FIBRINOLYTIC AND ANTICOAGULANT COMPLEXES OF LOW-MOLECULAR-WEIGHT HEPARIN WITH TUFTSIN

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Low-molecular-weight heparin (LMH) can inhibit activity of factor Xa [10] and it exhibits weak effects on coagulability of the blood in tests characterizing total activity of factors VIII, IX, XI, and XII [6]. Although it has low anticoagulant activity LMH does not possess a hemorrhagic action, but it is an effective antithromtobic agent. The antithrombotic activity of LMH does not necessarily correlate with its high anticoagulant activity [11]. High-molecular-weight heparin, by contrast with LMH, basically inhibits factor IIa and exhibits weak antifactor Xa activity [16]. On the other hand it has been shown that several peptides, containing the amino acid sequence Pro-Arg-Arg-Glu [12], exhibit anticoagulant activity. Peptides copied from the N-terminal region of the α-chain of fibrinogen, namely -Glu-Pro-Arg-, have protective anticoagulant and antipolymerization activity [7].

Tuftsin, a regulatory peptide with the amino-acid sequence Thr-Lys-Pro-Arg stimulates immunogenesis, influences behavioral reactions of animals [2, 4], and exhibits antipolymerization activity [3]. It can interact with unfractionated heparin with the formation of a complex; in this complex electrostatic forces arise between the heparin and tuftsin [8].

The aim of this investigation was to study complex formation of LMH with tuftsin, to obtain such a complex and to study its effect on the anticoagulant and fibrinolytic properties of the blood plasma in vitro and in vivo, after its intravenous injection into animals.

EXPERIMENTAL METHOD

Preparations of LMH were obtained from "Celsus" (USA), and tuftsin from "Serva" (Germany).

Complex formation between tuftsin and heparin was monitored by crossed electrophoresis [14]. A complex of tuftsin with heparin was obtained by the method [13], with our slight modification with respect to proportions by weight, namely 1:1, 1:3, and 3:1. The complexes obtained were freeze-dried and kept in the dry form at 20°C in an exsiccator over $CaCl_2$. In the experiments in vitro the effect of the LMH-tuftsin complex and its component parts in equivalent amounts on the anticoagulant properties of normal rat plasma was studied in the activated partial thromboplastin time [15], thrombin time, and recalcification time tests by the usual method.

Experiments in vivo were carried out on 40 male albino rats weighing 180-200 g. The effect of the LMH-tuftsin complex obtained with a proportion by weight of 3:1 on fibrinolytic and anticoagulant activity of rat

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TABLE 1. Effect of 0.1-0.01% Solutions of Low-Molecular-Weight Tuftsin Complex (LMH-T) on Some Parameters of Blood Clotting and Fibrinolysis in Vitro (M ± m, n = 10)

Specimens	Concentration percent	TFA, mm^2	NF, mm^2	RT, sec	TT, sec
LMH-T complex with undermentioned ratio of components:					
a) 1:1	0,1	43,3±2,4*,**	45±0*,**	>5 min*	>5 min*
	0,01	30,7±5,5*	36,7±2*,**	329±9*	>5 min*
b) 1:3	0,1	27,3±1*	26,7±3,4*	>5 min*	>5 min*
	0,01	27,4±0,7*	25,7±1*	212±10*	120±7,5*
c) 3:1	0,1	44,3±0,3*,**	38±4,1*,**	>5 min*	>5 min*
	0,01	38±0,7*	38±0,7*	430±12*	241±14*
Heparin in concentrations equivalent to its content in complex					
a)	0,1	2±2,3	0	>5 min*	>5 min*
	0,01	0	0	236±19*	53±7*
b)	0,1	0	0	>5 min*	>5 min*
	0,01	2,0±0,3	0	126,5±6	39,5±4,5*
c)	0,1	2±2,3	0	>5 min*	>5 min*
	0,01	0	0	280±10*	275±12*
Tuftsin in concentrations equivalent to its content in complex					
a)	0,1	20±1,4*	17±0,3*	—	—
	0,01	18±0,7*	18±0,7*	201±23*	22±4,5
b)	0,1	22±1,0*	21±1,7*	—	—
	0,01	20±0*	20±0*	162±10	21±3
c)	0,1	33,7±5*	30±5*	—	—
	0,01	25±0*	20±3,4*	147±9	21±1,0
	—	0	0	148±7	21,1±1,0

Legend. Significance of differences $p < 0.001$; *) compared with NaCl control, **) compared with tuftsin.

