Relationship between Antitumor Efficiency of Photodynamic Therapy with Photoditasine and Photoenergy Density

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We studied the effect of photodynamic therapy with photoditasine at different protocols of photoenergy exposure on morphofunctional parameters of M-1 sarcoma. It was found that proliferative activity of tumor cells (evaluated by immunostaining for PCNA) nonlinearly decreases after exposure to 150, 300, and 600 J/cm^2. The main form of cell death during the early period after photodynamic therapy was direct photocoagulation necrosis caused by destruction of sensitized cell structures and ischemic necrosis developing as a result of alteration of vascular network in the tumors. Photoenergy density was not essential for the intensity of induced apoptosis.

Key Words: *photoenergy; photoditasine; M-1 sarcoma; PCNA; necrosis*

Photodynamic therapy (PDT) of tumors is a therapeutic method including two components: photosensitizer and light. Photochemical reactions triggered during the interaction between these components cause generation of singlet oxygen and other biological oxidizers producing a cytotoxic effect [2,7,11]. The efficiency of PDT depends on photophysical characteristics and concentration of the photosensitizer in the tumor, photoenergy density, and irradiation protocol [5,6,12,13].

We previously demonstrated high efficiency of photodynamic therapy of M-1 sarcoma in rats with a new chlorine photosensitizer photoditasine (N-methylglucamine chlorine e_6 salt) [1].

Here we studied the effect of laser photoenergy density on the morphology and functional parameters of M-1 sarcoma after PDT with photoditasine.

MATERIALS AND METHODS

The study was carried out on random-bred male albino rats aged 3 months (160-180 g) with M-1 sarcoma implanted subcutaneously in the right thigh [1,3]. On days 12-14 after implantation the animals were intravenously injected with 2.5 mg/kg photoditasine and after 1.5 h PDT was carried out. Photoexposure was carried out on an Atkus-2 laser device (λ=661 nm, 0.48 W/cm^2 power density, 2 cm light spot diameter). The tumor was exposed to photoenergy of 150 J/cm^2 (n=15), 300 J/cm^2 (n=17), and 600 J/cm^2 (n=15). Tumor volume at the site of implantation was evaluated, tumor regression coefficient (TRC) after PDT and the integral tumor growth rate [1] were estimated.

Material for the analysis was collected 3 h and 1, 4, 12, and 21 days after PDT. The tumors were isolated under Nembutal narcosis. Tumor tissue was cut into 3-4-mm plates oriented along the long axis, fixed for 24 h in acid Bouin fixative, dehydrated, and embedded in HISTOMIX™. Microtome sections (6 μ) were put on slides covered with poly-L-lysine film (Sigma). Murine monoclonal antibodies to PCNA (proliferating cell nuclear antigen) diluted 1:50 (PC10 clone, Calbiochem) and biotin-streptavidin-peroxidase kit for detection of murine immunoglobulins (ICN) were used for immunostaining of proliferating cells. The nuclear organizer regions (NOR) were studied by the AgNOR method [14]. Apoptotic index of tumor cells was evaluated on hematoxylin and eosin-stained preparations impregnated by the method of Moser [1,3].

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Morphometric studies were carried out using IMSTAR computer analysis of microscopic images. The following stereological parameters were used: PCNA volume ratio (ρ_{PCNA}), *i.e.* the ratio of the total area of immunostaining for PCNA to the total area of tumor node section; quantitative density of tumor cell nuclei (N_{TCN}); volume density of NOR [1]. PCNA index (I_{PCNA}), *i.e.* the ratio of quantitative density of $PCNA^+$ nuclei to N_{TCN} stained by AgNOR method, was estimated for evaluation of cell proliferative activity. The relative fraction of proliferating cells (F_{PCNA}) was calculated by the formula:

$$F_{PCNA}=\rho_{PCNA}\times I_{PCNA}\times K_N,$$

where $K_N=N_{TCN}$ in experiment/N_{TCN} in control. Quantitative parameters for M-1 sarcoma in control animals and the size of sampling for morphometry of respective structures were presented previously [1]. The results were statistically processed using Mann—Whitney nonparametric *U* test.

