GENERAL PATHOLOGY AND PATHOPHYSIOLOGY

Emotional Stress in Rats Changes Concentration and Composition of Extracellular DNA Circulating in Blood Plasma under Normal Conditions and in Cerebral Ischemia

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We studied quantitative and qualitative characteristics of extracellular DNA circulating in the blood plasma of Wistar rats under normal conditions, in psychoemotional stress (after 18 hours of aggressive conflict situation), and in acute cerebral ischemia. It was found that animals predisposed to psychoemotional stress normally have increased levels of antibodies against low excreted fragment of transcribed region of ribosomal DNA repeat rich in cytosine-guanine (CpG). A sharp increase in the level of circulating extracellular DNA was noted. Its increase was more pronounced during ischemia against the background of psychoemotional stress than in the control. These data suggest that multiple stress exposures experienced during the life can result in accumulation of GpG-rich sequences in the plasma of individuals predisposed to psychoemotional stress.

Key Words: *emotional stress; free circulating DNA; CpG-DNA; blood plasma; cerebral ischemia*

Psychoemotional stress (PES) similarly to some physical and chemical factors produces a genotoxic effect. The degree of this effect depends on genotype and is higher in animals and people with low emotional resistance [4,9,11]. PES induces non-specific oxidative stress which causes multiple damages and oxidative modification of DNA, chromosomal aberrations and cell death [7,8,12]. DNA fragments released from dead cells and newly synthesized and excreted by viable cells enter the circulation and form a pool of circulating DNA (cDNA) [3]. It can be hypothesized that cDNA properties should reflect changes in emotional state.

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The present study was undertaken to evaluate quantitative and qualitative characteristics of cDNA in rats with different emotional reactivity under normal conditions and in cerebral ischemia as well as in normal animals and in animals subjected to emotional stress.

MATERIALS AND METHODS

The work was carried out in winter on Wistar male rats (n=76) weighing 320-380 g with strict adherence to International Guidelines for the Care and Use of Laboratory Animals. The animals were kept under natural light and had free access to food and water.

Individual emotional reactivity of rats was determined in the open field test using physical activity index (PAI) calculated as the ratio of sum of crossed

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central and peripheral sectors to the sum of latencies of the first movement and entry into the center [5]. Highly active animals with PAI ranging from 2 to 6 were considered as prognostically resistant to emotional stressors and passive rats with PAI 0.2-0.6 as prognostically predisposed to PES. Emotional stress was caused in the model of aggressive conflict behavior (tail fixation for 18 h without restriction of access to food and water [10]). The stress reaction was monitored by changes in the weight of thymus and adrenal glands isolated from sacrificed animals after the end of the experiment. Cerebral ischemia was reproduced under nembutal anesthesia (45 mg/kg) immediately after stress exposure by bilateral common carotid artery occlusion (CCAO). The blood (0.5 ml) was taken via the catheter inserted into the femoral artery before and 30 min after CCAO. The plasma was separated immediately by centrifugation (450*g* for 10 min), frozen, and stored at -70°C.

The properties of cDNA were evaluated using previously described methods [14]. cDNA was isolated from plasma the using a standard phenol-extraction method. Plasma was pretreated with RNase A and proteinase K. cDNA concentration was measured by fluorimetry on a Perkin Elmer LS-55 fluorescence spectrometer at excitation and emission wavelengths of 350 and 450 nm, respectively, at least twice for each sample. Fluorescent dyes bisbenzimide (Hoechst 33258; Serva) and RiboGreen® (Molecular Probes Europe BV) binding to DNA were used before and after exhaustive DNase hydrolysis. The size of cDNA fragments was determined by horizontal electrophoresis in 1% agarose gel. Size markers were purchased from MBI Fermentas. The level of cDNA fragmentation was determined as the ratio of cDNA concentrations in the same sample tested using two different dyes: RiboGreen® (determines the concentration of DNA fragments of all lengths; fluorescence signal only slightly depends on fragment size in the range from 0.1 to 21 kb) and Hoechst 33528 (low sensitive to short and highly sensitive to long fragments). Nuclease activity in the plasma was assayed by fragmentation of a standard human genome DNA sample (8 μl) after 2-h incubation with the plasma of experimental rats (2 μl) at 37°C. After incubation, the length of human DNA fragments was determined: the shorter are the fragments, the higher is nuclease activity of rat plasma. Human DNA standard incubated under the same conditions, but without rat plasma served as the marker. Anti-DNA antibodies in plasma were assayed by ELISA and expressed in relative units [15]. Standardized rat plasma in serial dilutions was used as the control. Activity of plasma in the corresponding dilution (measured by light absorption) was taken as an antibody concentration unit. The targets for antibodies were genomic rat DNA (free anti-DNA antibodies were determined) and linearized plasmid containing transcribed area of ribosomal repeat (TArDNA) insert; antibodies forming specific complexes with CpG–DNA were assayed.

