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Structural and Functional State of Erythrocyte Membranes in Mice at Different Stages of Experimental Parkinson's Disease Induced by Administration of 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine (MPTP)

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We studied some structural and functional parameters of erythrocyte membranes in mice at the late presymptomatic and early symptomatic stages of experimental Parkinson's disease induced by administration of MPTP (hemolysis, microviscosity of different regions of the lipid bilayer, LPO intensity, activity of antioxidant enzymes, and kinetic properties of acetylcholinesterase). At the presymptomatic stage, significant deviations of the studied parameters from the normal were observed; they were similar in direction and magnitude to those in humans with Parkinson's disease. At the early symptomatic stage, most parameters tended to normal. Microviscosity of bulk lipids increased at the presymptomatic stage and decreased after appearance of clinical symptoms. This dynamics probably reflects activation of compensatory mechanisms aimed at inhibition of oxidative stress triggered by the development of the pathological process.

Key Words: *experimental Parkinson's disease; erythrocytes; structure of membrane; oxidative stress; acetylcholinesterase*

Neurodegenerative diseases (NDD) occupy a top place among socially significant diseases. The cunning of NDD is that the first clinical symptoms of the disease appear only after degeneration of a considerable part of specific neurons and often reflect irreversible damage to the brain and exhaustion of compensatory resources. For timely and successful treatment of NDD, it is necessary to understand the events occurring at the preclinical stages of the disease, to identify markers of these stages, and to find targets and terms for preventive therapy.

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The leading role of oxidative stress (OS) in the pathogenesis and development of NDD, in particular, Parkinson's disease (PD), is now beyond doubt [9]. According to modern concepts, OS is the most important, though nonspecific pathogenic factor of NDD. Activation of LPO is the one of the main manifestations of OS. Intensification of LPO in the lipid phase of biological membranes modifies the composition and structure of the lipid bilayer and disturbs the function of membrane-bound proteins.

Cell membranes play an important role in the pathogenesis and development of NDD [10,14]. The search for "membrane markers" seems to be not less promising than identification of markers in the plas-

ma, cerebrospinal fluid, *etc*. The composition and biochemical, physicochemical, and structural properties of membranes components important for membrane functions and sensitive to OS (LPO level) can be informative markers.

In studies on NDD, much attention is paid to identification of extracerebral markers. Erythrocytes that are considered as a "reporter" of OS in the body are extensively studied.

The goal of this study is to characterize and compare structural and functional characteristics of erythrocyte membranes at the late presymptomatic and early symptomatic stage of PD modeled in mice and to reveal similarities and differences in parameters that could be used for early diagnosis and choice of targets for preventive therapy.

MATERIALS AND METHODS

We used a toxic model of PD (administration of 1-methyl-4-phenyl-1,2,3,6- tetrahydropyridine; MPTP) developed at the Laboratory of Nervous and Neuroendocrine Regulations of N. K. Koltsov Institute of Developmental Biology, Russian Academy of Science. This model allows simulation of different phases of preclinical and clinical stages of PD via administration of MPTP in different doses and schedules [1,13].

Erythrocytes of mice at the late presymptomatic and early symptomatic stages (stages I and II, respectively) were analyzed. The blood for the study was obtained from the laboratory of nervous and neuroendocrine regulations. Detailed description of the pathomorphological and biochemical changes in the nigrostriatal dopaminergic system and characterization of locomotor activity of animals at these stages of the pathological process are given in previous articles [1,13].

The work was performed on 2-3-month-old male C57Bl/6 mice weighing 22-26 g. MPTP dissolved in saline was administered subcutaneously in a dose of 12 mg/kg: 2 injections for modeling stage I and 4 injections for modeling stage II (with 2-h intervals). Controls received saline according to the same scheme. In 2 weeks (when the desired stage of PD developed), the mice were decapitated [1,13]. Each group included 9-10 mice.

The following parameters were used to characterize the state of the erythrocyte membrane: mechanical strength (% of spontaneous hemolysis that occurs during isolation of erythrocytes), content of LPO products (malonic dialdehyde, MDA) in erythrocytes, structural state of erythrocyte membranes assessed by microviscosity (s) of the surface regions of membrane near bulk-lipids (s1) and in areas of the lipid bilayer adjacent to proteins (s2). Functional state of the membranes was assessed by measuring kinetic parameters of the reaction catalyzed by membrane-bound acetylcholinesterase (AChE). Experimental methods used in the present study are previously described in detail [2,4].

Erythrocytes were isolated from the whole citrated venous blood. We used 5% erythrocyte suspension in a medium containing 0.1 M Tris-HCl buffer and saline (NaCl) (1:1).

The degree of hemolysis (%) during centrifugation was determined by measuring extinction (540 nm) of hemoglobin released into the medium. LPO intensity was evaluated by the content of TBA-reactive products (MDA) [4].

The state of the antioxidant system in erythrocytes was assessed by activity of Cu/Zn-SOD and glutathione peroxidase using routine techniques [2].

