EXPERIMENTAL BIOLOGY

Spontaneous and *In Vitro* Induced Apoptosis of Lymphocytes and Neutrophils in Patients with Alcohol Dependence

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Spontaneous and induced apoptosis of neutrophils and lymphocytes was studied in alcoholics during the abstinent syndrome and in healthy individuals. In alcoholics, the levels of lymphocyte and neutrophil apoptosis at the receptor and cellular levels were higher than in healthy subjects. Blood cells from alcohol addicts and normal individuals similarly react to stimuli (hyperthermia and synthetic glucocorticoid prednisolone) *in vitro*.

Key Words: *apoptosis; alcoholism; lymphocyte; neutrophil*

The problem of alcohol abuse remains extremely important all over the world. Studies of recent years are carried out in different directions, using a variety of methods and approaches. Ethanol abuse is associated with significant restructuring of the neuromediator, neuroendocrine, biochemical, and immunological processes, which disturbs the interactions between the nervous and immune systems and leads to failure of neurohumoral compensatory mechanisms, disorders of autoregulation, impairment of organism's reactivity, and immune disorders of different severity [9,12]. Under conditions of chronic alcoholization, ethanol can damage any organs and tissues because of its membranotropism and capacity to modify the key components of cell metabolism, which determines more intensive death of different cell types [1,3]. The role of apoptosis (programmed cell death) in the pathogenesis of ethanol-induced organ damage is proven [7].

We studied spontaneous and induced apoptosis of peripheral blood neutrophils and lymphocytes of *in vitro* in patients with alcoholism and somatically and mentally healthy subjects.

MATERIALS AND METHODS

Comprehensive clinical biological studies were carried out in 58 alcoholics aged 31-57 years (mean age 43.47±1.23 years). According to ICD-10, this disease is classified as mental and behavioral disorders resulting from ethanol abuse, the dependence syndrome (F10). Clinical data were collected with consideration for the clinical qualification and verification of patients' examinations in accordance with bioethical standards and protocol of the study approved by the ethic committee of Institute of Mental Health. Blood specimens from 22 somatically and mentally healthy men aged 18-60 years (mean age 31.33±2.36 years) without chronic diseases, not registered at health center, without signs of previous acute infections by the moment of examination, served as the control. Blood was collected on admission to Department of Addictions (Clinical Division of Institute of Mental Health) in the presence of pronounced abstinent syndrome before drug therapy. Alcohol abstinent syndrome was characterized by autonomic, dyssomnic affective and

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toxicogenic manifestations. Blood specimens from healthy individuals and alcohol addicts were collected from the ulnar vein after overnight fasting. A complex of methods characterizing apoptosis parameters was used. Lymphocytes were routinely isolated from heparin-stabilized blood by centrifugation in Ficoll-Paque density gradient (Pharmacia). The levels of cells with apoptosis marker were evaluated by indirect immunofluorescent method with monoclonal antibodies to CD95 antigen (Fas receptor; Sorbent company) [2]. Morphological changes in neutrophils and lymphocytes characteristic of apoptosis were evaluated in blood smears by light microscopy [6]. Spontaneous apoptosis was evaluated in smears prepared directly after blood collection. The smears were fixed (5 min) in azure-eosin methylene blue after May–Grunwald and stained (40 min) after Romanowskii–Giemza. Apoptosis induced by various factors was studied during cell incubation *in vitro*. Specimens of heparin-treated blood were incubated (4 h) at ambient temperature and at 41°C without or with 10 μM prednisolone and with 0.5% ethanol. The data were statistically analyzed and processed by nonparametric Kruskal–Wallis test using Statistica 6.0 software.

RESULTS

Stage 1 of programmed cell death is reception of a signal (apoptosis precursor) in the form of information entering the cell from the outside or generated inside it. The signal is perceived by the receptor and analyzed. By the present time, the inductor factor and its receptor have been described, for which other functions than apoptosis induction are not yet known [13]. These are Fas ligand (FasL) and Fas receptor (Fas, APO-1, CD95). Fas is expressed on many cells, its expression increasing with cell activation.

