Proliferation of Walker 256 Carcinosarcoma Cells: Effect of Whole-Body Hyperthermia and Antitumor Agents

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We studied the main biological characteristics of spontaneous growth of Walker 256 carcinosarcoma and under conditions of antitumor therapy. A combination of whole-body hyperthermia and cytostatic treatment (cyclophosphamide and melatonin) produced maximum suppression of the tumor growth, inhibition of mitotic activity of tumor cells, and stimulation of their necrotic and apoptotic death. The maximum decrease in mitotic activity of tumor cells was observed after combined exposure to whole-body hyperthermia and both cytostatic preparations; enhancement of apoptotic cell death and the decrease in the tumor node weight were also most pronounced under these conditions and practically no body weight loss was recorded in this case.

Key Words: *Walker 256 carcinosarcoma; tumor growth; whole-body hyperthermia; melatonin; cyclophosphamide*

Biological peculiarities of tumors are determined by the totality of vital characteristics, the most important of them are histogenesis of tumor cells, their proliferative activity as a factor of tumor tissue progression, differentiation potential, migration capacity (degree of metastasizing), mechanisms of tumor cell death, intensity and spread of cell death, and induction of neoangiogenesis in the tumor node. In modern clinical and experimental studies of the efficiency of antitumor therapy and development of new methods of therapy of malignant neoplasms, the main attention is focused on the effect of chemical agents and physical factors on stimulation of tumor cells death, suppression of their proliferation, metastatic activity, and neoangiogenesis [2,4]. The intensity of changes produced by different chemical and physical factors on the above-listed biological properties of tumors greatly varies. For choosing the most effective regimen of antitumor therapy, combinations of different factors are usually used and the search for new combinations of known and unknown agents with antitumor activity is now in progress.

Transplantable Walker 256 carcinosarcoma is often used in model experiments on rats. It was first identified by Prof. G Walker in 1928 as a spontaneous breast tumor in a pregnant albino rat (*Rattus norvegicus*) [10]. After transplantations over many years, several morphologically different substrains of this tumor were identified and classified as carcinoma, sarcoma, and mixed carcinosarcoma [10]. It can be hypothesized that Walker 256 carcinosarcoma initially appeared as a mixed tumor and consisted on SC giving rise to different cell types. The use of this tumor in preclinical studies of new methods of antitumor therapy allows evaluation of individual and combined effects of chemical and physical factors on different biological characteristics of tumor growth.

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Here we studied the main parameters of Walker 256 carcinosarcoma growth in Wistar rats after transplantation of tumor cells into thigh muscles and the effect of antitumor therapy with whole-body hyperthermia (WBH), cyclophosphamide (CP), melatonin (MT), and their combination on tumor growth.

MATERIALS AND METHODS

We used transplantable Walker 256 tumor strain maintained *in vivo* at the Laboratory of Physiological Genetics, Institute of Cytology and Genetics, Siberian Division of Sciences (Novosibirsk). Suspension of Walker 256 carcinosarcoma cells was injected into posterior thigh muscles in a dose of 10^6 cells in 0.1 ml isotonic NaCl. For macroscopic evaluation, the volume of the tumor (Fig. 1) was calculated from three perpendicular diameters measured with a caliper. Five days after tumor transplantation (when tumor volume attained 2.51±0.42 cm^3), the animals were divided into 12 groups for evaluation of the effect of high temperature and antitumor agents on mechanisms of cell death during tumor growth.

For modeling WBH, the animals were placed into hot water (45°C) up to their neck into a reservoir of a universal water thermostat BWT-U. This temperature is optimal for modeling WBH, because exposure to higher temperature leads to animal death. Heating was stopped after attaining rectal temperature of 43.5°C (heat shock).

