Cell Cycle Arrest in the Thymus and Spleen in Male Mice under Conditions of Chronic Social Defeat Stress: Effects of Diazepam

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 151, No. 4, pp. 391-394, April, 2011
Original article submitted March 1, 2010

The effects of chronic social defeat stress on the percentage of cells in different phases of the cell cycle and in apoptosis in the thymus and spleen of male mice were studied by the method of flow cytofluorometry. In stressed males, thymus weight decreased, the percent of proliferating thymocytes was significantly lower, and the percentage of G0-G1 cells was higher than in intact males. Stress substantially reduced the percentage of splenocytes in the G0-G1 phase and apoptotic cells, but the percentage of S and G2-M cells and proliferation index significantly increased. Chronic administration of anxiolytic diazepam prevented the majority of the changes in the percentage of cells in different phases of the cell cycle, but apoptosis in the thymus increased under these conditions. Possible association between cell cycle disorders, impairment of cell immunity, and chronic anxiety developing under conditions of long-term social defeat stress is considered.

Key Words: *social defeat stress; anxiety; diazepam; cell cycle; apoptosis; proliferation*

Chronic social defeat stress (CSDS) caused by repeated experience of social defeats in daily male-male confrontations leads to the development of high anxiety in male mice [13]. Many parameters of cellular and humoral immunity were also changed in these animals, which attests to the development of immunodeficient states: decreased immune response to sheep erythrocytes [8], changed lymphocyte subpopulation composition in immune organs, reduced cell count in the thymus and spleen [5], impaired general resistance [4,9], and enhanced metastasizing of Lewis lung carcinoma [2,14]. Anxiolytic diazepam is widely used in clinical practice [3]. It effectively relieves anxiety in animals and humans, prevents changes in the subpopulation composition of lymphocytes in the thymus and spleen [6], and reduces the rate of experimental metastasizing of transplantable tumors [11] in male mice.

The aim of the study was to verify the assumption that CSDS can affect apoptosis and proliferation of cells in immunocompetent organs and to examine the effects of diazepam on this process.

MATERIALS AND METHODS

Experiments were performed on 2.5-3-month-old mature male C57Bl/6J mice weighing 26-28 g; the mice were bred under standard vivarium conditions (Institute of Cytology and Genetics). The animals were maintained at 12:12 h light regimen with food (pellets) and water *ad libitum*.

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0007-4888/11/1514-0411

The consequences of CSDS were studied in male mice with repeated experience of social defeats in daily intermale confrontations (20 days) using sensory contact model [12]. Intact animals placed in individual cages for 5 days (to prevent the effect of group interactions) served as controls (group 1). Group 2 mice intravenously received saline and group 3 mice received diazepam (0.5 mg/kg, Polfa Tarchomin SA) starting from day 7 of agonistic interactions and throughout 2 weeks.

Analysis of the number of dividing and apoptotic cells in the studied organs was performed by flow cytofluorometry with DNA-specific dye propidium iodide [10] on a FACSCalibur Cytofluorometer (Becton Dickinson, filter 585/42). The method allows identifying cells in different phases of the cell cycle: resting (G0-G1), synthetic (S), post-synthetic and mitotic (G2-M) phases and apoptotic cells (A0). The data were presented as the percent of cells from their total number in the sample. The total percentage of proliferating cells (S+[G2-M]) and proliferation index (S+[G2-M])/G0-G1) were calculated.

Statistical data processing was performed using two-way ANOVA to identify the influence of factors "organ" (thymus, spleen) and "effect of stress" (intact and stressed males) and their interactions and the influence of factors "organ" and "effect of drug" (saline and diazepam) and their interactions in stressed animals. For paired comparisons of data between the experimental groups Student's *t* test was used ($p \leq 0.05$).

RESULTS

ANOVA revealed significant interaction between factors "organ" and "effect of stress" (Table 1) and between factors "organ" and "effect of drug" in animals after CSDS for all studied parameters. This indicates differences in apoptosis and proliferation processes in the thymus and spleen and differences in the effects of CSDS and diazepam on these processes in each organ.

