ROLE OF HEPARIN AND TISSUE TYPE PLASMINOGEN ACTIVATOR IN HIGH ALTITUDE ADAPTATION OF THE HEMOSTASIS SYSTEM

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The condition known as "mountain hypocoagulation," presenting as enhancement of the anticoagulant properties of the blood and stimulation of fibrinolysis [2], is a phenomenon observed during adaptation to high altitudes. The mechanisms of development and the biological significance of this phenomenon are insufficiently clear. Some authorities regard it as the result of a raised blood level of the principal endogenous anticoagulant, heparin [1], whereas others consider it to be due to a deficiency of plasma procoagulants, as a result of their utilization during intravascular blood clotting, followed by compensatory activation of fibrinolysis [4]. Previous investigations do not permit a sufficiently accurate assessment of the role of physiological regulators of blood clotting such as heparin and tissue type plasminogen activator (t-PA), which react rapidly to external influences, in the formation of "mountain hypocoagulation."

The aim of this investigation was to study the role of heparin and t-PA in adaptation of the hemostasis system to high-altitude conditions.

EXPERIMENTAL METHOD

Experiments were carried out on noninbred male albino rats weighing 220-250 g. The animals were divided into two groups, which were kept under animal house conditions in Frunze (group 1, altitude 760 m above sea level) and on Tuya-Ashu Pass (group 2, altitude 3200 m, Central Tyan'-Shan' Range). Samples of blood, organs, and tissues were taken from the rats of group 1 after a stay of 1 month in the animal house, and from the rats of group 2 on the 10th, 15th, 20th, and 25th days of adaptation to high altitudes. Blood was taken from the jugular vein and stabilized with 0.11 M sodium citrate, in the ratio blood/preservative of 9:1. Activity of heparin and of t-PA was determined in samples of platelet-deprived (1500 g, 20 min) blood plasma. Heparin activity was estimated spectrophotometrically by a method based on blockade, by plasma antithrombin III, of the catalytic activity of thrombin relative to N-Tos-Gly-Pro-Arg p-nitranilide, using the "Heparin Low Dose" kit (Boehringer Mannheim GmbH, Austria), and expressed in USP milliunits (mU) in 1 ml. Activity of t-PA and of the euglobulin fraction of the blood plasma was tested by an original spectrophotometric method (440 nm) based on hydrolysis of the chromogenic protein substrate plasmin azofibrin ("Diagnosticum" Kaunas Research and Production Combine) by an enzyme formed during activation of an excess of added Lys-plasminogen by t-PA from plasma in the presence of dye-modified fibrin, and expressed in international units (IU) in 1 ml plasma. To obtain samples of organs (heart, lungs, kidneys) and tissues the rats were anesthetized with ether and decapitated. The state of the mast-cell population of the animals was determined by a histochemical method [3] in film preparations of the serous membranes from the mesentery of the small intestine and the renal capsule, fixed in formalin and stained with toluidine blue. The content of secreted and inhibitor-bound (the storage pool) t-PA fractions, extracted from homogenates of the organs by different concentrations of KCl (0.15 and 2 M) was studied by the fibrin plate method, as described in [8], and expressed in

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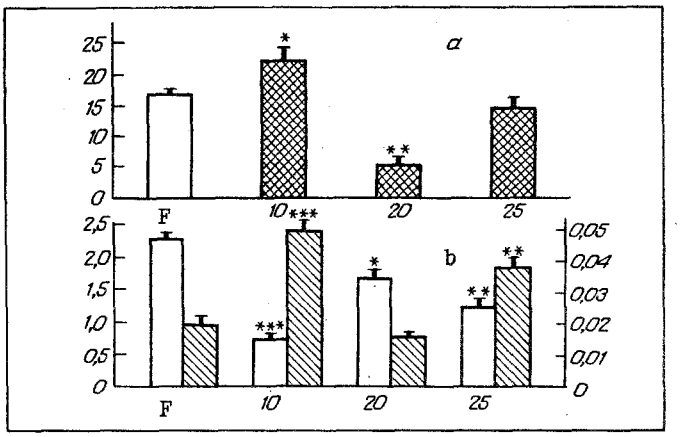

Fig. 1. Changes in heparin concentration (M ± m) in blood plasma (a) and mast cells (b) of rats kept in foothills (F) (n = 10) and during adaptation to high altitudes (n = 10). Abscissa, duration of stay in mountains (days); ordinate: a) heparin concentration in plasma (in USP mU/ml), b) heparin saturation index (unshaded columns) and degranulation index (shaded columns, in conventional units): *p < 0.05, **p < 0.01, ***p < 0.001. Significance of differences between values in rats during adaptation to high altitude calculated relative to their values in animals remaining in foothills.