Antitumor agents CP and MT were administered as monotherapy and in combination with WBH. CP (Biokhimik) was injected intraperitoneally in a single dose of 25 mg/kg in 0.1 ml isotonic NaCl on day 5 after tumor transplantation. MT (ICN Biomedicals Inc.) was injected intraperitoneally in a dose of 0.3 mg/kg for 14 days starting from day 5 after tumor transplantation. In case of combination with WBH, CP was injected 1 h before WBH due to some peculiarities of pharmacokinetics of its active metabolite; MT was injected according to the same scheme as without WBH. Tumor samples were taken on days 5, 12, and 19 after transplantation; in animals receiving complex treatment with WBH and antitumor drugs the tumor samples were obtained on days 3, 7, and 14 after the first exposure.

For light microscopy, the tumor samples were fixed in 10% neutral formalin, the sections were stained with hematoxylin and eosin. For preparing semithin sections, the samples were fixed in 4% paraformaldehyde and postfixed in OsO_4. The semithin sections were sliced on an LKB-8800 ultratome and stained with toluidine blue.

Morphometry was performed using an M200 microscope (Carl Zeiss) equipped with AxioCam HRc camera at a final magnification of ×630. The calculations were performed using Axio Vision Release 4.7.1" (Carl Zeiss) and a block of automatic measurements (Auto measure). Segmentation filter masks were used. At least 40 images were analyzed for each group; the area of each image was 39,437 μ². The volume density (Vv) of zones of tumor parenchyma, cells with necrotic changes, and degenerating tumor cells were evaluated. Mitotic index was calculated as the ratio of the number of tumor cells at different stages of mitotic division to the total number of tumor cells and expressed in ‰. Apoptotic index was calculated as the ratio of the number of tumor cells in the state of apoptosis (apoptotic bodies) to the total number of tumor cells and expressed in ‰. In both cases, at least 5000 cells were analyzed.

The results were processed statistically using Microsoft Excel software. The significance of differences between the means was evaluated using Student *t* test. The differences were significant at $p<0.05$.

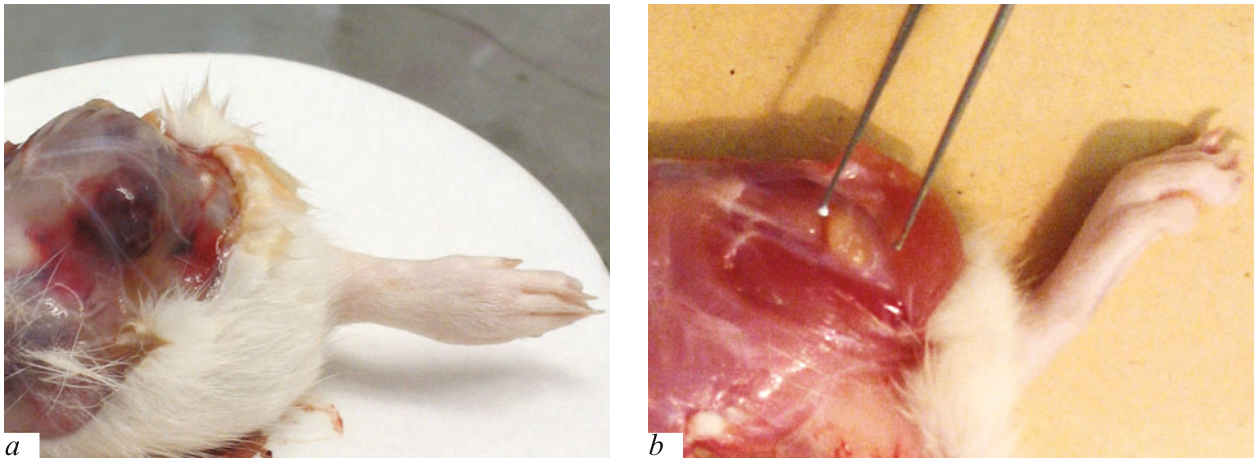

Fig. 1. Walker 256 carcinosarcoma after transplantation of tumor cells into the thigh muscles. *a*) 14 days after WBH; *b*) 14 days after WBH+CP+MT.