Microviscosity (s) of the lipid bilayer of erythrocyte membranes was evaluated by EPR-spectroscopy using of two paramagnetic probes synthesized at the N. N. Semenov Institute of Chemical Physics, Russian Academy of Science: stable iminoxyl radicals: 2,2,6,6-tetramethyl-4-capriloyl hydoxypiperidine-1-oxyl (probe 1) and 5,6-benzo-2,2,6,6-tetramethyl-1,2,3,4-tetrahydro-gamma-carboline-3-oxyl (probe 2). Probe 1 is located in the surface layer of bulk-lipids and probe 2 penetrates into the annular near-protein zone of lipids. The time of rotational correlation of the probes (τ_c) at room temperature served as the measure of microviscosity [4].

Activity of AChE was determined in hemolysate of erythrocytes by Ellman's method with acetylthiocholine (iodide) as the substrate. Kinetic parameters of the hydrolysis reaction of acetylthiocholine (maximum velocity (Vmax), apparent Michaelis constant (Km), and Vmax/Km) were calculated as parameters of the Michaelis—Menten equation describing the dependence of initial velocity of optical density increase on acetylthiocholine concentration [4].

Parameters of enzymatic AChE reaction were calculated and statistical data were processed using Sigma-Plot 11 software. Student's *t* was used to find the differences in the characteristics between the experimental and control groups because of the studied parameters of erythrocytes in mice had normal distribution.

RESULTS

At the late presymptomatic stage, no behavioral symptoms of PD were observed. Disorders in the dopaminergic nigrostriatal system typical of PD were revealed during both stages, but some differences in the severity and direction of these disorders were found [1,13].

At both stages of the pathological process, the hemolysis of erythrocytes that characterizes their me-

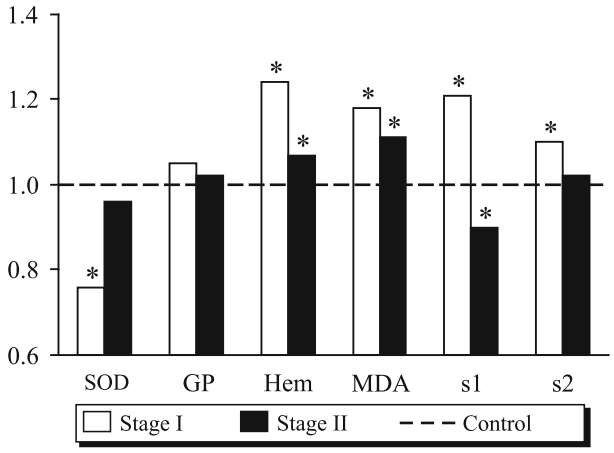

Fig. 1. Changes in the parameters of OS and structure of erythrocyte membranes in mice at the presymptomatic (stage I) and early symptomatic (stage II) stages of experimental PD (toxic model, injection of MPTP). GP: glutathione peroxidase, Hem: hemolysis, s1 and s2: microviscosity in regions of probes 1 and 2. *$p \leq 0.05$ in comparison with the control.

chanical strength increased (Table 1; Fig. 1); LPO is known to contribute to this process. The content of LPO products (MDA) was increased; similar to hemolysis, this increase was more pronounced during stage I.

SOD activity significantly decreased during stage I, but only a weak trend to a decrease was seen during stage II. Glutathione peroxidase activity did not differ from the control during both stages. The SOD/glutathione peroxidase ratio that reflects the balance in the system of ROS generation/detoxification decreased at the presymptomatic stage by 30% in comparison with the control that indicates disorders in the antioxidant defense system. During stage II, the SOD/glutathione peroxidase ratio did not differ from normal.

During stage I, microviscosity of the lipid bilayer in erythrocytes (s1 and s2) significantly surpassed the control level. During stage II, s1 was significantly reduced and s2 did not differ from the control. Thus, the presymptomatic and early symptomatic stages of experimental PD differ by microviscosity of the analyzed regions of the lipid bilayer of erythrocyte membranes and by direction of s1 changes in comparison from the control level.

One of the important features of the parkinsonism development is imbalance of dopaminergic and cholinergic activities. The key element in the functioning of the cholinergic system is AChE. The effect of AChE inhibitors (*e.g.*, environmental pollutants) is a risk factor that accelerates of PD development and complicates its course. The nigrostriatal toxicity of MPTP and its bioactive metabolite leads to parkinsonism and is partially realized through inhibition of AChE [15].

During stage I, kinetic parameters of erythrocyte AChE changed significantly in comparison with the control: enzyme efficiency decreased by 40% against the background of significantly increased peak reaction velocity (by 70%) and Km (by more than 200%) (Fig. 2). It should be noted that changes in AChE activity were not related to direct action of MPTP on AChE, because MPTP is completely eliminated from the blood within 2 days and the mice were sacrificed on day 15 after MPTP administration.

Hence, at the presymptomatic stage of the experimental PD, kinetic parameters of the erythrocyte AChE significantly differed from the norm. AChE activity normally (at physiological concentrations of acetylcholine) determined by Vmax/Km ratio was reduced. During stage II, Vmax and Km of erythrocyte AChE were almost the same in control and experimental animals. The efficiency of the enzyme was also within the normal limits. It cannot be ruled out that changes in the structure and properties of its membrane microenvironment can contribute to changes in the kinetic parameters of erythrocyte AChE.