Studies by ELISA showed that CD95 (apoptosis readiness) receptor is expressed in 11.63±0.31 healthy individuals and in 16.56±1.19% alcoholics (Table 1). These results are in line with published data [5]. A significant increase in the number of cells ready to trigger programmed cell death reactions indicates that ethanol and its metabolites are apoptosis inductors.

Study of the effect of 0.5% ethanol *in vitro* on the expression of CD95 receptor showed a significant increase of lymphocyte apoptosis in donors (17.00±0.85 and 11.63±0.31%, respectively, $p<0.05$; Table 1). A slight increase in CD95 receptor expression (20.05±1.72%) in response to addition of ethanol to the incubation medium was observed in cells from alcoholics in comparison with spontaneous apoptosis of cells from the same patients. Presumably, this indicates certain sensitization of lymphocytes to ethanol *in vivo* under conditions of chronic alcoholization.

Segmented neutrophils with morphological signs characteristic of cells subjected to apoptosis were detected in the blood smears from the patients. These neutrophils were smaller, round, in some cases with few large vacuoles at the same pole in the cytoplasm. The morphology of changes in the nuclear substance consisted in shrinkage of the nucleus with condensation and granulation of chromatin along its perimeter. Cytological analysis showed that spontaneous apoptosis of neutrophils reaches 1.14±0.36% in alcohol addicts, which is significantly higher than in healthy individuals (0.42±0.14%, $p<0.05$). Lymphocytes with fragmented nucleus were detected in 5.07±0.63% cases, which 5-fold surpassed the corresponding parameter in healthy individuals (0.90±0.18%, $p<0.001$). Degradation of the nuclear material in lymphocytes was noted; we observed chromatin fragmentation into several parts.

The lymphocyte apoptosis realization index (percentage of cells with morphological signs of apoptosis *vs.* total count of cells expressing apoptosis readiness receptors) was 7.74±1.65% in donors and 14.36±2.38% in patients with alcohol addiction ($p<0.05$). Chronic ethanol intoxication causes LPO activation, promotes cell membrane damage and impairs their permeability, leads to morphological changes and death of cells.

The effects of apoptosis modulators (synthetic glucocorticoid prednisolone, hyperemia, and ethanol) on the peripheral blood cells were studied in loading tests *in vitro* (Table 2).

Blood incubation at ambient temperature caused no appreciable changes in the counts of apoptotic neutrophils, both in donors (0.48±0.26%) and alcohol addicts (0.87±0.38%). Long-term hyperthermia sharply reduced viability of neutrophils from donors and alcoholics (8.31±2.73 and 17.32±3.93%, respectively, $p<0.05$). Culturing of blood specimens at high temperature with prednisolone reduced the percent of cells with granular chromatin, which attests to antiapoptotic effect of glucocorticoids on the neutrophils (Table 2).

Incubation of donor cells at ambient temperature significantly increased the number of lymphocytes with fragmented nuclei in comparison with spontaneous apoptosis (2.50±0.56 and 0.90±0.18%, respectively, $p<0.05$). Hyperthermia caused a 2-fold increase in the number of apoptotic lymphocytes in comparison with incubation at 20°C (Table 2). Lymphocytes from alcohol addicts reacted to the stimuli as follows. Incubation at ambient temperature did not lead to an appreciable increase in the counts of cells with fragmented nuclei. The maximum effect was observed in response to hyperthermia (7.33±2.24% *vs.* 5.13±0.68 in the control, $p>0.05$) and prednisolone (7.33±2.24% *vs.* 4.76±1.05% in the control, $p>0.05$).

TABLE 1. Effect of Ethanol on CD95 Receptor Expression in Alcohol Addicts and Healthy Individuals *in Vitro*

Experiment conditions	Healthy individuals	Alcoholics
No incubation	11.63±0.31	16.56±1.19*
Incubation with 0.5% ethanol	17.00±0.85^{+}	20.05±1.72

Note. $p<0.05$ compared to: *healthy individuals, $^{+}$spontaneous apoptosis (no incubation).