RESULTS

Histogenetically, Walker 256 carcinosarcoma is a mixed breast tumor consisting of sarcomatous (conventionally stromal) and epithelioid (conventionally parenchymatous) components and is characterized by infiltrative growth. Tumor cells form solid complexes, layers, and cords penetrating into muscular tissue

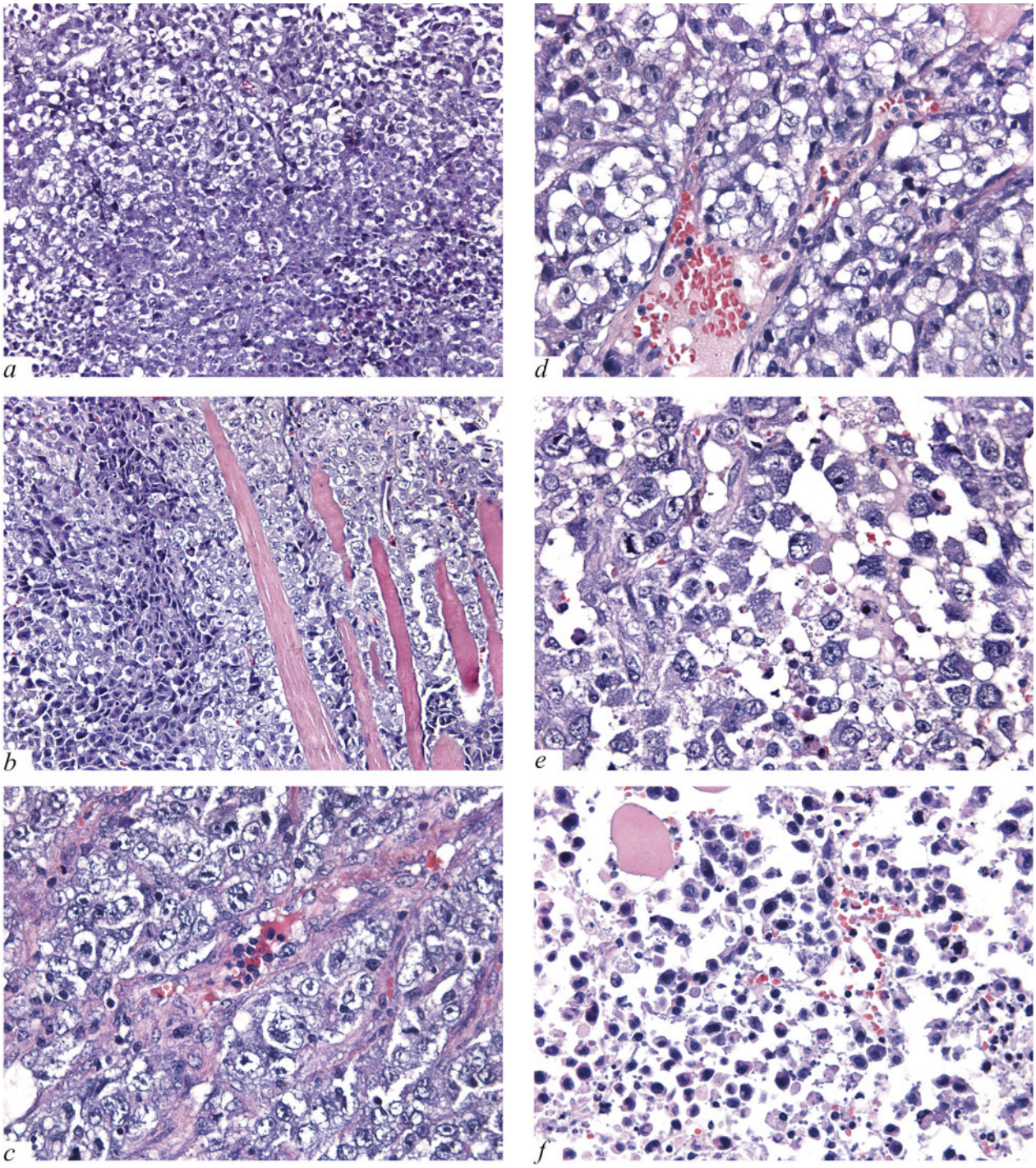

Fig. 2. Histogenesis of Walker 256 carcinosarcoma during spontaneous growth and after exposure to WBH and antitumor therapy. Hematoxylin and eosin staining. *a*) formation of pseudoglandular structures along the periphery of the tumor node 5 days after transplantation (×200); *b*) sarcomatous and epithelioid cords between preserved muscular fibers on day 12 after transplantation (×200); *c*) pronounced vasculogenesis and plethora in 3 days after WBH (×400); *d*) clusters of epithelioid cells between blood vessels in 3 days after WBH and MT treatment (×400); *e*) transition of epithelioid cells into ascetic state in 3 days after WBH+MT+CP (×400); *f*) changes in cell phenotype and their transition into ascetic state at the periphery of the tumor node in 3 days after MP administration (×400).

along the muscle fibers and blood vessels and disturbing architectonics and blood supply to the skeletal muscles. On day 5 after transplantation, the tumor was presented by a formed node (2.51±0.42 cm³). The stromal components were primarily presented by polymorphonuclear sarcomatous cells (type 1 cells; dark cells) of different shape and size with hyperchromatic nuclei. Epithelioid cells (type 2 cells; light cells) formed small nests (pseudoglandular structures) and solid layers along the periphery of the tumor node (Fig. 2, *a*). These formations usually had blurred boundaries, but somewhere clearly outlined epithelial structures separated from the sarcomatous component. In the center of the node, polymorphism of cells and nuclei was less pronounced and the tissue was less structured.

