalent complexes between AT and h_{16} and h_{12} had a significantly greater antithrombotic effect than the free fragments (Fig. 1), which may be explained by the finding that for equivalent amounts injected, the level of anti-Factor Xa activity in plasma was higher for the complexes than for the free fragments (Fig. 2). In addition, the complexes disappeared more slowly from the circulation.

To compare the specific antithrombotic activities of the free heparin moieties and their covalent complexes, the Factor Xa inhibition levels were calculated when the antithrombotic activity in plasma had reached an intermediate level (score 2). This occurred at a concentration of 0.5–1.0 IU/ml, both for the free and complexed forms of h_{16} and h_{12} (corresponding to concentrations of 0.2–0.4 μM), but at a 10-fold lower concentration for free or complexed H, indicating that the free and complexed heparin moieties have similar specific antithrombotic activities. The specific antithrombotic activity of H, however, was more than an order of magnitude greater than that of h_{16} or h_{12} , as expressed in both molar concentration and anti-Factor Xa activity, indicating that the antithrombotic effect of heparin in plasma does not only depend on its anti-Factor Xa activity. This lack of correlation between the antithrombotic and anti-Factor Xa activity of heparin fragments confirms previous reports (21, 27).

The different ratios between anti-Factor Xa activity and antithrombotic efficacy of H as compared with h_{16} or h_{12} may be due to differences in their kinetics of inhibition of thrombin and Factor Xa. Thus AT-H inhibits Factor Xa with a k of $2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ (15) and thrombin with a k of $3 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ (17). AT- h_{16} inhibits Factor Xa with a k of $1.3 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ and thrombin with a k of $2 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$, whereas AT- h_{12} inhibits Factor Xa with a k of $10^6 \text{ M}^{-1} \text{ s}^{-1}$ and thrombin with a k of $3 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ (15–17). Thus AT-H inhibits thrombin 100 times faster than Factor Xa. At a concentration of AT-H of 17 nM (score 2, Table II) thrombin will be inhibited with a $t_{1/2} = 0.5 \text{ s}$ and Factor Xa with a $t_{1/2} = 20 \text{ s}$. Likewise, at a concentration of AT- h_{16} of 220 nM (score 2, Table II), thrombin will be inhibited with a $t_{1/2} = 0.5 \text{ s}$ and Factor Xa with a $t_{1/2} = 2.5 \text{ s}$. At a concentration of AT- h_{12} of 370 nM, the half-life of thrombin will be 7 s and that of Factor Xa, 2 s. These half-life values are probably an underestimate of the real half-lives, because the free AT-H concentration is lower than the total concentration (28). They do, however, suggest that the antithrombotic effect of AT-H (and of H) is probably mainly due to its antithrombin activity, whereas that of AT- h_{16} and AT- h_{12} (and of h_{16} and h_{12}) seems to be due to their anti-Factor Xa activity.

The present study was carried out with the lowest concentrations of heparin moieties and their complexes, which resulted in a clear antithrombotic effect that disappeared when the

plasma concentration decreased two- to fourfold. Under the experimental conditions used, the disappearance of the anticoagulant activity is thus due both to turnover of the antithrombotic substances and to their equilibration with the extravascular space. The half-life in plasma after equilibration is, however, two- to threefold longer for the covalent complexes than for the free forms (15, 16), and 10-fold longer for h_{16} and h_{12} than for H (16). The half-life of AT- h_{16} and AT- h_{12} is 30-fold longer than that of H and 10-fold longer than that of AT-H. Thus, even with a 10-fold lower specific antithrombotic activity, AT- h_{16} and AT- h_{12} complexes could produce a stable antithrombotic state, requiring smaller amounts than AT-H; discontinuous injections would result in more stable plasma levels, and far less frequent injections would be required to maintain an antithrombotic concentration in plasma. Covalent complexes between AT and h_{16} or h_{12} could, therefore, be of potential advantage as antithrombotic agents in humans.

Acknowledgments

This study was supported by project 80-8513 of the Geconcerteerde Onderzoeksacties.

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