Antithrombotic and Thrombolytic Effects of a New Proteolytic Preparation Trombovazim (Russia)

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 147, No. 4, pp. 418-421, April, 2009
Original article submitted November 11, 2008

We studied the antithrombotic and thrombolytic effects of Trombovazim, a highly-purified proteolytic enzyme preparation obtained by immobilization of bacterial proteinases (*Bacillus*) on polyethylene oxide with a molecular weight of 1.5 kDa. Blood absorption of the preparation was evaluated after intragastric administration. *In vitro* experiments showed that Trombovazim produces anticoagulant and thrombolytic effects, which manifested in inhibition of fibrin clot formation and acceleration of its lysis. Drug concentration in the blood was elevated from the 4th to the 7th hour after intragastric administration of Trombovazim in a dose of 2250 U/kg, being maximum by the 5th hour (0.044±0.011 U/ml). Course treatment with Trombovazim (1000 U intragastrically, twice daily for 3 days) had a thrombolytic effect on rats with experimental intravascular thrombosis. This effect was manifested in a decrease in thrombus weight and increase in the percent of rats with recanalization of the occluded carotid artery.

Key Words: *proteolytic enzyme preparation; Trombovazim; antithrombotic and thrombolytic activity*

The development of nanomolecular drugs is directed to passage through the blood-tissue barriers, reduction of side effects (toxicity and allergic properties), and increase in the life-time of products in the body (prevention of enzymatic degradation and accumulation in the reticuloendothelial system; inhibition of renal filtration) [4,5,6]. A new preparation Trombovazim was developed at the Siberian Center of Pharmacology and Biotechnology. A highly-purified enzyme preparation was obtained by immobilization of *Bacillus* proteinases on polyethylene oxide 1500 [1,6]. Trombovazim is manufactured by the method of electron-beam synthesis. The formation of polymer—drug conjugates is induced by exposure to an electron beam. This treatment is followed by the formation of typical nanoparticles with a size of 20-100 nm. This process is designated as "molecular nanotechnology". Our previous studies showed that intravenous administration of Trombovazim has a strong thrombolytic effect in rats with experimental intravascular thrombosis [2].

Here we studied the *in vitro* anticoagulant and thrombolytic effects of Trombovazim. Blood absorption and thrombolytic properties of Trombovazim were evaluated after intragastric administration of this drug to rats.

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0007-4888/09/1474-0438

MATERIALS AND METHODS

The anticoagulant effect of Trombovazim was evaluated *in vitro* by inhibition of fibrin monomer (FM) polymerization. FM (3 mg) and urea (150 mg) were dissolved in 1 ml 0.02% acetic acid. A mixture of 0.1 ml FM solution and 0.6 ml Tris-HCl buffer served as the control. The samples contained 0.1 ml FM solution, 0.5 ml Tris-HCl buffer, and 0.1 ml Trombovazim in various concentrations. The time of fibrin clot formation in all samples was estimated in a thermostat at 37°C. Thrombolytic activity of Trombovazim was evaluated *in vitro* from the ability of this preparation to cause lysis of a dense fibrin clot obtained from purified FM. The samples contained 0.1 ml FM solution, 0.5 ml Tris-HCl buffer, and 0.1 ml Trombovazim in various concentrations. Trombovazim was added after clot formation. The time of fibrin clot lysis in all samples was estimated in a thermostat at 37°C.

The release of Trombovazim into the blood after intragastric administration of this preparation was studied on 70 male outbred rats weighing 350-420 g. Proteolytic activity of blood serum was measured. Trombovazim in a dose of 2250 U/kg was administered intragastrically. Blood samples were taken from the carotid artery (CA) of ether-anesthetized rats using a Teflon catheter. The blood was sampled 1, 2, 3, 4, 4.5, 5, 5.5, 6, 6.5, 7, and 8 h after drug treatment. After coagulation, the blood was centrifuged at 2700 rpm for 20 min. Azocasein served as a chromogenic substrate. Incubation of azocasein with blood serum was followed by proteolysis. The resulting fraction was not precipitated with trichloroacetic acid. Our experiments were performed with Azocasein (ICN Pharmaceuticals). The solution of azocasein (2.5 ml, 0.2%) in 0.05 M sodium phosphate buffer (pH 8.2) was added to 1 ml serum. Tubes were incubated on a water bath at 45°C for 2 h. Trichloroacetic acid (2.5 ml, 10%) was added. The non-reacting substrate was pelleted by incubation for 20 min. The mixture was filtered through paper filters. Absorption was measured on a Hitachi 557 spectrophotometer at 350 nm. The control sample consisted of blood serum from rats not receiving Trombovazim. The concentration of Trombovazim in blood serum was estimated from the calibration curve.

In vivo thrombolytic activity of Trombovazim was studied on 20 male Wistar rats (250-300 g) with experimental intravascular thrombosis [3]. In stage I, the common CA were prepared in anesthetized rats (60 mg/kg sodium ethaminal). Blood flow was recorded on MVF-1100 electromagnetic flowmeter (Nihon Kohden). Graphic data were registered using a KSP-4 recorder. Arterial thrombosis was induced by $FeCl_2$. A cotton tampon (1.5 mg) was moistened with 15% $FeCl_2$ solution and applied to the prepared left common CA. The tampon was removed after 10 min. The surgical field was washed 3 times with physiological saline. Blood flow was recorded over 40 min after application of $FeCl_2$.