Epithelioid cells were characterized by moderate polymorphism, contained primarily hypochromatic nuclei, and often underwent mitotic division. Some epithelioid cells looked like signet ring cells with the nucleus displaced to the periphery. Epithelioid cells are predominant cell population of Walker 256 carcinosarcoma cells (their volume density on day 5 after transplantation attained 88.5±1.8%). The progressive growth of carcinosarcoma was characterized by very high mitotic index (34.8±1.4‰) and low apoptotic index (0.7±0.2‰; Table 1). Apart from apoptotic death, foci of tumor cell necrosis of different size were seen in the tumor nodes; they were primarily located in central zones and were infiltrated by neutrophils and monocytes. The formation of blood vessels between the cells was noted.

Further growth and aging of the tumor node (days 12 and 19 after tumor transplantation) were accompanied by enhanced structuring, which manifested in enlargement of cords consisting of sarcomatous (dark) cells and formation of pseudoglandular structures (nests of epithelioid cells between the cords, Fig. 2, *b*). Intensive neoangiogenesis and growth of blood vessel into the tumor also attests to activation of morphogenetic processes. Somewhere, lysis and dissolution of preserved muscle fibers under the action of proteolytic enzymes synthesized by tumor cells were observed. Foci of tumor cell necrosis were seen in the tumor tissue, the number of apoptotic cells increased (Table 1).

