Ultrastructural and Stereological Analysis of Walker 256 Carcinosarcoma Cells at Various Stages of Their Differentiation

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Ultrastructural and stereological study of Walker 256 carcinosarcoma cells implanted into the femoral muscle of Wistar rats was performed. Two types of Walker 256 carcinosarcoma cells were distinguished and their ultrastructural analysis was carried out. Five differentiation stages were described for each of the two cell types. Differentiation of carcinosarcoma cells was associated with a decrease of the nucleus/cytoplasm ratio, enlargement of the cell, and hyperplasia of granular cytoplasmic reticulum elements, fixed ribosomes, and mitochondria. Differences in the ultrastructure of the two cell types at similar differentiation stages were detected.

Key Words: *Walker 256 carcinosarcoma cells; ultrastructure; stereology*

Tumor growth is a complex multi-staged process characterized by a progressive increase of autonomy and growth rate. One of the important characteristics of tumor growth is acquisition of specific properties by the tumor cells, such as capacity to autocrine regulation, infiltrative growth, metastasizing [2,7]. Walker 256 carcinosarcoma is a model object for studies of these characteristics of tumor cells. This tumor was isolated in 1928 by J. Walker from spontaneous tumors of the rat mammary glands. Later it was found by immunocytochemical analysis that Walker 256 carcinosarcoma cells carried markers of stem and/or hemopoietic cells and that tumor cells originated from monocytes [10]. Detailed study of Walker 256 carcinosarcoma growth pattern resulted in isolation and description of two tumor cell types [10] and detected some metabolic features of this tumor, specifically, its proteolytic activity and production of neoplastic cachexia mediators [5,9,11,12].

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Different types of Walker 256 carcinosarcoma cells are characterized by different sensitivity to drugs [6,10]. Therefore, Walker 256 carcinosarcoma is a good model for evaluating the efficiency of therapeutic approaches to blocking of tumor growth and metastases. Detection of stringent morphological criteria for identification of the tumor cell type and stages of its cell differentiation is essential for determining the targets for drug therapy.

We carried out an ultrastructural and stereological analysis of Walker 256 carcinosarcoma cells at different stages of their differentiation.

MATERIALS AND METHODS

Experiments were carried out on male Wistar rats (180-200 g). Manipulations on animals were carried out in accordance with the humane philosophy presented in directives of the European Community (86/609/EEC) and in Helsinki Declaration. Walker 256 carcinosarcoma strain supported *in vivo* was used (Laboratory of Physiological Genetics, Institute of Cytology and Genetics). Suspension of Walker 256 carcinosarcoma

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cells was transplanted into the hip muscle in a dose of 10^6 cells [3,8]. The tumor size was measured with a slide gage in three perpendicular directions 5 days after its transplantation and tumor volume was calculated. The animals were kept at fixed light:darkness regimen (12:12 h, light from 8.00 to 20.00) at standard temperature.

Tumor tissue specimens for electron microscopy were collected on day 5 after tumor transplantation; 5 specimens (1 mm^3) were collected from each animal. The fragments were fixed in 4% paraformaldehyde in 0.1 M Millonig phosphate buffer (pH 7.4) at ambient temperature for 2 h. After washing in cold Millinig buffer the samples were additionally fixed for 1 h in cold 1% osmium fixative on 0.2 M cacodylate buffer (pH 7.4) with 1.5% potassium ferrocyanide. After dehydration the samples were embedded in epon resin. Semithin sections (1 μ) were stained with toluidine blue, examined under a light microscope, and sites for examination under electron microscope were selected. Ultrathin sections (35-45 nm) were sliced on an LKB-8800 ultratome, contrasted with saturated aqueous solution of uranyl acetate and lead citrate, and examined under a JEM 1010 electron microscope.

Ultrastructural stereological analysis of tumor cells (50 cells per group) was carried out at a final magnification of ×32,000 in a closed test system with 144 points. Volume density (V_V) of the nucleoli, mitochondria, granular cytoplasmic reticulum, and lysosomes were evaluated. The number of profiles of ultrastructures in a test area (N_A) was evaluated for mitochondria, fixed and free ribosomes, and lysosomes. The proportion of surface density of the inner mitochondrial membrane to outer mitochondrial membrane and the nucleus/cytoplasm ratio were calculated. Quantitative data were processed using routine statistical methods [1]. The means (*M*), errors of the means (*m*), and level of significance of differences between the means (*p*) were calculated using Student's *t* test with Bonferroni correction for the 95% level of significance ($p<0.05$).

