THROMBOLYTIC ACTION OF UROKINASE COVALENTLY BOUND WITH MODIFIED THROMBIN

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Approaches suggested for the development of new fibrinolytic agents by chemical modification of enzymes have resulted in the obtaining of preparations with a more effective action in systems in vitro [2]. Tests of the action of such preparations in vivo constitute an important stage in further research. This naturally presupposes the transition from model enzymes (chymotrypsin, for example) to therapeutically important (urokinase, prourokinase, tissue plasminogen activator, etc.). The use of modified thrombin [3] as a polymer matrix with affinity for centers of thrombus formation has been reported to be promising. The action of the active principle in this preparation has been stimulated by chymotrypsin. We have obtained a preparation of modified thrombin by the suggested scheme [3] and urokinase was conjugated with it.

The aim of the present investigation was to obtain such a preparation of modified urokinase and to study its action in dogs with experimental thrombosis.

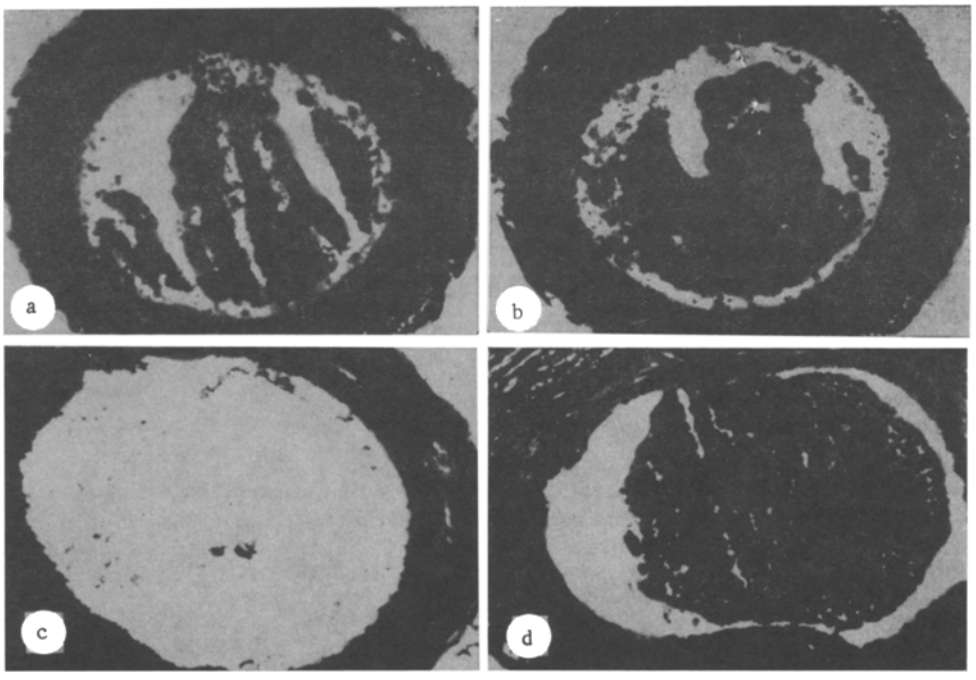

Fig. 1. Histological sections of thrombosed regions of dog's femoral arteries in control vessel (a) and 1.5 h after injection of native urokinase preparation in a dose of 2500 IU/kg (b); 1.5 h after injection of same dose of preparation of urokinase-modified thrombin conjugate (c), and in control vessel (d). Verhoeff–van Gieson's stain. 50 ×.

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TABLE 1. Enzymatic Parameters of AGLMe Hydrolysis by Native Urokinase and Urokinase Covalently Bound with Modified Thrombin (pH 7.5, 0.1 M KCl, room temperature, substrate concentration 10^{-3}-10^{-2} M, pH-stating method by initial reaction rates)

Preparation	Kinetic parameters			Retained catalytic activity after a 24-h incubation at 50°C (pH 7.5), % of original
	K_M, mM	k_{kat}, sec^{-1}	pH_{opt}	
Native urokinase	0,59	43,5	8,5	6
Urokinase covalently bound with modified thrombin	0,92	47	8,5	36

Note. For simplicity, the amount of apparent K_M and k_{kat} were calculated by the two-stage Michaelis–Menten scheme [1, 2].