IU in 1 ml of a solution of homogenate, prepared in the proportion of a 100 mg weighed sample of tissue to 1 ml of buffer. To plot calibration curves highly purified t-PA from pig heart (2500 IU/ml) was used, having been generously provided by S. A. Kudinov and E. M. Makogonenko, of the A. V. Palladin Institute of Biological Chemistry, Academy of Sciences of the Ukrainian using the multiple t test and the SAS software package.

EXPERIMENTAL RESULTS

During adaptation of the animals to high altitude marked fluctuations of the heparin level in the blood and mast cells were observed (Fig. 1). On the 10th day of the animals' stay in the mountains, heparin activity in the blood (Fig. 1a) reached its maximum, from which it gradually fell to a minimum by the 20th day, i.e., it was just over one-third of its value ($p < 0.001$) in animals of the control group, remaining in the foothills. By the 25th day of adaptation, however, the blood heparin concentration in rats kept in the mountains had returned to the level observed in the foothills.

Besides fluctuations of the blood heparin level, changes in the functional state of the mast cells also were found. The data in Fig. 1b show that the heparin saturation index of the mast cells fell more than threefold ($p < 0.001$) in the first 10 days of altitude adaptation, whereas lysis of the granules, which is connected with release of heparin from mast cells, increased by 2.5 times ($p < 0.001$). By the 20th day the heparin content in the mast cells and the state of degranulation were identical with those observed in animals of the control group in the foothills. By the 25th day of adaptation, however, release of the anticoagulant from the mast cells again intensified.

Comparison of the parameters reflecting mast cell function, namely the Fig. 1b not only reveals changes in the blood heparin concentration, but also provides evidence that this parameter is controlled and depends on the state of the producing cells (Fig. 1a). With an increase in heparin secretion, reflected in the degranulation index, the heparin concentration in the mast cells regularly falls but its circulating level rises. Toward the end of the halfway period of adaptation (25 days) of adaptation to high altitude the increase (by 90 ± 35%; $p < 0.01$) in secretory activity of the mast cells above its level in the control group of animals does not lead to any appreciable change in the blood heparin concentration. This may probably be connected with increased uptake of heparin from the blood stream by the mast cells or with stimulation of its de novo synthesis, i.e., with the formation of the new metabolic status of the mast cells arising during adaptation to high altitude.

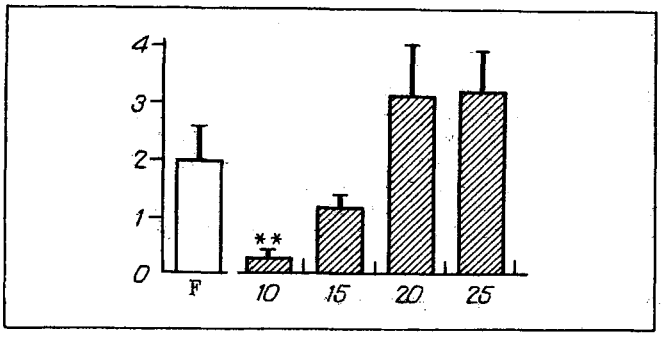

Fig. 2. Changes in concentration of tissue type plasminogen activator (M ± m, in IU/ml) in blood plasma of rats in foothills (F, n = 10) and during adaptation to high altitude (n = 10). Ordinate, concentration of plasminogen activator in blood plasma. Remainder of legend as to Fig. 1.

