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DISTURBANCE OF INTERACTION BETWEEN THROMBIN AND THE VESSEL WALL AND ITS INACTIVATION BY ANTITHROMBIN III IN AN EXPERIMENTAL NEPHROTIC SYNDROME

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In the modern view inactivation of thrombin in vivo is effected in several ways: through interaction of the enzyme with plasma inhibitors — chiefly with antithrombin III [3], highly specific reversible binding with receptor structures of the endothelium [6], and through activation of the anticlotting system, leading to the secretion of heparin, which catalyzes the inactivation of thrombin by antithrombin III [5], from mast cells into the blood stream. It has been suggested that the endothelium plays an important role in the secretion and inactivation of thrombin. Heparin-like binding sites of the enzyme located on the surface of the endothelium [11] and the membrane protein thrombomodulin evidently behave as cofactors in the action of inhibition of thrombin by antithrombin III [7]. In addition, thrombomodulin and an unidentified protein of endothelial cells with mol. wt. of 30 kD are unique receptor proteins, responsible for endocytosis of the enzyme by the endothelium [15, 13]. The change in the binding capacity of the vessel wall in relation to thrombin may probably lead to disturbances of thrombin clearance from the blood stream and its inactivation by plasma antithrombin III. The unusually high frequency of thromboembolic complications accompanying the nephrotic syndrome [12] suggests a disturbance of the mechanisms of biological inactivation and elimination of thrombin from the blood stream in this pathology. The investigation described below was carried out to test this hypothesis.

EXPERIMENTAL METHOD

Bovine α-thrombin was obtained by purification of a commercial preparation by ion-exchange chromatography on SP-Sephadex A-50 ("Pharmacia," Sweden) [4]. The α-thrombin was homogeneous on polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (PAG — SDS) and its molecular weight was 37 ± 1 kD. The clotting activity of the enzyme was 2000 NIH units/mg protein. The protein was labeled with ^{125}I ("Izotop," USSR) with the aid of "Iodo-Gen" (Pierce and Warriner, England), jointly with A. V. Rudin, by the method in [9]. To remove free ^{125}I and denatured protein, the ^{125}I-α-thrombin was subjected to chromatography on a column with Sephadex G-25. The ^{125}I-α-thrombin used in the subsequent experiments had specific radioactivity of 600 μCi/μM and its fibrinogen-clotting and amidase activity, determined spectrophotometrically by hydrolysis of D-phenylalanyl-pipecolyl-arginine paranitroanilide ("Serva," West Germany), was 1440 NIH units/mg and its molecular weight 37 ± 1 kD. Precipitation led to precipitation of 98% of the radioactivity together with protein.

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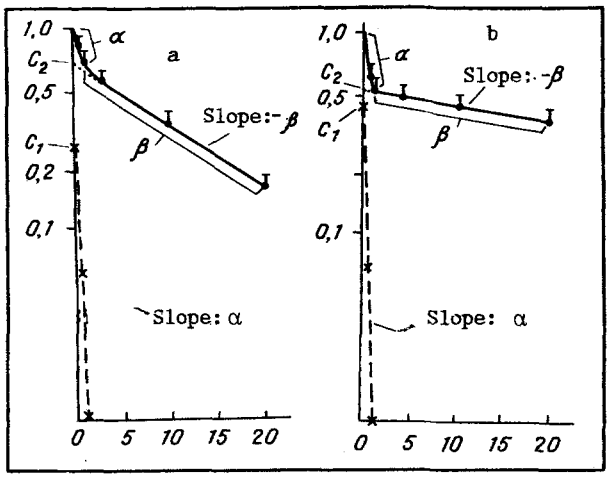

Fig. 1. Elimination of ^{125}I-α-thrombin from blood plasma of animals of control group (a) and rats with nephrotic syndrome (b). Abscissa, time after intravenous injection of ^{125}I-α-thrombin (in min); ordinate, radioactivity of blood plasma ($M \pm m$) Radioactivity of plasma 0.5 min after injection of ^{125}I-α-thrombin taken as 1.0. Values of coefficients (C_1 and C_2) and exponents (α and β) of elimination curve $x(t) = C_{1\exp}(-\alpha t) + C_{2\exp}(-\beta t)$ obtained by its graphic analysis between semilogarithmic coordinates. As a result of this, the elimination phase is described by a straight line with intercept of C_2 and slope of $-\beta$. The distribution phase is described by a straight line with intercept of C_1 and slope of $-\alpha$, plotted by subtraction of extrapolated values from their experimental values. Each experimental group consisted of five animals.