An interesting regularity was traced in incubation of blood specimens from alcohol addicts and donors with ethanol for 4 h (Table 2). Ethanol caused virtually no changes in lymphocytes, while the percent of neutrophils undergoing apoptosis under the effect of ethanol reached 2.06±0.40% in the specimens from alcoholics, which was significantly higher than in the controls (0.28±0.18%). It is known that neutrophils more intensely produce active oxygen metabolites than other cells. Ethanol, an inductor of free radical oxidation, affects neutrophils sensitive to it and triggers the mitochondrial pathway of apoptosis.

The mechanisms of apoptosis disorders in the peripheral blood immunocompetent cells in alcoholism can be explained by modification of the metabolic processes at the molecular biochemical level through stimulation of LPO processes and by direct effects of ethanol on cells [14]. *In vitro* incubation of cells with ethanol showed its effect on the neutrophils, but no lymphocyte death was observed. It can be hypothesized that under conditions of chronic alcoholization, the cells are exposed to the toxic effects of not only ethanol, but also its active metabolites, *e.g.* acetaldehyde.

In addition, imbalance of the pro- to antiapoptotic regulators can also stimulate apoptosis of peripheral blood cell in alcohol addicts. For example, ethanol causes an increase in the content of proapoptosic factors TNF-α, Fas ligands [10,12].

Hence, our study revealed an increase in CD95 receptor expression on lymphocytes of alcohol addicts. The levels of lymphocytes and neutrophils with morphological signs of apoptosis in alcoholics are significantly higher than in healthy individuals. *In vitro* tests showed similar reactions of peripheral blood neutrophils and lymphocytes of donors and alcoholics to hyperthermia and prednisolone. Incubation with ethanol did not lead to appreciable changes in the composition of apoptotic lymphocytes, but significantly stimulated the programmed death of neutrophils.

These results are in good agreement with the data of other authors on apoptosis disorders in various cells

TABLE 2. Effects of Hyperthermia, Prednisolone, and Ethanol on Leukocyte Apoptosis in Alcohol Addicts and Donors *In Vitro* (*M*±*m*)

Experiment conditions	Percentage of neutrophils with signs of apoptosis		Percentage of lymphocytes with fragmented nuclei	
	donors	alcoholics	donors	alcoholics
No incubation	0.42±0.14	1.14±0.36*	0.90±0.18	5.07±0.63**
Incubation at 20°C	0.48±0.26	0.87±0.38	2.50±0.56^{o}	6.32±1.17
Incubation at 20°C+prednisolone	0.66±0.36	0.82±0.36	3.96±0.87	9.21±1.70*
Incubation at 40°C	8.31±2.73^{x}	17.32±3.93^{x}*	5.13±0.68^{x}	7.33±2.24
Incubation at 40°C+prednisolone	2.19±1.19^{+}	2.09±0.50^{+}	4.76±1.05	6.39±1.32
Incubation at 20°C+ethanol	0.28±0.18	2.06±0.40*	3.43±0.89	2.19±0.79

Note. *$p<0.05$, **$p<0.001$ compared to donors; $p<0.05$ compared to: ^{x}incubation under the same conditions at 20°C, $^{+}$incubation under the same conditions at 40°C, ^{o}spontaneous apoptosis (without incubation).

in alcoholism [11,14]. This pattern of reaction with more intense apoptosis processes for immunocompetent cells explains low resistance of the immune system of alcoholics to infections and a high risk of tumors, viral and autoimmune diseases for this population, and is presumably one of the mechanisms of immunodeficiency developing in patients with addictions. The apoptotic mechanism of immunosuppression should be taken into consideration in the pathogenesis of addictive disorders. However, it remains unclear whether intensification of programmed cell death is characteristic of just some cell subpopulations or of all immunocompetent cells.

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