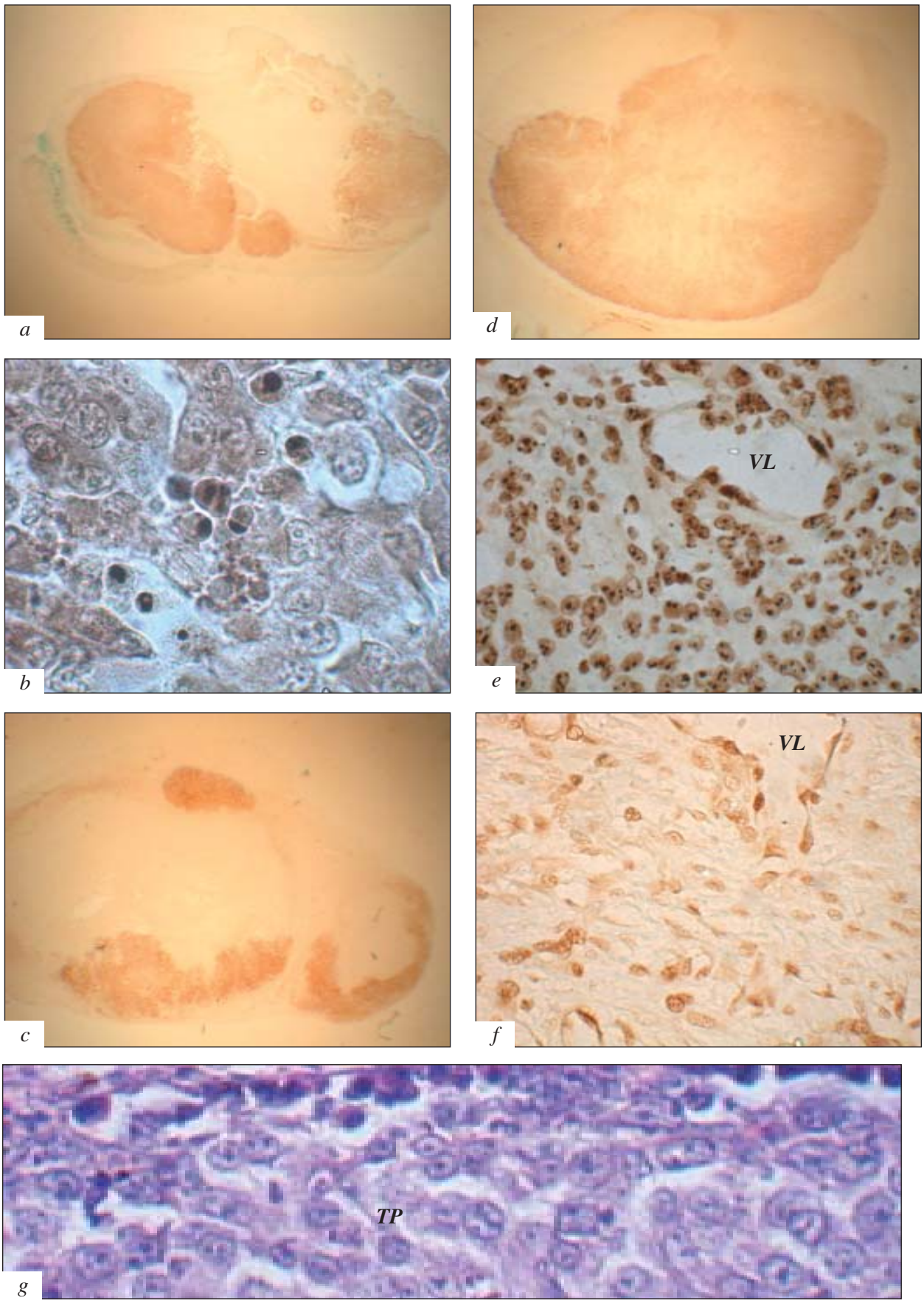
RESULTS

Tumor regression coefficient after exposured to 150 J/cm^2 photoenergy was -0.40±0.24 (n=15) and tumor growth rate was 0.52±0.23. Deviation from the average trends was observed only for a 0.61-cm^3 tumor poorly reacting to PDT. One tumor (0.36 cm^3) completely regressed by day 12. Increasing the photodose to 300 J/cm^2 led to more pronounced regression of sarcoma and apparent disappearance of the tumors on day 4 in 3 animals. TRC decreased to -0.86±0.08 (n=17; p<0.05) and the integral growth rate decreased to 0.37. After PDT with a dose density of 600 J/cm^2 the tumor nodes were not detected macroscopically on day 4 irrespective of the tumor volume on the day of PDT. TRC reached the minimum value (-0.99). In 2 rats small relapsing nodules appeared by day 12 and completely regressed by day 21.

