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Immunohistochemical Study for the Expression of Bcl-2 Family Proteins in Walker 256 Carcinosarcoma Cells under the Influence of Cytostatic Drugs

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Immunohistochemical study was performed to evaluate the expression of Bcl-2 family proteins (Bcl-2, Bax, and Bad) in Walker 256 carcinosarcoma cells after implantation into the thigh muscle of male Wistar rats (10^6 cells). The experiment was conducted under conditions of spontaneous tumor development and individual or combined treatment with melatonin and cyclophosphamide. The use of melatonin as monotherapy or in combination with cyclophosphamide was followed by a significant decrease in Bcl-2 expression in carcinosarcoma cells. The Bcl-2/Bax and Bcl-2/Bad ratio was significantly reduced under these conditions (particularly after combined treatment with cytostatic drugs). These changes were accompanied by a significant (by 93.61%) decrease in the volume of transplantable tumor on day 14. Daily treatment with melatonin was accompanied by significant changes in the structure of Walker 256 carcinosarcoma. It was manifested in the formation of connective tissue septa and pseudofollicles.

Key Words: *Walker 256 carcinosarcoma; apoptosis; proteins of the Bcl-2 family; melatonin; cyclophosphamide*

One of the mechanisms for tumor growth is an imbalance between proliferation, differentiation, and apoptosis of cells [1]. The expression and functional activity of Bcl-2 family proteins play a key role in dysregulation of apoptosis, which results in the progression of tumor diseases. These proteins are involved in the intrinsic (mitochondrial) pathway of apoptosis [9]. Proteins of the Bcl-2 family are classified into proapoptotic (Bax and Bad) and antiapoptotic proteins (Bcl-2, and Bcl-X_L). The biological role of these proteins is poorly understood. Published data show that Bcl-2, Bcl-X_L, and Bax play a role in the formation of ion-conducting channels in the lipid bilayer of mitochondria. Under these conditions, they appear as dimers or oligomers [13]. Some disorders (*e.g.*, oxidative stress) cause a significant decrease and/or loss of the mitochondrial membrane potential. These changes may be irreversible and result in the release of cytochrome C from the mitochondrial space to the cytosol, binding to apoptosis-activating factor-1 (Apaf-1), and

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complex formation with caspase-9 [12]. This enzyme triggers a cascade of proteolytic reactions and activates other caspases. Bcl-2 and Bax can form heterodimers and neutralize the effects of each other via direct interaction. However, both proteins probably have an independent modulatory effect on mitochondrial function and progression of apoptosis [5].

Recent studies showed that proteins of the Bcl-2 family are involved in the realization of other mechanisms for apoptotic death of cells. These mechanisms do not depend on the release of mitochondrial cytochrome C [15]. The cytoprotective effect of Bcl-2 can be also related to an increase in survivin expression and inhibition of p53 and p38MAPK accumulation [11]. Bcl-2 overexpression is associated with an increase in malignant transformation and metastatic dissemination of some tumors due to the induction of gene expression for matrix metalloproteinase-2 [7].

There is no agreement regarding the role of Bcl-2 family proteins in the realization of apoptosis and cytoprotection. Hence, much attention is paid to studying the involvement of Bcl-2 family proteins in tumor development, tumor growth, and antitumor effect of cytostatic drugs. To evaluate the antitumor effect of cytostatic drugs and other methods of antitumor treatment, it is important to develop new prognostic criteria for the efficacy of therapy. The dynamics of variations in Bcl-2 family proteins should be compared with the type and degree of morphological changes in tumor cells (*i.e.*, progressive or regressive tumor growth).

The present immunohistochemical study was performed to evaluate the expression of Bcl-2 family proteins in rats after transplantation of Walker 256 carcinosarcoma. The experiment was conducted under conditions of spontaneous tumor development and individual or combined treatment with melatonin and cyclophosphamide.