RESULTS

Two types of tumor cells, which were at different stages of differentiation, were distinguished by the results of ultrastructural analysis of developing Walker 256 carcinosarcoma. Type 1 was characterized by high electron density of the cytoplasm and nucleus (Fig. 1). Type 2 cells were larger and with lesser electron density of the cytoplasm and nucleus (Fig. 2). Five stages of cell differentiation were distinguished for each cell type by the nuclear-cytoplasmic proportion according to the results of morphometric analysis (Table 1).

Some type 1 cells were small electron-dense cells with numerous microvilli, small cytoplasm volume, with large heterochromatin lumps in the round nucleus (Fig. 1, *a*). The cytoplasmic organelles in the cytoplasm were poorly developed. This cell type was referred to differentiation stage 1. Cells of differentiation stage 2 had a larger cytoplasm and a virtually 2-fold lesser nucleus/cytoplasm ratio in comparison with stage 1 cells (Table 1). The nucleus was slightly elongated, with large lumps of heterochromatin (Fig. 1, *b*). Volume density of the mitochondria in these cells was increased 2.7 times, of granular cytoplasmic reticulum 1.9 times, and the numerical density of fixed ribosomes was 1.9 times increased.

Cells of differentiation stage 3 were large electron-dense tumor cells with numerous mitochondria, lysosomes, free ribosomes, and polysomes in the cytoplasm and with an elongated electron-dense nucleus (Fig. 1, *c*). The volume and numerical density of the mitochondria was most markedly increased in these cells (by 2.3 and 3.5 times, respectively), as well as the volume density of granular cytoplasmic reticulum (by 3.2 times) and numerical density of fixed ribosomes (3.6 times; Table 1). The numerical density of free ribosomes decreased by 1.7 times.

Cells of differentiation stage 4 were characterized by elongated electron-dense nucleus with large nucleolus (Fig. 1, *d*) and higher (compared to differentiation stage 1 cells) volume and numerical density of mitochondria (6 and 4.7 times, respectively). Volume density of granular cytoplasmic reticulum and numerical density of fixed ribosomes were significantly increased in these cells (by 5.5 and 4.6 times, respectively), while numerical density of free ribosomes was 2-fold decreased. It is noteworthy that the nucleus/cytoplasm ratio was minimum at this differentiation stage (by 6.9 times lower than at stage 1) and the volume density of the nucleoli was maximum (by 15.6 times higher). The cells of stage 5 differentiation were even larger, with electron-dense cytoplasm (Fig. 1, *e*); the nucleus/cytoplasm ratio decreased by 4.6 times compared to differentiation stage 1 cells. Volume and numerical densities of the mitochondria were even more increased in these cells (by 7.7 and 5 times, respectively), as well as the volume density of granular cytoplasmic reticulum (by 6.3 times). The volume and numerical densities of lysosomes were also increased significantly in cells of this differentiation stage (by 3.9 and 2 times, respectively).

Type II tumor cells included small cells with moderate electron density of the cytoplasm and nucleus and with numerous free ribosomes and polysomes. These cells were referred to differentiation stage 1 (Fig. 2, *a*). Cells of differentiation stage 2 were elongated with an elongated nucleus and a large nucleolus