Comparative structural analysis of reorganization of the tumor tissue after WBH and antitumor treatment with the picture of natural pathomorphosis was performed on days 3, 7, and 14 after exposure, *i.e.* on days 5, 12, and 19 after tumor transplantation without treatment, respectively. The changes in the architectonics of the tumor observed on day 3 after WBH and antitumor treatment were similar. Large light cells were located along the node periphery and formed grape-like accumulations, while the central zone was occupied by cells with moderate polymorphism. Solitary somatic muscle fibers undergoing lytic changes were also seen at the node periphery. Foci of necrotic tumor cells were found in the central and peripheral zones. Dark cells were rare after all exposures; they formed small clusters or cords. Marked edema and plethora of the tumor tissue were revealed after WBH (Fig. 2, *c*), while after WBH in combination with CP and MT treatment these phenomena were less pronounced. Enhanced structuring and the formation of pseudoglandular structures lying between the blood capillaries was observed after MT treatment (Fig. 2, *d*). This picture was typical of all treatment schemes including MT. MT is included into antitumor protocols due to its antioxidant activity and the capacity to inhibit spontaneous and induced tumor growth [3,5,8]. More or less intensive transition of tumor cells to ascitic state was observed along the periphery of the tumor node (Fig. 2, *e, f*).

After 7 days, marked changes in the architectonics of Walker 256 carcinosarcoma were revealed in animals receiving CP alone or in combination with WBH and MT. Practically no light cells were found in the tumor node, small round or oval cells with hyperchromatic nuclei predominated (Fig. 3, *a*). Persisting light cells (often multinuclear) segregated and their cytoplasm formed processes; these morphological transformations reflect migration capacity of cells, *i.e.* their metastasizing potential (Fig. 3, *b*). Preserved muscle fibers underwent lytic changes (Fig. 3, *c*). In animals receiving MT, the tumor retained its pseudoglandular structure with well-developed vascularization (Fig. 3, *d*).

Structuring of the tumor node remained unchanged 14 day after all treatments: light cells forming clusters were located along the node periphery, the central zone was occupied by clusters of monomorphic cells and necrotic foci of different size. The tumor was often incapsulated, lymphatic vessels of the sinusoid type developing in the loose connective tissue capsule contained tumor cells. The structure of the tumor node in animals receiving CP recovered; light cells predominated again and were arranged in typical clusters (Fig. 3, *e*), dark cells were rare. In some cases, few tumor nodes were formed and their structure repeated the structure of the tumor node. Lytic changes in muscle fibers surrounding the tumor node were in progress. Tumor cells sometimes invaded the muscle fibers, gradually resorbed them, and filled their volume leaving unchanged only small subsarcolemmal muscular zones (Fig. 3, *f*).

Mitotic index of tumor cells decreased at all terms after treatment with MT and CP and their combinations with WBH; it was most pronounced on days 3 and 7 (Table 1). Monotherapy with CP and MT more markedly reduced mitotic index than WBH. The combina-

TABLE 1. Morphometric Analysis of Walker 256 Carcinosarcoma in the Dynamics of Tumor Growth and during WBH and Antitumor Therapy (*M*±*m*)