Three hours after PDT with a dose density of 150 J/cm^2 extensive foci of tumor photodestruction were seen on the preparations; PCNA reaction in these foci was negative (Fig. 1, *a*). F_{PCNA} was 19.1±2.2%, which was 4-fold lower than in tumors not exposed to PDT [1]. Vascular reaction (sharp plethora) was detected in the retained parenchyma. Tumor cell lysis with destruction of the plasma membranes and "denudation" of cells nuclei was observed along capillaries. Small accumulations of tumor cells in the early apoptosis stage appeared in the subcapsular zone and around the capillaries (Fig. 1, *b*). After 24 h the greater part of the parenchyma was presented by necrotic areas surrounded by polymorphonuclear leukocytes, lymphocytes, and macrophages. The reaction of cell nuclei to PCNA was observed only at the periphery of the tumor nodes and presented as narrow interrupted strips adjacent to the capsule. F_{PCNA} decreased to 7.72±4.20%. On day 4 small fragments with clearly seen PCNA reaction appeared at the periphery of the tumor nodes (Fig. 1, *c*) surrounded by connective tissue streaks and numerous capillary loops of newly formed blood vessels. F_{PCNA} decreased to 4.22±3.90%, but I_{PCNA} increased. According to morphometry data, the quantitative parameters of sarcoma on days 12 and 21 after PDT with a dose density of 150 J/cm^2 were similar to those in the control group [1].

Three hours after PDT with a dose density of 300 J/cm^2 the intensity of $PCNA^+$ reaction appreciably decreased (Fig. 1, *d*) and the estimated F_{PCNA} was 2-fold lower than at 150 J/cm^2. Extensive foci of coagulation necrosis and hemorrhages were seen in the tumor parenchyma. Tumor cells were shrunk and had hyperchromatic cyto- and karyoplasm. Proliferating cells remained as small islets at the base of the tumors. The majority of tumor cells were in a state of phototoxic shock even in zones with retained reaction of the nuclei to PCNA. Cell nuclei shrank and their karyoplasm was condensed. The apoptotic index was 0.85±0.09%. One day after PDT weak $PCNA^+$ reaction was observed mainly at the lower interface of the tumors and virtually the entire neoplasm was presented by dead tissue. On day 4 a universal picture of subtotal tumor necrosis was observed. Sarcoma cells which survived by this term were usually located near blood vessels (Fig. 1, *e*) and retained their proliferative potential (Fig. 1, *f*). On days 12 and 21 there were no significant differences in the morphofunctional characteristics of M-1 sarcoma in comparison with repeatedly growing tumors in group with PDT at 150 J/cm^2. At the same time, we observed intensive lymphocyte infiltration in marginal zones. Sometimes 1-2 tumor cells wide strips were seen along the tumor perimeter at the interface with normal tissue, these tumor cells were in a state of lysis, fragmentation, and apoptosis (Fig. 1, *g*). Apoptotic index during this period was 0.33-0.51%; apoptotic cells were scattered at the periphery in zones of M-1 sarcoma growth and active proliferation.