MATERIALS AND METHODS

Experiments were performed on 28 male Wistar rats weighing 180-200 g. The study was conducted in accordance with the requirements of the European Community (86/609/EEC) and Helsinki declaration. The strain of transplantable tumor Walker 256 was maintained *in vivo* at the Laboratory of Physiological Genetics (Institute of Cytology and Genetics, Siberian Division of the Russian Academy of Sciences). The cell suspension of Walker 256 carcinosarcoma was injected into the thigh muscle (10^6 cells in 0.1 ml isotonic solution of NaCl) [3,10]. Five days after tumor transplantation (tumor volume 2.51±0.42 cm^3), the animals were divided into the following 4 groups: control group 1 (n=7), spontaneous tumor development; group 2 (n=7), intraperitoneal injection of cyclophosphamide (CP, Biokhimik) in a single dose of 25 mg/kg in 0.1 ml isotonic solution of NaCl; group 3 (n=7), intraperitoneal injection of melatonin (MT, ICN Biomedicals Inc.) in a dose of 0.3 mg/kg for 14 days; and group 4 (n=4), combined treatment with MT and CP.

For immunohistochemical study, tumor tissues were sampled 5 days after tumor transplantation (group 1), 7 and 14 days after the start of therapy (groups 2 and 3), or 14 days after the start of therapy (group 4). Macroscopic analysis was performed to evaluate tumor volume. The size of tumor was measured in three perpendicular planes with a trammel. Carcinosarcoma volume was estimated during tissue sampling and 14 days after tumor transplantation (group 1).

Immunohistochemical study of the expression of Bcl-2 family proteins was performed by the indirect streptavidin-avidin method on paraffin sections [4]. Mouse monoclonal antibodies (dilution 1:100, BD Biosciences) were used as primary antibodies for the detection of Bcl-2 and Bad. Bax was identified using rabbit polyclonal antibodies to the domain, which plays an important role in the formation of homodimers and heterodimers with antiapoptotic proteins of the Bcl-2 family (BD Biosciences). The sections were additionally stained with hematoxylin and eosin. The specific binding of antibodies to antigens was confirmed by disperse fine-granular staining of the cell cytoplasm (pale yellow to dark brown color). Stronger staining was considered to correspond to a greater amount of cell antigens.

The expression of antigens in tumor cells was estimated from the intensity of immunohistochemical staining. Micrographs were obtained using a M200 motorized microscope (Carl Zeiss) and AxioCam HRc camera (Carl Zeiss) at a final magnification of 630. The intensity of staining was evaluated on an automatic measurement device (Auto measure, Sigma) with Axio Vision 4.7.1 software. The program was prepared with a Segmentation filter mask (Sigma). The percentage area of positively stained tumor cells (test area 39,437 μ^2) was introduced into the program of calculation. Forty images were analyzed in each group. The quantitative data were analyzed statistically [2]. The significance of differences between mean values was evaluated by Student's t test.

RESULTS

Chemotherapy for local tumors allows us to evaluate tumor chemosensitivity from changes in its size. The decrease in tumor weight or volume by at least 50% is designated as partial remission [8]. The volume of transplantable tumor (Walker 256 carcinosarcoma) in animals was significantly reduced on day 14 of treatment with CP (by 72.8%, p<0.05) or combined ad-

ministration of MT and CP (by 93.61%; Table 1). The volume of transplantable Walker 256 carcinosarcoma tended to decrease by the end of treatment with MT (as compared to the control group).

Microscopy of Walker 256 carcinosarcoma in rats was performed on day 5 after transplantation into the thigh muscle. The major types of cells were presented by large "light" cells with a large nucleus and large nucleolus and small cells with a basophilic nucleus. Nuclear polymorphism was particularly pronounced in the population of "light" cells. Mitoses were often found. The use of antibodies to antiapoptotic protein Bcl-2 allowed us to identify brown and dark-brown granules that were regularly distributed in the cytoplasm. The cells were heterogeneous by the content of immunohistochemical products. Some cells contained single granules, while others consisted of numerous and densely packed granules (Fig. 1, *a*).

Immunohistochemical study of the expression of proapoptotic protein Bax showed that this protein was regularly distributed in the cytoplasm of carcinosarcoma cells. The cells were heterogeneous by protein content (Fig. 1, *b*). It should be emphasized that treatment with anti-Bax antibodies allowed us to identify the immune-specific "contouring" of microvessels and some tumor cells. These results reflect differences in the intracellular localization of Bax. Immunohistochemical reaction products were found during the identification of Bad and Bcl-2. These products appeared as granules regularly distributed in the cell cytoplasm.