The data were processed by the Mann–Whitney *U* test using Statistica 6.0 software. When the distribution of data did not fit the normal law, the results were presented as medians and interquartile range (Me[25%;75%]).

RESULTS

In normal random animal sample (n=34), cDNA concentration varied from 44 to 227 ng/ml (77 [58;140] ng/ml). Rats were subdivided into 2 groups by cDNA levels. The concentrations were normally distributed in each group (Fig. 1, *a*). In group 1 (n=18), cDNA concentration was below the median (60.3±8.7 ng/ml); electrophoresis revealed clear-cut low-molecular-weight fraction (Fig. 1, *b*). This was accompanied by high endonuclease activity of the blood plasma:

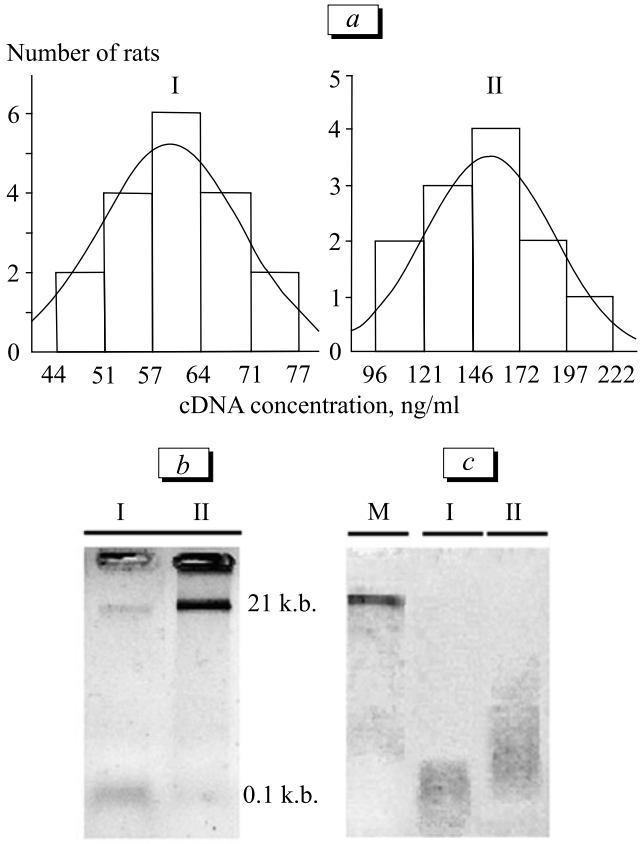

Fig. 1. cDNA characteristics of rats in groups 1 and 2. *a)* frequency distribution of concentrations; *b)* electrophoregram: example of cDNA pattern in group 1 (I) and 2 (II); *c)* electrophoregram: fragments of human genomic DNA after 2-h incubation with plasma samples of the same rats. M: marker (human genomic DNA incubated without rat plasma). The figures indicate the size of the fragments.