Experiments were carried out on noninbred albino rats weighing 230-250 g. An experimental nephrotic syndrome (ENS, autologous immunocomplex nephritis of Heymann) was produced in some animals by a single immunization with homogenate of rat renal cortex in Freund's complete adjuvant, as described previously [2]. Rats in the experimental group were chosen 3-4 months after immunization, by the possession of similar manifestations of disturbance of renal function, monitored as the protein concentration in the urine and the blood urea. Animals of the control group received injections of adjuvant without the immunogen. The rats of both groups received $1 \cdot 10^{-7}$ M ^{125}I-α-thrombin (4 NIH units, 60 μCi) in 0.2 ml of physiological saline, pH 7.4, by transcutaneous puncture of the jugular vein. Samples of 0.2 ml blood were taken from the opposite vein before injection of the enzyme and 0.5, 1.0, 4.0, 10, and 20 min after its injection, during short-term (up to 5 min) interrupted immobilization. The blood was stabilized with 3.8% sodium citrate solution in the ratio of 9:1. The volume of blood taken was replaced by an equal volume of physiological saline. Preliminary experiments showed that platelet-deprived blood plasma (1500 g, 20 min) had the same radioactivity after injection of the labeled enzyme as whole blood. The radioactivity of the plasma was connected with its proteins, as shown by the 99% precipitation of labeled material after treatment of the plasma with 10% TCA. It was therefore possible to determine radioactivity in the platelet-deprived plasma by studying clearance of the labeled enzyme. The half-life of ^{125}I-α-thrombin ($T_{1/2}$) was determined by graphic analysis of the curve showing changes in radioactivity of the plasma, using a two-component mathematical model [10]. Radioactivity of the samples was counted on an "LKB-Wallac 1282" gamma-counter (LKB, Sweden). The distribution of radioactivity in samples of plasma was studied by PAG-SDS electrophoresis, as described in [14]. In addition, the activated partial thromboplastin time, the concentration of soluble complexes of fibrin-monomers, and the heparin-cofactor activity of antithrombin III, were determined in the blood plasma as described previously [1]. The experimental results were subjected to statistical analysis by the Fisher–Student method.

EXPERIMENTAL RESULTS

The ENS, characterized by proteinurea (2.3 ± 0.7 mg/ml) and by elevation of the blood urea (by 47 ± 11%, $p < 0.01$) compared with values found in animals of the control group, was accompanied by the development of a prethrombotic state. This was shown by lengthening of the activated partial thromboplastin time by 111 ± 28% ($p < 0.01$), caused, as shown previously [2],