The presymptomatic and early symptomatic stages of experimental PD differed by the observed changes in the studied parameters of OS and structure and function of erythrocyte membranes relative to the control.

Agreeing that the experimental model of the PD used by us adequately characterizes the course of the disease in humans [1,13], we considered it possible to compare the observed changes in erythrocytes with those reported at the advanced clinical stages of PD.

The content of LPO products (MDA) and oxidative hemolysis in patients with PD significantly surpass the normal [11]. Since the development and specific features of NDD are associated with the region

TABLE 1. Parameters of OS, Membrane Structure, and AChE Activity in Control Mice (*M±SE*).

Parameter		Control	
		stage I	stage II
AChE	Vm, arb. units	0.17±0.01	0.23±0.02
	Km, $\times 10^5$ M	3.5±0.1	7.3±1.2
	Vm/Km, arb. units	49.0±1.2	34.0±2.7
Activity of SOD, arb. units		84.0± 0.5	53.3±2.8
Activity of GP, arb. units		90.0±7.0	84.0±1.4
Hemolysis, %		15.1±0.6	15.9±0.3
MDA, μmol/liter of erythrocytes		48.7±2.5	50.1±1.2
Microviscosity s1, $\tau_c \times 10^{10}$ sec		0.28±0.01	0.30±0.01
Microviscosity s2, $\tau_c \times 10^{10}$ sec		0.98±0.01	1.01±0.01

Note. GP: glutathione peroxidase.

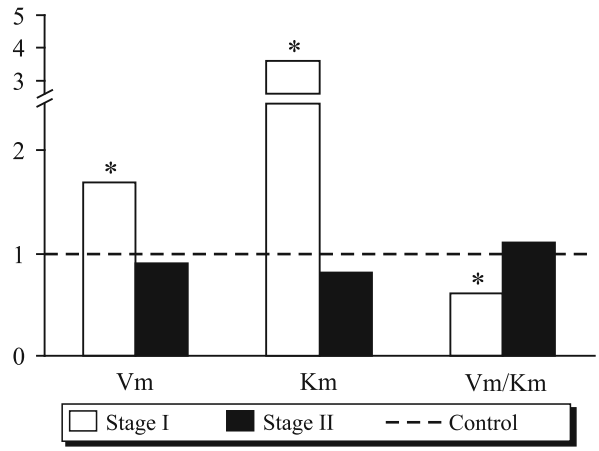

Fig. 2. Changes in the kinetic parameters of AChE of mouse erythrocytes at the presymptomatic (stage I) and early symptomatic (stage II) stages of experimental PD (toxic model, injection of MPTP) in comparison with the corresponding parameters in the control. *$p<0.05$ in comparison with the control. Absolute values of kinetic parameters for control animals: $Vm_{cI}=0.17\pm0.01$ arb. units, $Vm_{cII}=0.23\pm0.02$ arb. units, $Km_{cI}=(3.5\pm0.1)\times10^{-5}$ M, $Km_{cII}=(7.3\pm1.2)\times10^{-5}$ M.

of residence, the data obtained for Russian patients are of special interest. It was shown that during stages II and III of PD (Hoehn and Yahr scale), MDA content in erythrocytes of patients was higher by more than 60% than in healthy subjects [3].

The data about the enzymatic antioxidant defense in erythrocytes in PD are ambiguous and contradictory. In particular, data about SOD range from a decrease to lack of changes or an increase in activity. Nevertheless, from published reports it can be concluded that imbalance in the system of antioxidant enzymes is observed during all symptomatic stages of PD [5,7,11,12].

Changes in the microviscosity of membranes at clinical PD were described in [3]. Analysis with the use of a fluorescent probe revealed changes in the structure of blood cell membranes in patients with stage II and III of PD. Microviscosity in the surface area of the lipid bilayer of erythrocyte membranes increased by 23% in comparison with the control, microviscosity of annular lipids by 18%.

According to some reports, AChE activity in erythrocytes was slightly increased in patients with clinically manifest PD [6]. It should be noted that activity is usually assessed by the reaction rate at optimal substrate concentration, *i.e.*, essentially Vmax. In our case, we also observed an increase in Vmax during stage I.

Comparing the data characterizing different stages of PD including the presymptomatic, we can note that changes in the parameters of OS and structural and functional characteristics of the membranes even at the preclinical stage were similar and comparable with those observed during "advanced" stages of the disease. Early symptomatic stage (stage II) deserves special attention. The analyzed parameters at this stage had either tended to "normal", or demonstrated opposite changes in comparison with the presymptomatic and advanced stages (as shown it for s1). These shifts in the studied parameters at the early symptomatic stage of PD can reflect activation of compensatory mechanisms aimed at inhibition of OS caused by the pathological process.

It is not excluded that this stage of the disease is the most promising target for the therapeutic effect of antioxidants, inhibitors of free radical oxidation (in particular, tocopherol that used for NDD in clinical practice), by which, according to the idea [8] about the LPO regulation system in the membrane, targeted modification of structure and functions of membranes *in vivo* became possible.

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