TABLE 1. cDNA Characteristics in Rats Prognostically Predisposed and Resistant to Emotional Stress after CCAO (*M*±*m*)

Rats	cDNA concentration, ng/ml		Degree of fragmentation, rel. units	
	baseline	ischemia, 30 min	baseline	ischemia, 30 min
Passive (*n*=12)	64.7±13.8^{+}	286.2±114.2*	3.4±0.2^{++}	2.3±0.4*
Highly active (*n*=12)	187.0±54.2	132.4±20.9	2.1±0.2	1.7±0.8

Note. $^{+}p<0.001$, $^{++}p<0.001$ between the groups; *$p<0.05$ relative to the baseline values (*U* test).

standard DNA sample was hydrolyzed to short fragments (<150 b.p.) after incubation with their plasma (Fig. 1, *c*). In group 2 (*n*=16), cDNA concentration exceeded the median (154.9±18.5 ng/ml) and almost three-fold surpassed that in group 1. In this case, low-molecular-weight DNA fraction was detected as trace amounts (almost all DNA fragments were ≥21 k.b.) and endonuclease activity of the blood plasma was lower than in group 1. After incubation, fragments of standard DNA sample had a greater length (from 4000 to 100 b.p.; Fig. 1, *c*). Comparison of cDNA properties in passive (*n*=12) and highly active (*n*=12) animals showed that 8 passive rats (67%) refer to group 1, and 10 highly active rats (83%) to group 2. Both quantitative evaluation (Table 1, column "background") and electrophoresis (Fig. 2) showed higher degree of cDNA fragmentation in passive rats ($p=0.036$). High endonuclease activity in blood plasma and low cDNA concentration under normal conditions are probably a result of their greater sensitivity to narcosis. The facts that passive rats recover worse after cerebral ischemia [1] and hypoxia-sensitive rats are known to be more sensitive to nembutal anesthesia [6] may confirm this assumption. The possibility of more severe consequences of surgical intervention cannot also be excluded. Enhanced DNA damage and increased apoptosis in blood cells characteristic of post-traumatic period [7] is apparently more pronounced in emotionally reactive animals. Clarification of this issue requires further investigation.

Increased endonuclease activity of blood plasma after the increase in cDNA concentration contributes to its cleavage into low-molecular-weight fragments further reutilized by cells and excreted by kidneys. As a result, cDNA level can be reduced even below the initial level despite enhanced cell death. In this case, low excreted genome sequences accumulating in cDNA pool can serve as the marker of cell death. It may be, for example, resistant to fragmentation CpG-rich transcribed region of the ribosomal repeat (TArDNA) [14]. Our study showed that plasma levels of anti-DNA antibodies in passive (*n*=7) and highly active (*n*=7) rats were similar (0.55±0.02 and 055±0.01 arb. units, respectively) with significantly higher level of antibodies against TArDNA in passive animals (1.10±0.04 and 0.90±0.06 arb. units, $U=6$, $p=0.032$). Revealed sensitization of predisposed to PES normal rats to TArDNA attests to accumulation of CpG-rich sequences resistant to fragmentation in the plasma. We hypothesized it to be the result of their high emotional reactivity and exposure to various stressors. To test this hypothesis, we compared cDNA properties in normal and experienced aggressive conflict ambivalent rats with PAI 0.55-0.75 and 1.50-1.85. In control (*n*=9) and experimental (*n*=9) groups, the proportions of animals with comparable PAI values were similar. Changes in the weight of target organs in rats that had undergone PES indicated stress development. Thymus weight in the control and experimental groups was 65.9±1.9 and 53.6±3.8 g, respectively ($p<0.02$) and weight of the adrenal glands was 6.7±0.4 and 7.4±0.5 g. cDNA level 2.5-fold surpassed the control (187 [134;251] and 74 [51;127] ng/ml, respectively, $U=16$, $p=0.041$; Fig. 3, *a*) with minor changes in degree of fragmentation (2.5 [1.8;3.6] and 1.6 [1.5;2.2] ng/ml, respectively). As a result, significant number of short cDNA fragments was revealed in blood plasma (Fig. 3, *b*). These data indirectly suggest that TArDNA fragments can accumulate in cDNA pool during the stressfull life of passive rats.

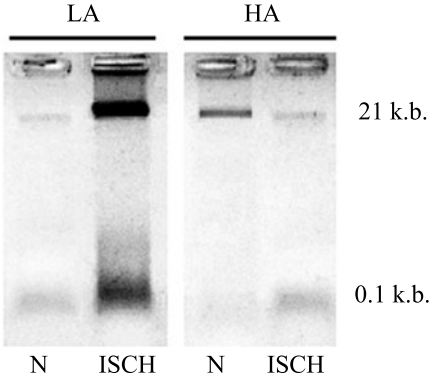

Fig. 2. Electrophoregram of cDNA in highly active (HA) and passive (LA) rats in norm (N) and 20 min after CCAO (ISCH). The figures indicate the size of the fragments.