Quantitative analysis was performed to evaluate the intensity of immunohistochemical reactions on day 5 after tumor transplantation into the thigh muscle. Bad expression in spontaneous carcinosarcoma was higher than the expression of Bcl-2 and Bax (by 1.6 and 1.7 times, respectively; Table 1). No differences were found in the expression of Bcl-2 and Bax. It was difficult to perform quantitative analysis for the expression of Bcl-2 family proteins in carcinosarcoma cells on day 14 of spontaneous tumor development due to severe tumor lysis and presence of cell detritus.

The Bcl-2/Bax ratio reflects proliferative activity of cells, cell survival, and possibility for the induction of apoptosis. According to modern notions, the Bcl-2/Bax ratio is a key factor of apoptosis regulation. A dynamic equilibrium exists in the Bcl-2/Bax ratio. These proteins form homodimers and heterodimers, which results in the induction of cell apoptosis [6]. Low the Bcl-2/Bax ratio probably contributes to the induction of apoptosis. By contrast, high Bcl-2/Bax ratio correlates with cell resistance to apoptotic factors [14]. The Bcl-2/Bax and Bcl-2/Bad ratios on day 5 of spontaneous development of Walker 256 carcinosarcoma were 1.04 and 0.61, respectively.

Previous studies showed that high concentration of Bcl-2 in Walker 256 carcinosarcoma cells cam protect them from the damaging effect of Ca^{2+} [15]. H_2O_2 was used to induce oxidative stress in carcinosarcoma cells with high value of the Bcl-2/Bax ratio. Apoptosis was realized through activation of calcineurin and did not depend on variations in the mitochondrial membrane potential or release of cytochrome C [15]. A decrease in the mitochondrial membrane potential and release of cytochrome C were revealed in cells with low value of the Bcl-2/Bax ratio (tumor cells SCC-25). Activation of caspase-3 was observed under both conditions. These data indicate that high Bcl-2/Bax ratio contributes to the prevention of mitochondrial damage. However, another mechanism of apoptosis cannot be excluded.

TABLE 1. Volume of Transplantable Walker 256 Carcinosarcoma and Immunohistochemical Staining of Bcl-2 Family Proteins in Wistar Rats after Individual or Combined Treatment with MT and CP (*M±m*)

Group, period, days		Carcinosarcoma volume, cm^3	Relative area of immunohistochemical staining, %		
			Bcl-2	Bax	Bad
1 (spontaneous development)	5th	2.51±0.42	19.86±1.52	19.13±2.05	32.59±1.81
	14th	37.67±3.48	—	—	—
2 (CP)	7th	12.25±1.14*	18.92±1.73	28.56±2.73	21.62±0.99*
	14th	10.25±1.52*	23.07±1.76	25.54±1.71	16.12±1.16*
3 (MT)	7th	14.53±1.52*	5.93±1.49*	36.09±2.56*	35.00±2.57
	14th	26.43±4.12	5.04±0.54*	12.97±1.41	9.28±1.47*
4 (MT+CP)	14th	2.41±1.54*	3.03±0.34*	25.45±0.73	24.91±3.17

Note. *$p<0.05$ compared to the control (group 1).