In the thymus and spleen of group 1 animals, the percents of cells in different phases of the cell cycle and apoptotic cells were different (Table 2). In the spleen, the percent of resting cells (G0-G1) was significantly higher ($p<0.0003$) and percent of dividing cells (S and G2-M) was considerably lower (both $p<0.0002$) than in the thymus. Index of proliferation in the spleen was significantly lower ($p<0.0001$) than in the thymus. In group 1 animals, the percent of splenocytes in the state of apoptosis in the spleen was substantially higher than in the thymus ($p<0.0021$).

CSDS-induced changes in the percentage of cells attested to disturbances in proliferation and apoptosis processes in immune organs. As in our previous experiments [5], CSDS reduced weight index of the thymus ($p<0.01$), which suggests the development of accidental involution of the organ (*e.g.* atrophy), a typical manifestation of stress. CSDS also reduced the percentage of G2-M thymocytes ($p<0.042$), the total percentage of S and G2-M thymocytes ($p \leq 0.050$), and proliferation index ($p \leq 0.050$); the percentage of G0-G1 cells increased ($p<0.049$). Similar changes were found in animals with iron deficiency [15]. In both studies, the decrease in thymus weight was associated with impairment of cellular immunity and inhibition of cell proliferation. Our findings support the hypothesis that the decrease in thymus weight is determined by reduced cell proliferation. This suggests that this phenomenon can be related to the antiproliferative effect of glucocorticoids. Taking into account the fact that CSDS reduces the percent of G2-M cells, one can assume possible dysregulation in the control point of G2-phase (preparation for mitosis).

The weight index of the spleen remained within normal limits (Table 2), but the percentage of all phases of the cell cycle changed. In contrast to the thymus, the percent of G0-G1 splenocytes decreased ($p<0.013$) and the percent of S- and G2-M-phase splenocytes ($p<0.005$ and $p<0.011$, respectively) and proliferation index ($p<0.008$) increased under the

TABLE 1. Two-Factor Analysis of Interactions between Factors "Organ" and "Effect of Stress" and the Factors "Organ" and "Effect of Drug"

Parameter	Interaction of "organ" and "effect of stress"	Interaction of "organ" and "effect of drug"
A0, %	$F(1,31)=6.40$; $p<0.017$	$F(1,31)=7.60$; $p<0.010$
G0-G1, %	$F(1,31)=11.83$; $p<0.002$	$F(1,31)=5.72$; $p<0.023$
S, %	$F(1,31)=14.09$; $p<0.001$	$F(1,31)=10.68$; $p<0.003$
G2-M, %	$F(1,31)=12.19$; $p<0.001$	$F(1,31)=9.06$; $p<0.005$
(S+[G2-M]), %	$F(1,31)=15.10$; $p<0.001$	$F(1,31)=11.07$; $p<0.002$
(S+[G2-M])/G0-G1	$F(1,31)=13.61$; $p<0.001$	$F(1,31)=9.84$; $p<0.004$

TABLE 2. Impact of CSDS on the Percentage of Cells in Different Phases of the Cell Cycle and Apoptotic Cells in the Thymus and Spleen (*M*±*m*)

Parameter	Group 1	Group 2	Group 3
Thymus			
Index mg/g body weight	1.54±0.11	0.86±0.07**	1.24±0.06^{++}
A0, %	0.07±0.02	0.07±0.01	0.34±0.11*$^{++}$
G0-G1, %	87.08±0.81	89.31±0.62*	88.38±0.75
S, %	9.10±0.83	7.56±0.53	8.12±0.60
G2-M, %	3.75±0.20	3.07±0.19*	3.16±0.24
(S+[G2-M]), %	12.85±0.80	10.62±0.62*	11.28±0.81
(S+[G2-M])/G0-G1	0.15±0.01	0.12±0.01*	0.13±0.01
Spleen			
Index mg/g body weight	3.86±0.21	4.94±0.73	4.38±0.5
A0, %	1.71±0.40^{xx}	0.74±0.20*	3.30±1.12^{++}
G0-G1, %	94.76±1.20^{xx}	87.26±1.85**	93.09±1.23^{+}
S, %	2.69±0.74^{xxx}	8.45±1.20**	2.91±0.51^{++}
G2-M, %	0.84±0.15^{xx}	3.55±0.68**	0.70±0.14^{++}
(S+[G2-M]), %	3.53±0.86^{xxx}	12.00±1.84**	3.61±0.57^{++}
(S+[G2-M])/G0-G1	0.04±0.01^{xxx}	0.14±0.02**	0.04±0.01^{++}