Group	Body weight, g	Tumor node weight, g	Volume density, %			Mitotic index, ‰	Apoptotic index, ‰
			tumor parenchyma	degenerative cells	necrotic cells		
Intact Wistar rats	302.1±2.9	-	-	-	-	-	-
3 days after treatment							
Walker 256 (day 5 after tumor transplantation), control	284.0±2.2^{+}	3.6±0.2	88.5±1.8	16.2±1.9	5.4±2.6	34.8±1.4	0.7±0.2
Walker 256+WBH	256.8±3.6^{+*}	15.5±1.2*	69.4±1.6*	30.8±3.5*	12.6±3.4	28.6±1.3**	2.8±0.5*
Walker 256+CP	265.3±2.9^{+*}	7.0±1.0*	72.3±3.8*	30.2±5.5	13.0±5.3	24.3±0.8**	4.4±0.3*
Walker 256+MT	273.7±2.3^{+*}	7.8±0.6**	79.6±2.4**	29.6±1.8**	9.7±3.2	22.5±1.8**	6.5±0.8**
Walker 256+WBH+CP	268.0±2.5^{+*}	7.0±0.5**	70.0±3.2*	40.0±1.4**	12.2±1.8	23.1±0.9**	7.2±0.4**
Walker 256+WBH+MT	263.8±5.7^{+*}	9.7±0.6**	72.4±2.7*	40.5±1.7**	14.2±4.4	20.9±1.7**	6.3±0.8**
Walker 256+WBH+CP+MT	268.9±3.3^{+*}	8.7±0.7**	68.7±3.7*	42.3±1.5**	14.9±2.4*	18.9±2.4*	8.2±0.6**
Walker 256+CP+MT	279.6±2.1^{+}	10.4±1.3**	70.3±2.8*	44.5±2.3**	16.4±1.9*	19.6±1.0**	7.5±0.3**
7 days after treatment							
Walker 256 (day 12 after tumor transplantation), control	258.6±4.4^{+}	15.4±0.9	86.4±2.3	18.8±1.6	5.9±1.8	30.0±1.2	1.2±0.5
Walker 256+WBH	238.5±2.2^{+*}	40.0±1.4**	66.6±2.8**	41.5±3.4	15.5±2.3*	24.4±2.5	3.3±0.3*
Walker 256+CP	291.0±2.9*	8.8±0.3*	73.6±3.4*	38.5±1.8**	13.9±2.7	22.3±1.6*	6.0±0.5*
Walker 256+MT	274.7±2.4^{+*}	10.2±0.5*	76.8±2.7*	32.5±1.4*	11.4±3.3	19.9±0.8**	7.7±1.2*
Walker 256+WBH+CP	275.8±4.1^{+*}	3.4±0.2**	66.7±5.2*	43.1±1.9**	18.4±3.4*	19.2±1.7**	7.4±0.8*
Walker 256+WBH+MT	261.6±3.0^{+}	7.1±0.5**	64.3±3.3**	41.2±3.6**	16.9±1.9**	19.5±1.4**	9.7±0.4*
Walker 256+WBH+CP+MT	266.6±3.1^{+}	1.6±0.1**	62.3±2.7**	44.5±2.8**	17.6±2.2	17.8±0.8**	11.4±0.2**
Walker 256+CP+MT	290.0±1.3^{+*}	1.9±0.2**	64.4±2.5**	48.6±2.2**	15.8±1.8	16.4±1.2**	13.2±0.8**
14 days after treatment							
Walker 256 (day 19 after tumor transplantation), control	274.5±3.8^{+}	30.4±1.2	82.4±1.9	20.2±5.5	7.0±2.4	29.5±1.3	2.5±1.2
Walker 256+WBH	209.5±3.5^{+*}	49.5±3.5*	68.3±2.2**	26.8±4.3	15.2±3.5	30.5±0.8	4.2±1.7
Walker 256+CP	299.0±3.4*	7.8±0.9**	75.8±2.8	32.2±3.5*	16.2±2.2*	24.8±1.7*	4.8±0.8
Walker 256+MT	269.0±3.6^{+}	21.3±1.3*	77.4±3.3	33.8±2.7*	11.0±3.7	24.5±0.5*	8.2±0.7*
Walker 256+WBH+CP	279.3±3.3^{+}	2.3±0.3**	71.3±1.8*	37.0±2.3**	16.3±2.8*	25.9±0.8	8.6±0.5*
Walker 256+WBH+MT	249.5±2.3^{+*}	29.7±1.1	73.2±3.6	39.8±4.4*	12.4±2.4	21.2±1.8**	9.6±0.4*
Walker 256+WBH+CP+MT	280.7±3.0^{+}	1.9±0.1**	65.3±3.2**	43.9±1.8**	12.6±2.7	19.0±0.7**	13.8±0.6**
Walker 256+CP+MT	286.3±3.5^{+}	2.0±0.1**	68.9±2.6*	44.0±1.5**	11.5±3.5	21.4±1.4*	14.2±0.5**

Note. $^{+}p<0.05$ in comparison with intact animals; *$p<0.05$, **$p<0.01$ in comparison with the control.