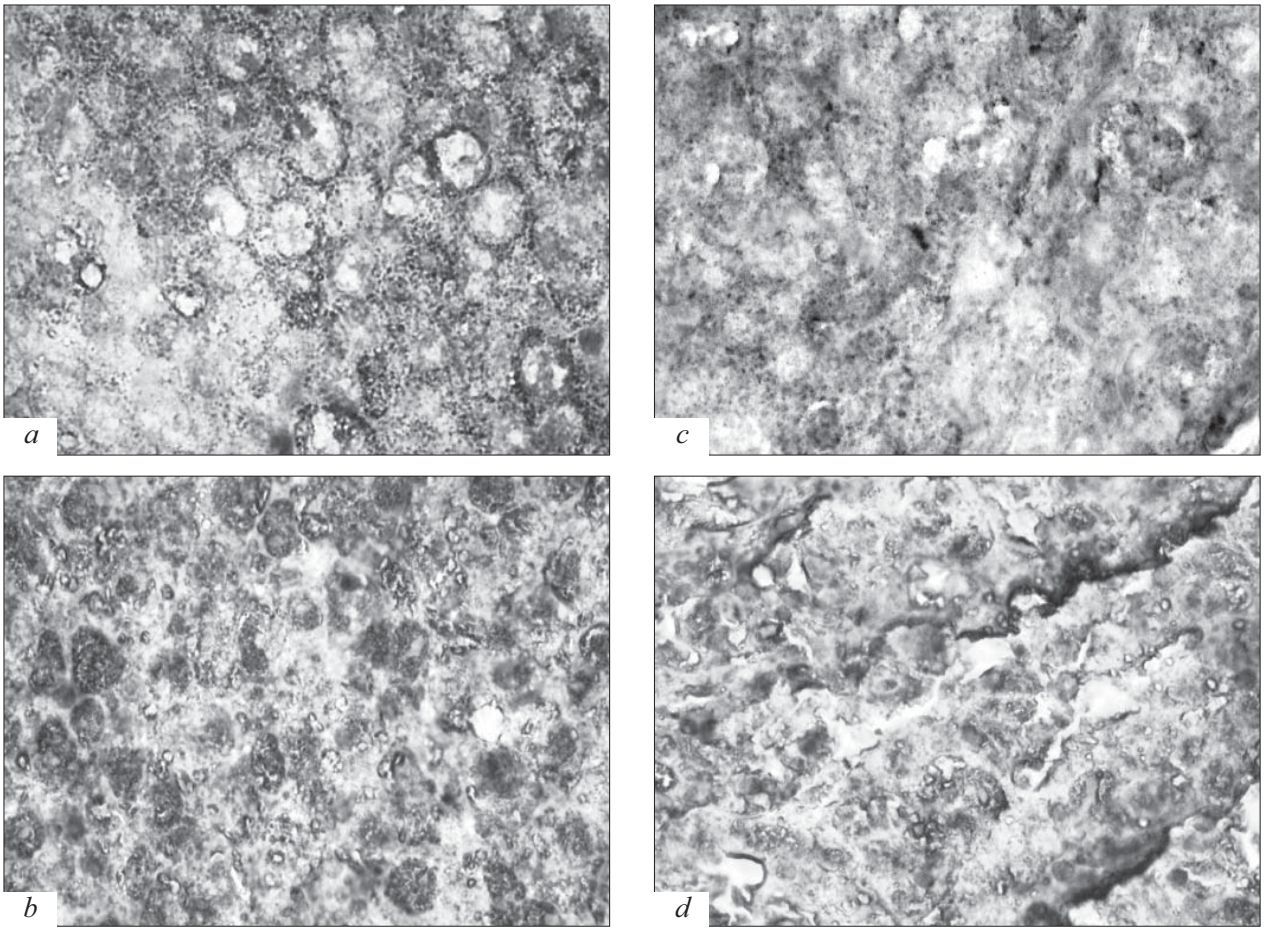

Fig. 1. Immunohistochemical detection of Bcl-2 family proteins in Walker 256 carcinosarcoma cells after treatment with CP (×1000). High content of Bcl-2 protein in carcinosarcoma cells on day 5 after transplantation into the thigh muscle (*a*); distribution of Bcl-2 protein in carcinosarcoma on day 5 after transplantation (*b*); mitotic division of carcinosarcoma cells and diffuse distribution of Bcl-2 on day 14 after treatment with CP (*c*); increase in Bax content in carcinosarcoma cells on day 14 after treatment with CP (*d*).

A considerable number of small cells with basophilic nuclei were found in carcinosarcoma on days 7 and 14 after single injection of CP. The peripheral zone of tumor was infiltrated with leukocytes. Inflammatory cell infiltration sometimes spread to the surrounding muscle tissue. Single muscle fibers were preserved in some samples of tumor tissue.