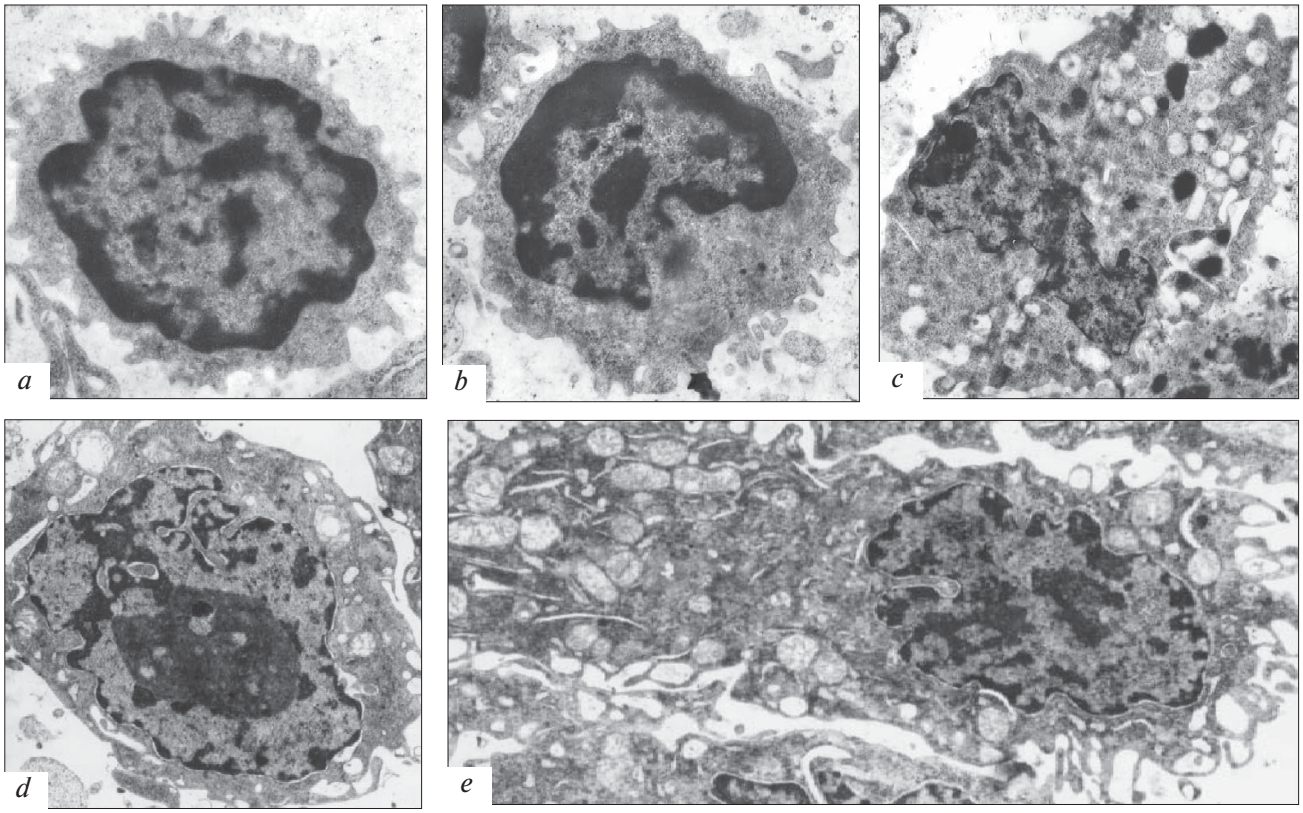

Fig. 1. Differentiation stages of Walker 256 carcinosarcoma type I cells. *a*) stage 1: round nucleus with slightly irregular edges, large lumps of heterochromatin, numerous microvilli; *b*) stage 2: bean-shaped nucleus with marginal heterochromatin, high density of cytoplasmic organelles; *c*) stage 3: elongated electron-dense nucleus with nucleolus, numerous mitochondria in the cytoplasm; *d*) stage 4: elongated nucleus with marginal heterochromatin and large nucleolus, increased number of mitochondria, lysosomes, elongated microvilli; *e*) stage 5: increased number of mitochondria, lysosomes, polysomes, elongated microvilli. ×8000 (*a*, *b*), ×6000 (*c*-*e*).

(Fig. 2, *b*), with its volume density increased by 2.3 times. The volume density of the granular cytoplasmic reticulum of these cells was by 2.3 times increased. The reticulum cisterns were located at the periphery. The numerical density of fixed ribosomes was 3-fold increased, while numerical density of free ribosomes was 14% decreased. Cells of differentiation stage 3 were represented by large elongated flat forms with elongated nuclei and large nucleoli (Fig. 2, *c*). The cytoplasm of these cells contained numerous cisterns of the granular cytoplasmic reticulum, free ribosomes and polysomes (Table 1). Differentiation of cells of this type was associated with an increase in the granular endoplasmic reticulum membranes.

Type II cells of differentiation stage 4 were large (Fig. 2, *d*), with numerous cisterns of the granular cytoplasmic reticulum, whose volume density was by 4.4 times higher than in cells of differentiation stage 1. The numerical density of fixed ribosomes was increased significantly (by 2.8 times), while that of free ribosomes was lower (by 2.2 times). The nuclei were elongated, of irregular shape, with lacerated edges. The nucleus/cytoplasm ratio was reduced significantly (by 8.2 times) at this stage of differentiation, similarly as in type I cells. Volume density of the nucleoli (in contrast to type I cells) was reduced significantly (by 4.3 times; Table 1). The volume density of the mitochondria was lowered (by 1.8 times) in these cells. Cells of differentiation stage 5 were even larger and contained more cisterns of the granular cytoplasmic reticulum (volume density increased by 6.3 times; Fig. 2, *e*). Volume and numerical density of mitochondria were also higher in these cells (by 2.6 and 1.8 times, respectively), and the proportion of surface density of internal mitochondrial membrane to external one was 1.5-fold increased. Similarly as in type I cells, the volume and numerical density of lysosomes was increased (by 2 and 2.6 times, respectively).