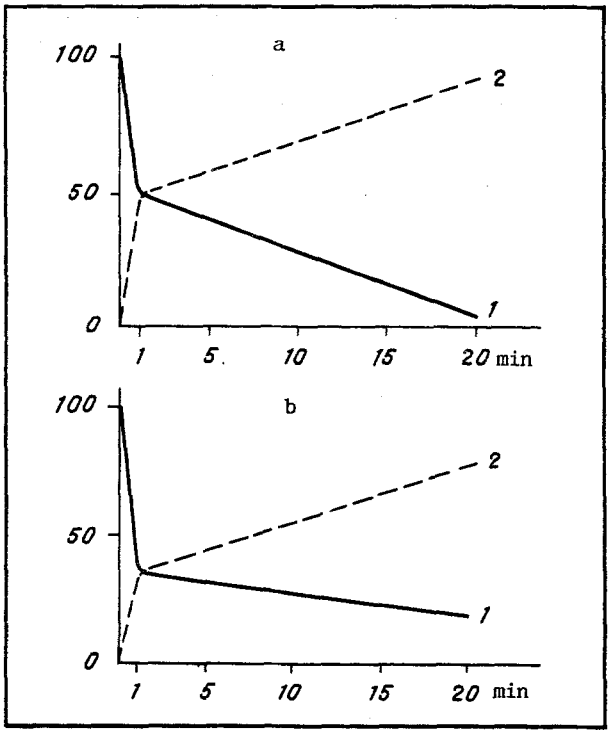

Fig. 2. Distribution of radioactivity in samples of blood plasma from animals of control group (a) and rats with nephrotic syndrome (b), receiving ^{125}I-α-thrombin, according to results of PAG—SDS electrophoresis. Abscissa, time after injection of ^{125}I-α-thrombin (in min); ordinate, percentages of components of ^{125}I-α-thrombin with different molecular weights. Total radioactivity of gel tube after electrophoretic fractionation of blood plasma, obtained at different times of the experiment, taken as 100%. Plasma from three rats tested in each experimental group. 1) Mol. wt. 37 ± 1 kD; 2) mol. wt. ≥100 kD.

by consumption of factors of the prothrombin complex for the purpose of excessive thrombinogenesis, as shown by an increase of 200 ± 37% ($p < 0.01$) in the concentration of soluble complexes of fibrin monomers. The antithrombin III level was depressed by 40 ± 11% ($p < 0.01$). Thus ENS was accompanied by activation of the clotting system and by an acquired antithrombin III deficiency.

The study of ^{125}I-α-thrombin clearance revealed significant differences in the rate of elimination of the enzyme from the blood stream of animals of the control group and rats with a fully developed phase of ENS. The results in Fig. 1 show that the clearance curve of ^{125}I-α-thrombin from the blood plasma of animals of both groups consisted of two components, corresponding to the phases of distribution (α) of labeled protein in the blood stream and its elimination (β). In vivo these processes took place with high velocity. For instance, in the blood stream of animals in the control group (Fig. 1a) $T_{1/2}$ of ^{125}I-α-thrombin in the distribution phase ($T_{1/2}\alpha$) was 0.33 ± 0.1 min, and in the elimination phase ($T_{1/2}\beta$) it was 8.15 ± 0.2 min. During ENS (Fig. 1b) clearance of the enzyme in the distribution phase took place with $T_{1/2}\alpha$ = 0.22 ± 0.1 min, and in the elimination phase with $T_{1/2}\beta$ = 28.9 ± 1.1 min. consequently, in ENS a more than threefold inhibition ($p < 0.01$) of clearance of the enzyme in the elimination phase was observed. Phasic changes in radioactivity of the blood plasma after injection of labeled thrombin into animals are explained on the grounds that a more rapid fall in the enzyme concentration in the blood stream takes place in the distribution phase, due mainly to its dilution in the blood. The phase of elimination reflects to a greater degree the process of interaction of labeled thrombin with binding sites on the endothelium [14]. During ENS a disturbance of binding of ^{125}I-α-thrombin with specific structures of the vascular endothelium evidently was observed, leading to inhibition of clearance of the enzyme from the blood stream.

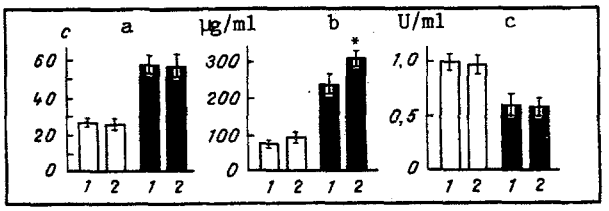

Fig. 3. Changes in parameters of hemostasis system of animals of control group and rats with nephrotic syndrome after injection of $1 \cdot 10^{-7}$ M ^{125}I-α-thrombin. Abscissa: 1) value of parameters before injection of ^{125}I-α-thrombin, 2) 5 min after injection of enzyme; ordinate: a) activated partial thromboplastin time; b) concentration of soluble complexes of fibrin-monomers; c) activity of antithrombin III. In each group values for five animals were studied. $^*p < 0.05$. Unshaded columns denote intact rats, shaded — nephrotic syndrome.