TABLE 2. Total Fibrinolytic Activity (TFA), Nonenzymic Fibrinolysis (NF), Level of Plasminogen Activator (AP), Thrombin Time (TT), and Recalcification Time (RT) in Rat Blood Plasma 10 min after Intravenous Injection of LMH-Tuftsin Complex (3:1) and its Component Parts in Equivalent Doses (M ± m, n = 10)

Experimental conditions	TFA, mm^2	NF, mm^2	AP, mm^2	TT, sec	RT, sec
Injection of 0.1% solution of LMH-tuftsin complex (0.4 ml/200 g)	52±7,8*	31±1,5*	34±1,9*	42±7,9*	96±8,4
Injection of 0.025% tuftsin solution (0.4 ml/200 g)	44±0,3*	29±1,5*	28±1,5*	18,5±2,0	102,5±12,3
Injection of 0.075% solution of LMH (0.4 ml/200 g)	44±3,4*	26±0,5	27,4±2,1*	26±3,8	110±10,5
Injection of 0.85% NaCl solution — control (0.4 ml/200 g)	32±1,6	20±1,2	3±0	16,2±0,1	88,3±3,1

Legend. *$p < 0.001$ compared with corresponding tests of control with NaCl.

blood plasma was studied. Animals of group 1 received the complex in a dose of 0.4, those of groups 2 and 3 received low-molecular-weight heparin (0.3) and tuftsin (0.1 mg/200 g) in doses equivalent to their content in the complex; animals of group 4 received 0.85% physiological saline. Solutions were injected intravenously.

Blood was taken from the animals' jugular vein 10 min after a single injection of the complex and of its components. A 3.8% solution of sodium citrate was used as the preservative for the blood.

Total fibrinolytic activity (TFA), nonenzymic fibrinolysis (NF) on unstabilized fibrin plates by the method of Kudryashov and co-workers [5], fibrinogen concentration by the method in [1], activity of plasminogen activator [9], and anticoagulant properties according to the thrombin time and recalcification time, by the usual method, were determined in blood plasma.

The numerical results were subjected to statistical analysis by the Fisher–Student method.

EXPERIMENTAL RESULTS

The first step was to determine that complex formation had taken place between tuftsin and LMH, by means of the crossed electrophoresis method. At the point of intersection of tuftsin and LMH during their movement in an electric field, staining for acid sulfate or carboxyl groups of heparin disappeared, evidence that the acid evolved in its interaction with tuftsin.

LMH-tuftsin complexes obtained with different relative weights of their components (1:1, 1:3, and 3:1) exhibited different anticoagulant and fibrinolytic properties in vitro also.

As Table 1 shows, the highest TFA and NF activity was possessed by the complex obtained with LMH and tuftsin in the ratio of 3:1. Under these circumstances zones of lysis of 0.1% solutions reached 44-45, and of 0.01% solutions of the complex – 36-38 mm^2.

It will be clear from Table 1 that the highest TFA and NF activity was possessed by the complex obtained with LMH and tuftsin in the ratio of 3:1. Zones of lysis of 0.1% solutions of the complex reached 44-45 mm^2, and with 0.01% solutions of the complex – 36-38 mm^2. Tuftsin in a dose equivalent to its content in the complex caused lysis of unstabilized fibrin both in the presence of 0.85% NaCl solution (zones of lysis not less than 33 mm^2) and in the presence of 3% ε-aminocaproic acid (zones of lysis 30 mm^2), an inhibitor of enzymic fibrinolysis. Heparin in a dose equivalent to the complex revealed no zones of lysis. However, both heparin and the LMH-tuftsin complex, in the ratio indicated above, exhibited quite high anticoagulant activity. When a 0.1% solution of the complex and 0.075% heparin were used, clot formation did not occur during more than 5 min, as shown by the recalcification time and thrombin time tests. Only with the test preparations in a concentration 10 times less, did heparin possess higher anticoagulant activity in the thrombin test (antifactor IIa activity) than the complex; by the recalcification time test the opposite picture was found: the LMH-tuftsin complex had activity 1.8 times greater than the equivalent dose of heparin. Tuftsin in an equivalent concentration (2.5 μg/ml) had virtually no effect on the anticoagulant properties of plasma in vitro.

It follows from the results obtained by the study of the anticoagulant properties of the LMH-tuftsin complex (with all three different ratios of LMH to tuftsin) that all these complexes possessed anticoagulant activity in all the tests used, and their activity was 1.5-4 times greater than that of the equivalent dose of heparin.