In stage II, the rats were repeatedly anesthetized after 48 h. Blood flow in the left common CA was recorded. The animals were killed by narcotic overdose. The segment was excised from the left common CA. Thrombus was removed, washed with physiological saline, treated with a filter paper to remove excess water, dried to a constant weight at 60°C, and weighted on an analytical balance. Trombovazim in a dose of 1000 U/kg was administered intragastrically 4 h before and 2 h after application of $FeCl_2$. In the follow-up period, Trombovazim was administered twice daily. Control animals received an equivalent volume of distilled water.

The results were analyzed by Student's *t* test.

RESULTS

In vitro experiments showed that Trombovazim had a strong anticoagulant and thrombolytic effect, which depended on drug concentration (Table 1). Increasing the concentration of Trombovazim in a FM-containing incubation medium by 2 or 4 times was followed by a proportional increase in the time of fibrin clot formation. The process of fibrin polymerization was suppressed in the presence of 4 U/ml Trombovazim in the incubation medium. Addition of Trombovazim to fibrin clot was accompanied by an increase in the rate of its dissolution, which depended on drug concentration in the incubation medium.

Trombovazim concentration in the blood of rats increased insignificantly 1-3 h after intragastric administration of this preparation in a dose of 2250 U/kg (Fig. 1). Starting from he 4th hour blood Trombovazin concentration increased significantly, re-

TABLE 1. *In Vitro* Effect of Trombovazim on the Time of Fibrin Clot Formation and Lysis (*M*±*m*)

Experimental conditions		Time of fibrin clot formation, sec	Time of fibrin clot lysis, sec
Control		16.7±3.2	No lysis
Trombovazim, U/ml	0.5	26.3±4.8	27.8±3.5
	1	53.1±12.7	14.7±2.9
	2	100.6±21.2	14.2±2.2
	4	No coagulation	9.8±2.2

TABLE 2. Effect of the Course of Intragastric Treatment with Trombovazim on Thrombus Formation after Application of $FeCl_2$ to Common CA in Rats (*M*±*m*)

Parameter	Control (*n*=7)	Trombovazim (*n*=7)
Stage I		
Baseline blood flow in CA (before application of $FeCl_2$), ml/min	4.6±0.6	4.1±0.4
Initiation of thrombus formation, min	13.4±1.4	13.5±1.0
Time to complete cessation of blood flow, min	17.5±1.5	17.0±1.1
Stage II (48 h after occlusion)		
Blood flow in CA, ml/min	0.6±0.6	3.1±1.2*
Rats with complete occlusion of CA, %	86	43*
Rats with CA thrombus, %	86	71
Thrombus weight (in animals with thrombus), mg	0.89±0.05	0.67±0.09*

Note. *$p<0.05$ compared to the control.

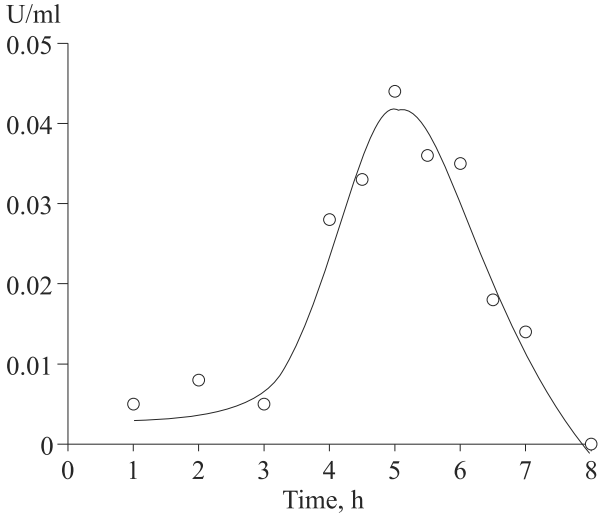

Fig. 1. Trombovazim concentration in the blood of rats after intragastric administration of this drug in a dose of 2250 U/kg.

ached maximum by the 5th hour, and decreased by the 7th hour. These data suggest that Trombovazim is mainly absorbed from the intestine. Eight hours after intragastric administration of Trombovazim, proteolytic activity of blood serum in treated rats did not differ from the control. Therefore, intragastric administration of Trombovazim is followed by its absorption into the blood. These changes are followed by a significant and long-term (3 h) increase in proteolytic activity of the blood.

Studying the thrombolytic activity of Trombovazim on the model of intravascular thrombosis revealed no differences between animals of the control and treatment groups during stage I (Table 2). However, 48 h after occlusion the ratio of Trombovazim-receiving rats with complete occlusion (3 of 7 specimens) was much lower compared to the control (6 of 7 specimens). Among animals of the treatment group with blood flow recovery in CA, 2 rats had a small thrombus in the vessel lumen and in 2 rats no thrombus was found. The weight of thrombus in Trombovazim-receiving rats (0.67± 0.09 mg) was 25% lower than in the control. Mean blood flow in the left CA (side of occlusion) of treated animals was 3.1±1.2 ml/min. This parameter in control specimens was 5-fold lower compared to the treatment group.

Our experiments showed that the course of intragastric administration of Trombovazim in a dose of 1000 U/kg has a thrombolytic effect on rats with intravascular thrombosis. Treatment with Trombovazim is followed by recanalization of the common CA and decrease in thrombus weight in the vessel lumen of rats.

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