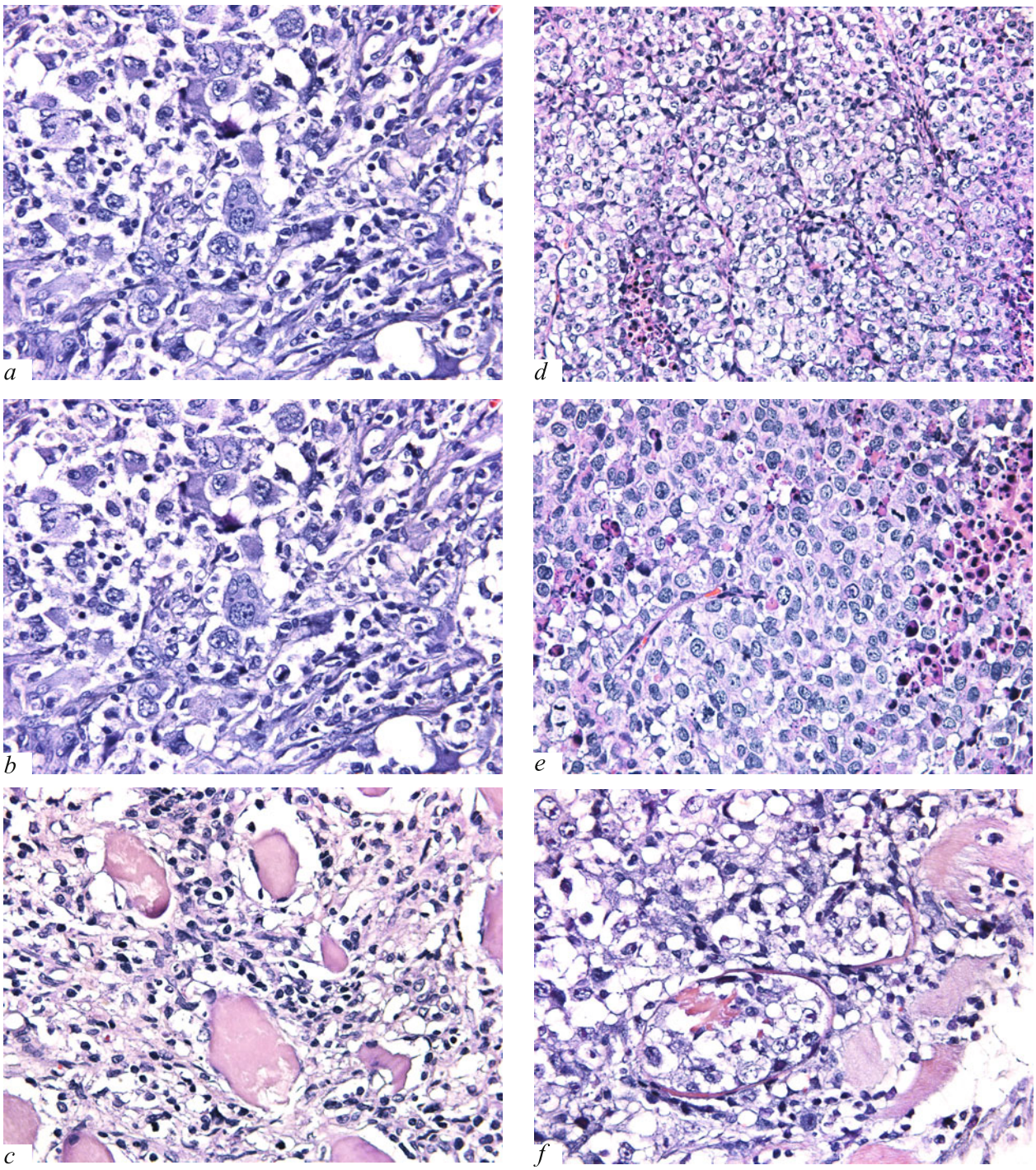

Fig. 3. Histogenesis of Walker 256 carcinosarcoma induced by WBH and antitumor treatment. Hematoxylin and eosin staining. *a*) pronounced reduction of epithelioid cells and their segregation 7 days after CP treatment (×400); *b*) reduction of epithelioid cells and growth of sarcomatous tissue 7 days after WBH and CP treatment (×400); *c*) reduction of epithelioid cells and lytic changes in muscle fibers 7 days after WBH and CP treatment (×400); *d*) pseudoglandular structure of carcinosarcoma 7 days after MT treatment (×200); *e*) recovery of tumor node structure 14 days after CP treatment (×400); *f*) invasion of tumor cells into muscle fibers and their resorption (×400).

tion of WBH with each antitumor agent potentiated the inhibitory effect produced by the individual influence. Proliferative activity of tumor cells was maximally suppressed in case of combinations WBH+CP+MT (by more than 1.8 and 1.6 times compared to the control on days 3 and 7, respectively) and CP+MT (by 1.8 times), which correlated with deceleration of tumor growth.

The apoptotic index increased in all groups, but the effect was more pronounced at all terms, if MT was in-

cluded in the treatment scheme (Table 1). The apoptotic index was elevated by 11.7 times in WBH+CP+MT group on day 3 and by 11 and 5.7 times in CP+MT group on days 7 and 14, respectively.

Intensification of apoptotic and necrotic death of tumor cells after WBH and administration of antitumor drugs reduced the volume density of the parenchymatous component of carcinosarcoma (Table 1) and was accompanied by a decrease in the volume of the tumor node, which confirmed the efficiency of antitumor therapy. In our study we observed a decrease in the volume of Walker 256 carcinosarcoma only when protocols with CP were used. On day 7 and by the end of the experiment, the tumor volume decreased after CP treatment by 2.6 and 3.7 times, respectively, by 3.4 and 16.9 times after WBH+CP; by 12.3 and 15.6 times after CP+MT treatment; and by 22.6 и 18 after WBH+CP+MT.