Single injection of CP had little effect on the expression of antiapoptotic protein Bcl-2 at various stages of study. However, the intensity of staining in sections was slightly reduced after 7 days (Fig. 1, *c*). The expression of proapoptotic protein Bax was elevated on days 7 and 14 (by 49 and 34%, respectively; Fig. 1, *d*). The expression of proapoptotic protein Bad in treated animals was much lower compared to the control. These differences were particularly pronounced on the 14th day (by 1.51 and 2.02 times, respectively, Table 1). On day 7, the expression of Bax and Bad was higher than that of Bcl-2 (by 51 and 14%, respectively). No differences in the expression of Bcl-2 and Bax were found after 14 days. However, Bad expression was 30% lower than Bcl-2 expression. The Bcl-2/Bax ratio decreased to 0.66 on day 7, but increased to 0.90 after 14 days (similarly to the control). The Bcl-2/Bad ratio on days 7 and 14 was 0.88 and 1.43, respectively. These data suggest that apoptotic death of carcinosarcoma cells occurs at the initial stage of tumor development. Necrosis is the major mechanism of cell death in the follow-up period.

Daily treatment with MT was followed by the most significant changes in structural characteristics of Walker 256 carcinosarcoma. On day 14 the tumor was divided into pseudofollicular structures by well-developed and thickened connective tissue septa (Fig. 2, *a*). Bcl-2 expression was significantly reduced on days 7 and 14 (by 3.35 and 3.94 times, respectively, compared to the control; Table 1). We revealed a decrease in the number of positively stained cells and intensity of cell staining (Fig. 2, *b*).

The expression of proapoptotic proteins Bax and Bad was elevated on day 7. The increase in Bax ex-