The investigation showed that tuftsin also had an anticoagulant action, but only in doses equivalent to its content in the LMH-tuftsin complex, obtained in the ratio of 1:1 and 3:1, and only by the recalcification time test. It was shown that tuftsin, in all concentrations studied, showed ability to dissolve unstabilized fibrin, but to a rather lesser degree than the complexes.

Since the highest nonenzymic fibrinolytic and anticoagulant activity was possessed by the LMH-tuftsin complex obtained in the ratio of 3:1, this complex was used subsequently in experiments on animals. An increase in the anticoagulant properties of the blood plasma by the thrombin time test by 2.6 times, an increase in plasminogen activator activity by more than 10 times, and an increase in TFA and NF of the plasma by 1.6-1.55 times compared with the same parameters in control animals receiving NaCl (Table 2) were established 10 min after a single intravenous injection of 0.1% solution of the complex in a volume of 0.4 ml/200 g body weight.

As Table 2 shows, 10 min after injection of tuftsin in a dose equivalent to its content in the complex, an increase of TFA and NF of the rat plasma by 1.34 and 1.45 times respectively and the appearance of plasminogen activator in the rat plasma (zones of lysis were increased by 9 times) compared with the same parameters in control rats receiving 0.85% NaCl solution were observed. Under these circumstances, a slight increase in anticoagulant activity was observed in the plasma of rats receiving the above dose of tuftsin. Injection of LMH in a dose equivalent to that in the complex led to some increase in the anticoagulant properties of the plasma 10 min after injection, as shown by an increase in the thrombin time from 16.2 (control) to 26 sec, and the recalcification time from 88.3 (control) to 110 sec. Incidentally, both after injection of tuftsin and after intravenous injection of LMH, plasminogen activator was found in the blood along with an increase in TFA and NF of the plasma by 1.37 and 1.25 times respectively, compared with the same parameters in the control rats receiving physiological saline.

It can thus be concluded from the results of this investigation that the immunopeptide tuftsin possessed fibrinolytic and anticoagulant activity both in vitro and in vivo, and in the form of a complex with low-molecular-weight heparin, these activities were intensified several times. The complex of low-molecular-weight heparin with tuftsin obtained with the components in the ratio by weight of 3:1 possessed the highest nonenzymic fibrinolytic and anticoagulant effects.

This complex can evidently prevent thrombus formation when threatened, and this is a matter for further study.

REFERENCES

1. G. V. Andreenko, Lab. Delo, No. 5, 3 (1962).
2. I. P. Ashmarin, Patol. Fiziol., No. 3, 3 (1988).
3. I. P. Ashmarin, V. E. Pastorova, L. A. Lyapina, et al., Izv. Akad. Nauk SSSR, No. 2, 302 (1991).
4. A. A. Kamenskaya, N. Yu. Sarycheva, N. B. Voroshilina, et al., Zh. Vyssh. Nerv. Deyat., **39**, No. 4, 767 (1989).
5. B. A. Kudryashov, L. A. Lyapina, and I. P. Baskova, Vestn. Mosk. Univ., Ser. Biol. Pochvoved., No. 5, 41 (1974).
6. V. P. Baluda, Z. S. Barkagan, E. D. Gol'dberg, et al., Laboratory Methods of Investigation of the Hemostasis System [in Russian], Tomsk (1980), pp. 249-253.
7. N. G. Levitskaya, A. N. Kleimenov, M. T. Petrosyan, et al., Byull. Éksp. Biol. Med., No. 7, 190 (1987).
8. M. G. Petrosyan, M. A. Rozenfel'd, A. K. Petrov, et al., Byull. Éksp. Biol. Med., No. 2, 57 (1983).
9. T. Astrup and S. Müllertz, Arch. Biochem., **40**, 346 (1952).
10. P. Bianchini, B. Osima, B. Parma, et al., Arzneimittel-Forsch., **35**, No. 8, 1215 (1985).
11. J. Fareed, J. M. Walenga, K. Williamson, et al., Sem. Thromb. Haemost., **11**, No. 1, 5 (1985).
12. A. P. Laudano and R. F. Doolittle, Biochemistry, **19**, 1013 (1980).
13. V. Mansfeld and J. Hladovec, Coll. Czechosl. Chem. Commun., **21**, No. 5, 1209 (1956).
14. S. Nakamura, K. Takeo, K. Tanaka, and T. Ueta, Hoppe-Seylers Z. Physiol. Chem., **318**, 115 (1960).
15. E. Perlick, Gerinnungslaboratorium in Klinik und Praxis, Leipzig (1960), pp. 65-66.
16. J. Pieters and T. Lindhouf, Blood, **72**, No. 6, 2048 (1988).