After WBH, the volume of Walker 256 carcinosarcoma increased by 9.8, 1.7, and 1.1 times after 3, 7, and 14 days, respectively. After treatment with MT, the tumor volume on day 3 of the experiment was higher by 5.8 times in comparison with the control; after 7 and 14 days this parameter tended to decrease. After combined treatment with WBH and MT, the volume of tumor changed in a wave-like manner starting from day 3 and to the end of the experiment: on day 3 it considerably surpassed the control level, on day 7 decreased by 2 times, and on day 14 increased again and practically attained the control level. In all cases, the increase in carcinosarcoma volume was determined by significant tissue edema.

Walker 256 carcinosarcoma belongs to model tumors determining the development of anorexia/cachexia syndrome, which in turn is associated with high mortality [6] due to catabolism of skeletal muscle proteins [11]. Some peculiarities of this tumor concerning proteolytic activity and production of mediators of neoplastic cachexia [9,12]. Oxidative stress is considered to be a trigger of signal transduction for activation of proteosome system and protein oxidation [1,7]. Under conditions of natural growth of Walker 256 carcinosarcoma, rat body weight (without tumor node) decreased by 19% on day 19 after transplantation. The body weight drop was most pronounced on day 14 after WBH (by 47%); significant decrease in body weight was also observed after WBH+MT (by 27%) and MT (by 18%); in other cases this parameter changed insignificantly.

Our findings suggest that the combination of WBH and cytostatic treatment (CP and MT) produced maximum suppression of the tumor growth, inhibition of mitotic activity of tumor cells, and stimulation of their necrotic and apoptotic death. The maximum decrease in mitotic activity of tumor cells was observed when CP was included into the treatment protocols, especially in WBH+CP+MT and CP+MT groups; enhancement of apoptotic cell death and the decrease in the tumor node weight were also most pronounced under these conditions. Body weight of experimental animals in these groups decreased by only 6-8%, which attests to the absence of body weight loss.

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