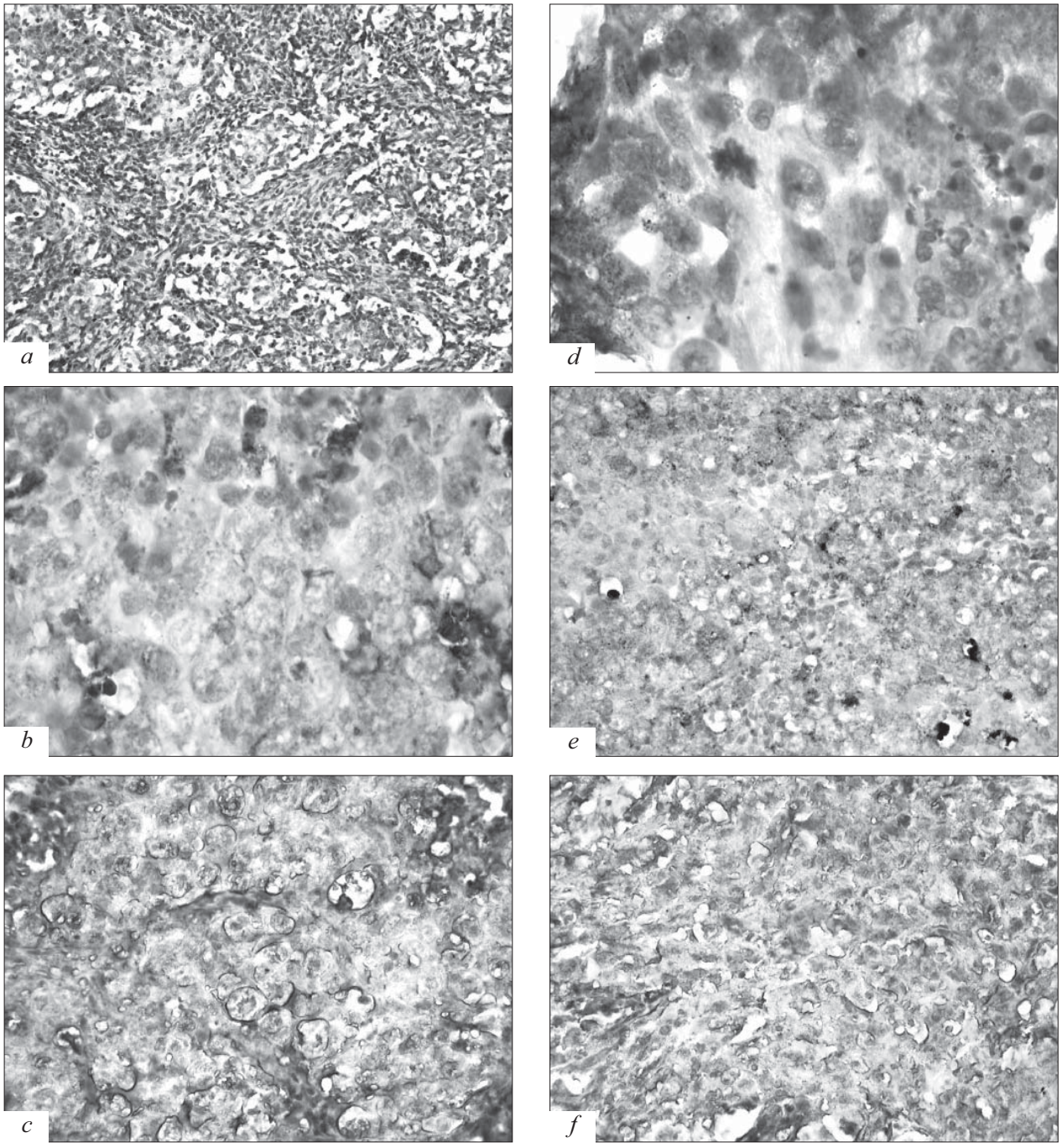

Fig. 2. Immunohistochemical detection of Bcl-2 family proteins in Walker 256 carcinosarcoma cells on day 14 after treatment with MT (*a-d*) and CP (*e*, *f*). Formation of thickened connective tissue septa and pseudofollicular structure of carcinosarcoma (×200, *a*); low content of Bcl-2 in carcinosarcoma cells and high content of Bcl-2 in blood cells (×1000, *b*); distribution of Bcl-2 in carcinosarcoma (×400, *c*); low content of Bad in mitotically active cells of carcinosarcoma (×1000, *d*); immunophenotypic heterogeneity of carcinosarcoma cells by Bcl-2 protein (×400, *e*); distribution of Bax in carcinosarcoma (×400, *f*).

pression was particularly pronounced in this period (by 89% compared to the control). The expression of proapoptotic proteins Bax and Bad was reduced on day 14 (by 32 and 72%, respectively, compared to the control; and by 2.78 and 3.77 times, respectively, compared to day 7; Fig. 2, *c*, *d*). The Bcl-2/Bax ratio decreased significantly on day 7 (0.16), but increased after 14 days (0.39). However, Bcl-2/Bax ratio in these animals was much lower than in specimens with spontaneous tumor development. Bcl-2/Bad ratio also decreased on day 7 (0.17), but increased after 14 days. It should be emphasized that Bcl-2/Bad ratio under these conditions (0.54) was lower compared to the control.

Administration of MT alone or in combination with CP was followed by similar changes in the cytoarchitectonics of carcinosarcoma. Connective tissue septa and pseudofollicles were found in the tumor. The expression of Bcl-2 protein after combined treatment with CP and MT was 6.6-fold lower than in the control. Tumor cells were strongly heterogeneous by Bcl-2 content (Fig. 2, *e*). However, the expression of proapoptotic proteins Bax and Bad did not differ from the control (spontaneous development of Walker 256 carcinosarcoma). As differentiated from Bcl-2, Bax and Bad were regularly distributed in tumor tissue (Fig. 2, *f*). Bcl-2/Bax and Bcl-2/Bad ratios were lowest on day 14 (0.12).

We conclude that monotherapy or combined antitumor therapy with MT is accompanied by a significant decrease in the expression of Bcl-2. The significant decrease in the Bcl-2/Bax and Bcl-2/Bad ratios under these conditions was accompanied by reduction of tumor volume and structural changes. These data suggest that MT not only has a modulatory effect on apoptotic death of carcinosarcoma cells, but also serves as an inductor of morphogenetic processes and contributes to the changes in tumor cytoarchitectonics (formation of pseudofollicular structures). The efficacy of therapy was highest after combined administration of drugs with various mechanisms of action.

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