Samples of plasma obtained at different times after injection of ^{125}I-α-thrombin were subjected to PAG-SDS electrophoretic analysis in order to study the behavior of the labeled enzyme in the blood stream. Results of analysis of the distribution of radioactivity in samples of plasma from animals of both groups are given in Fig. 2. It was found that 1 min after injection into the control group of animals (Fig. 2a) 50% of the ^{125}I-α-thrombin had electrophoretic mobility corresponding to mol. wt. ≥100 kD, and 50% the electrophoretic mobility of native α-thrombin (mol. wt. 37 ± 1 kD). At the 20th minute of the experiment a progressive increase was observed in the content of the ^{125}I-α-thrombin component with mol. wt. ≥100 kD (up to 95%), identified on the basis of molecular weight as a complex of the labeled enzyme with antithrombin III (mol. wt. 60), whereas only 5% of the ^{125}I-α-thrombin was not bound with the inhibitor and had mol. wt. = 37 ± 1 kD. During the ENS (Fig. 2b), 1 min after injection the component of the labeled enzyme with mol. wt. ≥100 kD accounted for 35% of the total radioactivity of the plasma and 65% was accounted for by the component with the molecular weight of native α-thrombin. Compared with animals of the control group, in the rats with ENS a slower formation of the component of ^{125}I-α-thrombin with mol. wt. ≥100 kD was observed, i.e., the thrombin—antithrombin III complex. Thus at the 20th minute of the experiment, this component accounted for 80% of the radioactivity of the blood plasma, and the component with mol. wt. of 37 ± 1 kD accounted for 20%. Thus 20 min after injection of ^{125}I-α-thrombin, in rats with ENS compared with animals of the control group the fraction of the component of the labeled enzyme with mol. wt. ≥100 kD was 152 less ($p < 0.05$), whereas the fraction of the component with the molecular weight of native α-thrombin was increased fourfold ($p < 0.001$). On the basis of these results it can be postulated that α-thrombin is eliminated too slowly from the blood stream and is ineffectively inactivated by plasma inhibitors in rats with ENS.

This hypothesis was confirmed in a series of experiments to study the effect of ^{125}I-α-thrombin on the state of the hemostasis system of the two groups of animals (Fig. 3). In a dose of $1 \cdot 10^{-7}$ M the enzyme was found not to cause any significant changes in activated partial thromboplastin time, antithrombin III activity, and the concentration of soluble complexes of fibrin monomers in animals of the control group. Consequently, the injected dose of the enzyme was not thrombogenic and did not activate protein C or the anticlotting system in the course of 5 min of observation, corresponding in duration to the phase of distribution and the beginning of the phase of elimination of the labeled enzyme from the blood stream. In animals with ENS, accompanied as stated previously by a prethrombotic state, the same dose of enzyme 5 min after its injection caused an increase in concentration of the molecular markers of thrombinogenesis, namely soluble complexes of fibrin monomers, by 25% ($p < 0.05$), evidence of interaction between α-thrombin and fibrinogen. No significant changes were observed under these circumstances in the other parameters studied. Consequently, the dose of α-thrombin used, while not thrombogenic in animals of the control group, was thrombogenic in rats with ENS.

To sum up the above observations, it can be considered that in ENS two basic processes responsible for clearance of thrombin from the blood stream were disturbed: binding of the enzyme with the vessel wall and its inhibition by antithrombin III. The possible causes of these disturbances are: antithrombin deficiency or the presence of molecular defects in it, and also anomalies of the vessel wall. Since against the background of acquired antithrombin III deficiency, accompanying the nephrotic syndrome, even during the first minutes after injection of ^{125}I-α-thrombin the molar ratio of enzyme:antithrombin III in the plasma was not less than 1:100, i.e., the concentration of the inhibitor was sufficient to effectively inactivate the thrombin, it is

acceptable to suppose that disturbance of inhibition of the enzyme was connected with a defect or deficiency of the catalysts of this process. Since binding of thrombin by the endothelium and its inhibition by antithrombin III in vivo are effected by glycosaminoglycans of the glycocalyx of the endothelial cells [7, 8, 11], the most likely cause of the observed changes is a functional defect of the vascular wall.

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