Besides changes in the endogenous heparin levels in the blood and mast cells of animals kept in the mountains, significant fluctuations of the t-PA level also were found in the systemic circulation and tissues (Fig. 2; Table 1). During the animals' stay at a high altitude, blood heparin activity initially fell to one-seventh of the value characteristic of the foothills, and then rose to reach a maximum toward the end of the period of observation. It must be emphasized that the blood t-PA level in rats adapted to conditions in the foothills and mountains was significantly higher (by 4.3 times, $p < 0.05$, and by 7 times, $p < 0.001$ respectively) than in animals living in the plains.

Analysis of the causes of fluctuation of the blood t-PA level, by determination of the secreted fraction (0.15 M KCl) and the reserve pool (2 M KCl) of the enzyme in the tissues showed (Table 1) that the increase in the t-PA concentration in the systemic circulation during adaptation to a mountain ecology was due chiefly to increased release of t-PA from the lung vessels. Secretion of the enzyme from the vascular endothelium of the heart and kidneys was virtually indistinguishable from that found in animals kept in the foothills.

Besides secretion of t-PA into the blood stream, an important influence on the intensity, primarily of local fibrinolysis, is exerted by its synthesis in the endothelium, in which it is present as a "storage" fraction or pool, meaning enzyme bound with a specific inhibitor, namely type I plasminogen activator inhibitor [11]. At high altitude the strongest stimulation of t-PA synthesis by comparison with the control group has been found to take place in the vessels of the heart (Table 1).

Thus the leading cause of activation of fibrinolysis in the systemic circulation during adaptation to high altitude, and which other investigators also have recorded [6], is the secretion of t-PA, chiefly from the endothelium of the mountains, with its lowered partial oxygen pressure. Meanwhile the strongest stimulation of t-PA synthesis de novo was found in the vessels of the heart. However, because of the area of the vascular endothelium of different organs and the specific content of t-PA in the tissues, it must be concluded that the chief source of secretion of the enzyme into the blood in the lungs.

The results suggest that the development of the phenomenon of "mountain hypocoagulation" during adaptation to mountain conditions is the result of a rise of the blood levels of heparin and t-PA, which is connected with their secretion into the systemic circulation by the producing cells. Because of the high heparin concentration in the blood (and even in catalytic amounts it accelerates inactivation of thrombin and certain other enzymes of the hemostasis system by plasma antithrombin III) [14], the development of a syndrome of disseminated intravascular blood clotting at high altitudes is unlikely.

The mechanism of the change in the blood levels of heparin and t-PA, the most important physiological regulators of the hemostasis system, during adaptation to high altitude, and also the biological significance of these changes, are not clearly understood. It can be tentatively suggested that changes in the level of this principal anticoagulant and key fibrolytic enzyme during adaptation to a mountain ecology are closely linked with stimulation of angiogenesis in the mountains: not only are they the result of angiogenesis, but also an important factor regulating vascular growth. The blood heparin level is determined by the number of mast cells, with a mainly perivascular distribution, and by their functional activity – by the ratio of the intensities of synthesis, secretion, and uptake of heparin. In the early period of adaptation, activation of mast

TABLE 1. Changes in Content of Tissue Type Plasminogen Activator (in IU/ml) in Heart, Lung, and Kidney Homogenates from Rats in the Foothills and during Adaptation to High Altitude

Oxygen	Statistical parameter	Foothills		Mountains							
				Time of stay in mountains, days							
				10		15		20		25	
		fraction									
		2	1	2	1	2	1	2	1	2	1
Heart	n	10	10	4	4	4	4	4	4	4	4
	M	1,36	0,50	1,07	0,39	1,68	0,96	2,21	0,39	2,14	0,36
	$\pm m$	0,11	0,06	0,10	0,02	0,24	0,20	0,22	0,02	0,25	0,05
	p	—	—	>0,1	>0,5	>0,1	>0,1	<0,05	>0,5	<0,05	>0,5
Lungs	n	10	10	4	4	4	4	4	4	4	4
	M	3,0	1,61	3,79	2,64	3,46	2,43	4,29	3,71	4,79	3,82
	$\pm m$	0,31	0,22	0,10	0,12	0,67	0,66	1,0	0,53	0,84	0,59
	p	—	—	>0,1	>0,05	>0,5	>0,1	>0,1	<0,05	>0,1	<0,05
Kidneys	n	10	10	4	4	4	4	4	4	4	4
	M	0,61	0,89	0,41	0,42	0,42	0,79	0,29	0,93	0,57	0,89
	$\pm m$	0,08	0,18	0,02	0,02	0,04	0,09	0,03	0,16	0,05	0,09
	p	—	—	>0,1	>0,5	>0,5	>0,5	>0,5	>0,5	>0,5	>0,5