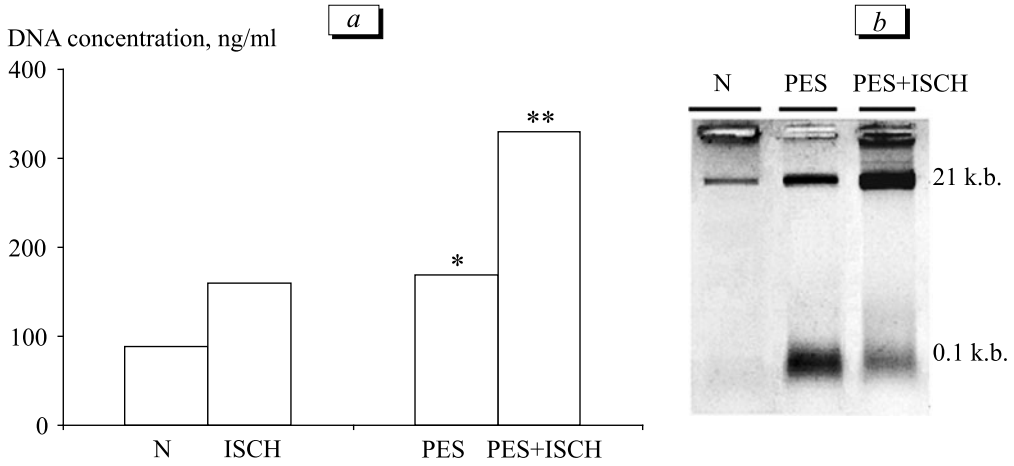

Fig. 3. cDNA characteristics of ambivalent rats. *a)* concentration of cDNA in normal rats (N); after CCAO (ISCH); after 18-h PES and CCAO; *b)* electrophoregram of cDNA in rats under the same conditions. The numbers indicate the size of the fragments. *$p<0.05$, **$p<0.02$ relative to the control (*U* test).

During the most acute period of cerebral ischemia, cDNA concentration in highly active rats remained within the normal range or decreased with minor changes in the degree of fragmentation. This suggests the maintenance of the balance between activity of cell death processes and cDNA elimination and is consistent with the data on relative stability of the brain of highly active rats during sharp restriction of blood flow [1]. In contrast, cDNA levels in passive animals increased 4.5-fold, which was accompanied by accumulation of short fragments in the blood plasma. These data indicate that cell death significantly predominates over cDNA elimination (Fig. 2; Table 1) and that the examined model is more sensitive to cerebral ischemia. The degree of cDNA fragmentation in this case was reduced by 1.5 times (p=0.042), which apparently can be explained by that necrosis predominated over apoptosis in the early posttraumatic period [7]. In controls (n=6), blood sampling did not affect significantly examined characteristics of cDNA; its concentrations before and 20 min after the procedure were 107.8±23.7 and 89.6±17.9 ng/ml, respectively, the degree of fragmentation, 1.9±0.1 and 2.0±0.3. Animals earlier subjected to PES showed post-CCAO cDNA level surpassing the control by 2 times (330 [257;352] and 160 [136;353] ng/ml, respectively, U=14, p=0.011) indicating increased damaging effects of cerebral ischemia under PES.

Hence, cDNA level sharply increases under conditions of emotional stress, which can result in accumulation of low excreted cpG-rich sequences during the life. We have previously shown that the sequences may dose-dependently either enhance or suppress NO synthesis by endothelial or immunocompetent cells [2]. On the other hand, their constituent bases, in particular guanine, can be oxidized under conditions accompanied by the activation of radical reactions [13]. We can assume that under physiological conditions, stress-induced changes in DNA promote the adaptive response. However, in pathology (for example, cerebral ischemia), these changes can trigger a cascade of events aggravating the pathological process.

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