Qualitative and quantitative ultrastructural changes in types I and II Walker 256 carcinosarcoma cells during their differentiation suggest distinguishing the stages of progressive and regressive (starting degradation) growth for each cell type. The progressive growth stages for type I cells are differentiation stages 1-4, while stage 5 characterizes the regressive growth. For type II cells these are stages 1-3 and 4-5, respectively. Important ultrastructural criteria of progressive growth are the decrease in the nucleus/cyto-

TABLE 1. Results of Morphometric Analysis of Walker 256 Carcinosarcoma Cells, Types I and II (*M*±*m*)

Parameter		Cell type	Tumor cell differentiation stages				
			1	2	3	4	5
Nuclear/cytoplasmic proportion		I	2.15±0.09	1.11±0.08*	1.22±0.07*	0.31±0.08*	0.46±0.12*
		II	1.07±0.08	0.71±0.09*	0.51±0.06*	0.13±0.07*	0.25±0.08*
Surface density of internal/ external mitochondrial membrane, μ^2/μ^3		I	2.2±0.23	1.8±0.12	3.1±0.17*	2.7±0.14	2.4±0.15
		II	2.1±0.07	2.4±0.11	2.6±0.14	2.2±0.18	3.2±0.12*
Volume density of structures, %	nucleolus	I	3.5±0.15	4.3±0.22*	29.8±0.12*	54.5±0.16*	6.1±0.25*
		II	17.8±0.21	40.1±0.18*	13.9±0.15*	4.1±0.09*	—
	mitochondria	I	4.7±0.19	12.5±0.17*	10.9±0.28*	28.5±0.78*	36.4±0.43*
		II	4.8±0.12	3.6±0.42	8.8±0.26*	2.7±0.15*	12.5±0.34*
	granular cytoplasmic reticulum	I	4.5±0.11	8.7±0.28*	14.5±0.16*	24.9±0.12*	25.1±0.34*
		II	11.9±0.14	26.8±0.32*	44.1±0.12*	52.4±0.16*	74.8±0.61*
	lysosomes	I	2.1±0.18	2.5±0.42	2.2±0.13	2.3±0.12	8.3±0.72*
		II	2.3±0.45	2.0±0.18	5.9±0.41*	3.2±0.24	4.6±0.12*
Numerical density of structures	mitochondria	I	1.8±0.14	1.5±0.34	6.3±0.11*	8.4±0.46*	9.2±0.36*
		II	2.4±0.17	2.2±0.15	3.1±0.28	2.1±0.44	4.3±0.25*
	fixed ribosomes	I	5.6±1.48	10.4±1.35*	20.3±1.19*	25.7±1.36*	22.4±1.16*
		II	10.3±1.26	30.6±1.44*	35.9±1.37*	28.6±1.53*	22.4±1.19*
	free ribosomes (polysomes)	I	43.2±1.76	48.2±1.26	25.8±1.66*	20.3±1.18*	28.1±1.35*
		II	56.1±2.18	48.2±1.53*	41.5±1.22*	25.4±1.18*	24.6±1.54*
	lysosomes	I	1.3±0.45	1.2±0.15	1.3±0.25	1.0±0.09	2.6±0.12*
		II	1.2±0.34	1.1±0.13	3.2±0.18*	2.3±0.19	3.1±0.42*

Note. *$p<0.05$ for comparison of stage 1 cells with subsequent stages of types I and II cells.

plasm ratio, increase of the volume density of granular cytoplasmic reticulum cisterns and of numerical density of fixed ribosomes. Signs of regressive growth are increased volume and numerical density of lysosomes. Our results indicate the phenotypical heterogeneity of Walker 256 carcinosarcoma cells, characterized by specific differentiation potential and degradation rate.

These differences in the intracellular organization of Walker 256 carcinosarcoma cells can be explained by different gene expression in the two cell types. It has been shown, for example, that variants of Walker 256 carcinosarcoma (aggressive and regressive) differ by the level of some cytokines' gene expression (transforming growth factor-β, IL-12, γ-IFN, and TNF-α). This fact is essential for development of effective chemotherapy protocols.