TABLE 2. Thrombolytic Action of Urokinase Preparation Covalently Bound with Modified Thrombin

Angiography		Histology	
A	B	A	B
Before administration: Complete thrombosis After administration: Artery completely patent	Before administration: Complete thrombosis After administration: Contrast material does not pass	Complete absence of thrombus	Vessel thrombosed
Before administration: Long thrombus, partially patent After administration: Complete passage of contrast material	Before administration: Mural thrombus After administration: thrombosis	Complete absence of thrombus	Vessel occluded by tissue folds, remainder of lumen thrombosed
Before administration: Contrast material does not pass After administration: Artery completely patent	Before administration: Contrast material does not pass After administration: contrast material does not pass	Tissue folds cover lumen of vessel, no thrombotic mass present	Complete occlusion of vessel
Small thrombus, contrast material passes	Small thrombus, contrast material passes	Complete absence of thrombus	Mural thrombus
Before administration: Complete occlusion of vessel After administration: Passage of contrast material	Mural thrombus	Complete absence of thrombus	Mural thrombus covering one-third of lumen of vessel
Before administration: Complete occlusion After administration: Percolation of contrast material	Before administration: Complete occlusion After administration: contrast material does not pass	Thrombosis absent, lumen of vessel half covered by tissue	Complete occlusion of vessel

Legend. The use of native urokinase in the same dose (2500 IU/kg) does not induce lysis of the thrombus in either treated or control artery. Histological picture similar to that given in this table for control vessel. A) left (treated) femoral artery; B) right (control) femoral artery.

EXPERIMENTAL METHOD

Chemical modification of α-thrombin ("Sigma", USA) by 1,12-dodecamethylenediamine ("Fluka," Switzerland) was carried out by the method described previously [3]. Urokinase was conjugated with modified thrombin in the same way [3]. The urokinase was obtained from the Institute of Biomedical Technology, Ministry of Health of the USSR. Modified urokinase derivatives were isolated by gel chromatography on a Sephadex G-150 column and lyophilized. The catalytic activity of urokinase was determined potentiometrically, relative to hydrolysis of the methyl ester of acetylglycyllysine (AGLMe, from "Sigma") [1] and spectrophotometrically relative to lysis of the fibrin clot [2]. Platelet aggregation induced by native

or modified thrombin or by urokinase–modified thrombin conjugate was monitored as described previously [3]. The thrombolytic action of the enzyme preparations were studied in mongrel dogs weighing 10-20 kg, anesthetized with pentobarbital (30 mg/kg, intraperitoneally). Experimental femoral arterial thrombosis was induced by suturing a flap of its wall into the lumen of the vessel, with the adventitia facing the blood flow [4]. The presence of a thrombus was determined angiographically. The urokinase preparations were injected through the catheter, taken from the right carotid artery directly into the zone of thrombosis of one of the femoral arteries. A thrombus on the artery of the opposite side served as the control. The preparations were injected in a dose of 2500 IU/kg in 150 ml of physiological saline in the course of 50-60 min. Thrombolysis was estimated angiographically and histologically. The blood vessels of the animals were excised 1.5 h after the beginning of injection of the preparations and were fixed at 4°C for 24 h in a 4% solution of formaldehyde in phosphate-buffered saline (pH 7.4). Frontal sections were cut on a "Reichert-Jung Mod 2700-Frigocut" freezing microtome (Austria) and stained for collagen and elastic tissue by the Verhoeff–van Gieson method.

EXPERIMENTAL RESULTS

Gel-chromatographic separation of the incubation mixture of urokinase with modified thrombin (after carbodiimide covalent binding) showed that 56% of the urokinase was attached to thrombin. The residual esterase activity of the modified urokinase was 42% of the original value and the molecular weight of the product was about 100 kilodaltons. The kinetic parameters of AGLMe hydrolysis by native or modified urokinase did not differ significantly (Table 1), but the thermostability of the latter was increased. The content of urokinase by weight in the modified preparation was 20-25%, its specific activity was 100-500 IU/mg of the preparation, and the conjugate did not induce platelet aggregation. The fibrinolytic action of preparations of native and modified urokinase (taken in catalytically equal amounts) in the model system in vitro [2] was the same.

The thrombolytic action of these preparations in dogs with experimental femoral arterial thrombosis differed. Injection of native urokinase did not induce lysis of the thrombi. The dose used (about 2500 IU/kg body weight) is quite low and the result is in agreement with data in the literature [5]. The use of a preparation of modified urokinase led to lysis of the thrombi in the course of 1.5 h after injection (Table 2). The typical histological picture of sections through the thrombosed region of the vessel is illustrated in Fig. 1. The results are evidence of the effective thrombolytic action of a conjugate of urokinase covalently bound with modified thrombin. Such a preparation induces complete thrombolysis in doses at least ten times smaller than for the native enzyme. The probable reason for this may be increased affinity of the modified urokinase for the site of the thrombus and an increase in fibrinolytic potential of the blood [3].

LITERATURE CITED

1. A. V. Maksimenko, V. P. Torchilin, and V. N. Smirnov, Biokhimiya, 47, No. 3, 405 (1982).
2. A. V. Maksimenko and V. P. Torchilin, Vopr. Med. Khimii, No. 4, 12 (1985).
3. A. V. Maksimenko, A. N. Rusetskii, and V. P. Torchilin, Byull. Éksp. Biol. Med., 103, No. 1, 35 (1987).
4. S. É. Ragimov, A. A. Belyaev, M. A. Bragin, et al., Byull. Vses. Kardiol. Nauch. Tsentra, No. 2, 100 (1985).
5. D. Collen, Blood and Vessel, 12, 502 (1981).