Legend: p) significance of difference in values for rats during adaptation to high altitude, calculated relative to animals kept in foothills. 1) fraction extracted with 0.15 M KCl; 2) with 2 M KCl.

cells under the influence of an unknown stimulus, catecholamines perhaps [5], probably leads to an increase in secretion of heparin from them into the blood. Heparin, which has marked polyanionic properties, binds growth factors [10], the majority of which are cationic proteins and inhibit angiogenesis, which takes place especially rapidly in the early period of adaptation in the mountains. Heparin also has been shown to interact with thrombospondin, a protein synthesized by platelets, megakaryocytes, and endothelial cells, which have a heparin-binding domain and a permissive action toward stimulation of proliferation of vascular smooth-muscle cells by epidermal growth factor [12]. Since the thrombospondin receptors on the cell surface probably have a carbohydrate component, a heparin sulfate, and heparin may prevent the binding of this protein with the cells and (or) disturb the formation of other multimolecular protein complexes, which act as stimulators of proliferation of vascular cells [7]. Later during the stay in the mountains and with improving neovascularization the intensity of secretion of the anticoagulant into the blood stream decreases, possibly because of its increased uptake by mast cells, but at the end of the period of adaptation it again increases a little, this time because of an increase in the number of functionally perfect cells along the newly formed vessels or with an increase in their functional activity.

The relatively steady rise of the t-PA level in the blood after its initial sharp decline, which other workers also have found [13], evidently is also linked with the stimulation of angiogenesis, leading both to an increase in the number of endothelial cells producing this enzyme and to the release of t-PA into the blood stream under the influence of the basic fibroblast growth factor [9].

Probably several components of the hemostasis system not only regulate the liquid state of the blood and its clotting, but also participate in the control of angiogenesis, on the intensity of which they, in turn, are dependent.

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SOME ASPECTS OF THE USE OF LIPOSOMES TO STORE NEUROTROPIC DRUGS

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Administration of biologically active substances to animals in liposomal form has a marked effect on many pharmacologic parameters, such as the dose—effect relationship, toxic and side effects, pharmacokinetics and pharmacodynamics, and so on [2-4].

The degree and site for binding of molecules of neurotropic drugs of the liposomal membrane depend on the electrostatic and hydrophobic interactions. The strength of binding of these molecules with liposomes can be estimated by means of the nuclear magnetic resonance (NMR) method.

The line width of ^{1}H-NMR of amphiphilic substances in the composition of the phospholipid membrane, because of the reduced mobility of their molecules, is much greater than the line width of molecules in the aqueous phase. In the case of rapid exchange between the membrane-bound and free states the molecules of these substances will have a certain intermediate line width, proportional to the coefficient of distribution between membrane and water [1].

The use of this method to determine the strength of binding of molecules of neurotropic drugs with the lysosomal membrane has been made much easier by the fact that the aromatic region of the ^{1}H-NMR spectra of natural phospholipids is transparent, and molecules of most preparations used contain aromatic groups [1]. It is an interesting fact that the molecules of most compounds contain tertiary amino groups, which carry a positive charge at physiological pH values. Addition of negatively charged phospholipids to neutral phospholipids significantly increases the membrane — water partition coefficient for these compounds [6, 7]. Besides the phospholipid composition of the liposomes, their structural organization also affects the rate of release of the preparations. For instance, small single-layered sonicated liposomes ought to give up the preparations faster than large, stratified liposomes of the same composition. By modifying the above parameters, it is possible to vary at will the degree and character of interaction of preparations with liposomes, and thus to act together on their pharmacokinetics.

The aim of this investigation was to study the ability of liposomes to prolong the action of neurotropic drugs when administered locally.

EXPERIMENTAL METHOD

Liposomes were prepared from egg phosphatidylcholine obtained from Khar'kov Bacterial Preparations Factory and azolectin, obtained from "Sigma" (USA). The therapeutic preparations were used in the form of pharmacopoeial

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