Hence, the basic criteria and differentiation stages of Walker 256 carcinosarcoma cells were determined by the results of ultrastructural stereological analysis. Two types of cells and five differentiation stages were distinguished for each type of tumor cells. Differentiation of each cell type was associated with a decrease of the nuclear/cytoplasmic proportion, enlargement of the cell, increase of volume density of granular cytoplasmic reticulum cisterns and of numerical density of fixed ribosomes and mitochondria.

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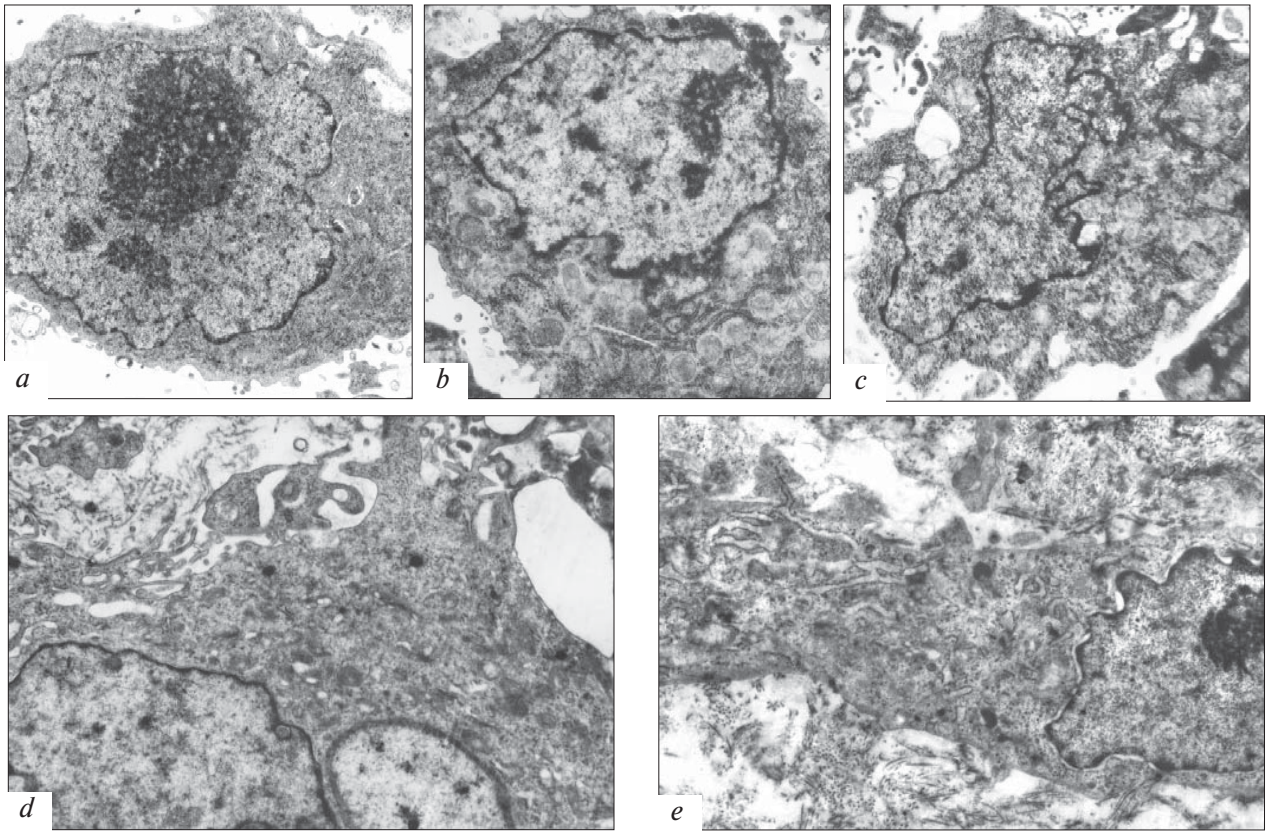

Fig. 2. Differentiation stages of Walker 256 carcinosarcoma type II cells. *a*) stage 1: the nucleus contains mainly euchromatin and a large nucleolus; *b*) stage 2: increased content of marginal heterochromatin in the nucleus, greater irregularity of edges; greater number of fixed ribosomes in the cytoplasm; *c*) stage 3: modified shape and elongation of the nucleus, low content of marginal heterochromatin, large nucleolus, hyperplasia of the granular cytoplasmic reticulum; *d*) stage 4: well-developed granular cytoplasmic reticulum; *e*) stage 5: nucleus with predominating euchromatin and a large nucleolus, pronounced irregularity of nuclear membrane, hyperplasia of granular cytoplasmic reticulum cisterns, increased number of microvilli. ×8000 (*a*), ×6000 (*b*-*e*).

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