Note. $^{x}p<0.05$, $^{xx}p<0.1$, $^{xxx}p<0.001$ compared to the corresponding parameter in the thymus; *$p<0.05$, **$p<0.01$ compared to group 1; $^{+}p<0.05$; $^{++}p<0.01$ compared to group 2.

influence of CSDS. We can assume intensification of the process of cell division, which may indicate partial or complete release of cell cycle block at the stages of DNA synthesis and/or preparation for mitosis. The percentage of apoptotic cells in stressed animals decreased ($p<0.027$). Since apoptosis is a genetically programmed defense mechanism triggering self-destruction of altered cells containing defective DNA [1], it can be assumed that apoptosis insufficiency caused by CSDS against the background of high levels of proliferating cells and reduced activity of cellular and humoral immunity [5] may be a decisive predisposition factor to cancer development. This hypothesis is supported by numerous reports on more intensive growth of Lewis adenocarcinoma and significantly higher number of metastases in the lungs in animals subjected to CSDS compared to controls [2,14]. Since apoptosis provides the basis of many important processes such as positive and negative selection of T- and B-lymphocytes, glucocorticoid-induced lymphocytes death, and cell death caused by the exposure to adverse environmental factors [1], changes in the percent of apoptotic cells in the state of apoptosis can be responsible for the formation of cellular immune deficiency in male mice under the influence of CSDS.

CSDS is accompanied by the development of negative mental and emotional state, the main component of which is high level of anxiety [13]. In similar studies, chronic administration of diazepam relieving anxiety [3] reduced high metastatic activity in mice [10]. In this experiment, diazepam administered chronically under stress conditions prevented virtually all changes in the percentage of cells at different phases of the cell cycle (Table 2); in the thymus, the percent of G0-G1, S, G2-M phase cells and proliferation index did not differ from those in intact mice. The exception was the percent of apoptotic cells, which exceeded the corresponding parameter in stressed ($p<0.004$) and intact ($p<0.038$) mice. In the spleen, the percent of G0-G1 phase splenocytes ($p<0.046$) and A0 splenocytes ($p<0.009$) in animals receiving diazepam was higher than in the group of stressed males receiving saline, while the percent of S phase and G2-M phase splenocytes ($p<0.005$ and $p<0.008$, respectively), the total percentage of proliferating cells (S+[G2-M], $p<0.005$), and proliferation index ($p<0.008$) were lower than in stressed males receiving saline. In this case, all the parameters did not differ from those in the control animals. These results are consistent with the data on the effects of diazepam on proliferation of lymphocytes *in vitro* [7].

Since chronic administration of diazepam has a pronounced anxiolytic effect [3] and inhibits the effects of CSDS on parameters of cellular immunity [6], we can conclude that expressed anxiety developing under these conditions is the pathogenic factor forming immunodeficient state with changes in subpopulation composition of lymphocytes and cell cycle disturbances in immunocompetent organs. However, the study revealed a possible negative effect of diazepam: we observed increased apoptosis in the thymus, which, in turn, can lead to the loss of immune cells. The question to be solved is whether these processes are interrelated, or the effects of diazepam on the parameters of immunity, cell cycle, and psychoemotional status have different mechanisms, as well as what are the molecular mechanisms of the effects of stress on the processes of cell cycle. However, it is obvious that it is necessary to control the negative psycho-emotional consequences of CSDS for preventing immunosuppression and cancer development.

We are grateful to L. V. Omelyanchuk for productive discussion of our results while working on this article. The study was conducted with the support of Basic Sciences to Medicine Foundation (grant No. 21.28) and Molecular and Cellular Biology Program of the Russian Academy of Sciences (grant No. 22.16).

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