Three hours after PDT with a dose density of 600 J/cm^2 a photodynamic burn developed on the skin above the tumor, massive exudation formed in the subcutaneous fat (Fig. 1, *h*), erythrocyte clots formed in vessels surrounding the tumor, and proliferative activity of cells in the tumor virtually collapsed. The parenchyma along dead vessels was presented by fields of tumor cells in a state of coagulation, with tissue fragments containing shrunk and hyperchromatic cells (Fig. 1, *i*). In contrast to groups exposed to PDT with dose densities of 150 and 300 J/cm^2, there were no signs of massive plasmatic and hemorrhagic impreg-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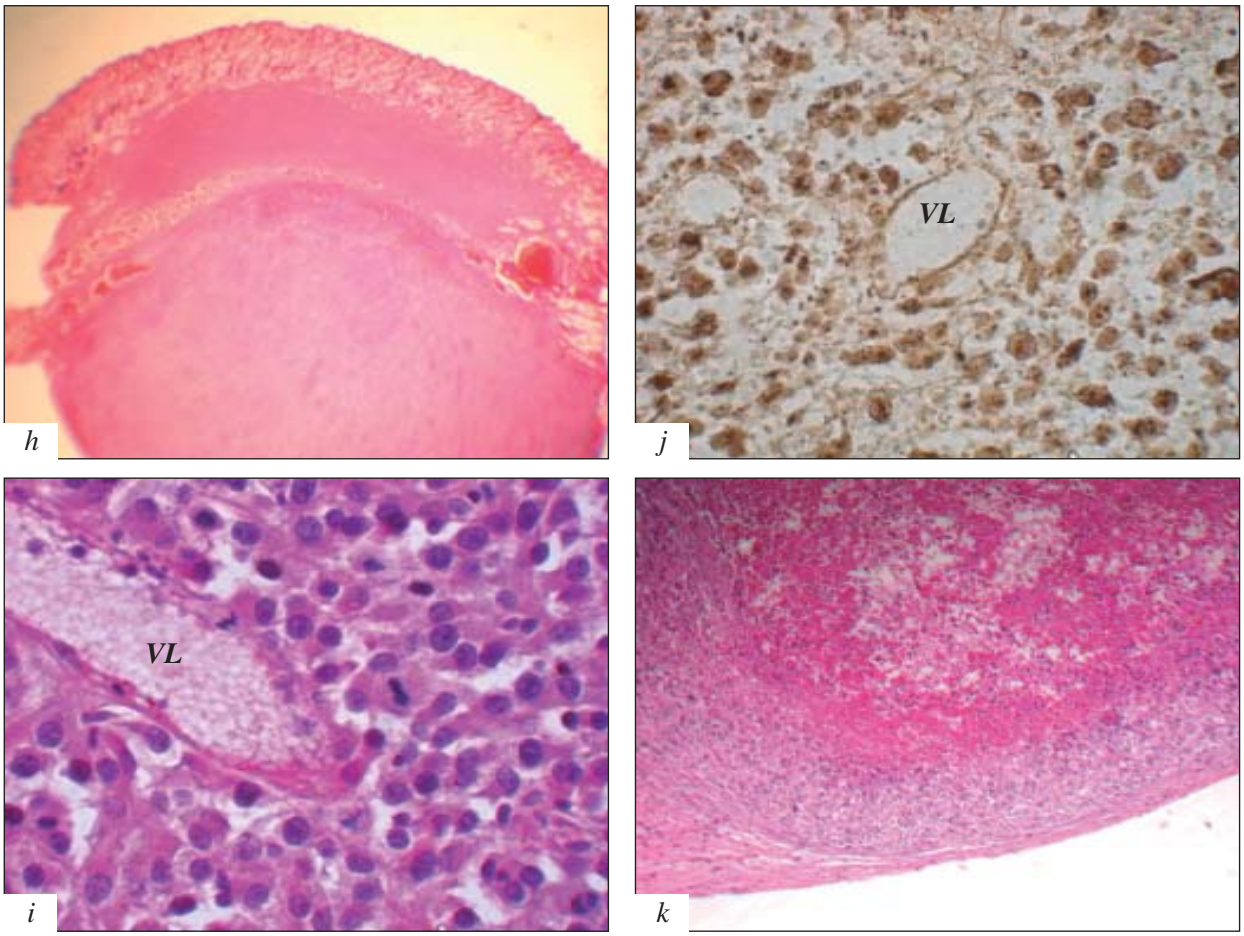

Fig. 1. Effect of photodynamic therapy with photoditasine with a dose density of 150 (*a-c*), 300 (*d-f*), and 600 J/cm^2 (*h-k*) on functional morphology of M-1 sarcoma 3 h (*a*, *b*, *d*, *h-j*), 1 (*k*), 4 (*c*, *e*, *f*), and 12 days (*g*) after irradiation. *a*, *c*, *d*, *f*: immunohistochemical reaction of cell nuclei with antibodies to PCNA; *b*: impregnation by the method of Mozer; *e*, *j*: staining by AgNOR method; *g-i*, *k*: hematoxylin and eosin staining, ×7.5 (*a*, *c*, *d*, *h*); ×1200 (*b*, *g*); ×470 (*e*, *f*, *i*, *j*); ×40 (*k*). VL: vessel lumen; SF: subcutaneous fat; TP: tumor parenchyma.

nation of the parenchyma. The walls of some vessels looked destroyed, but hemorrhages were not extensive. This indicates hemodynamic arrest in tumor nodes. The reaction of cell nuclei to PCNA was retained only in the basal subcapsular zone of the tumors. Estimated F_{PCNA} value decreased to 2.4%. The volume content of NOR in areas with positive PCNA reaction increased to 15.91±0.84% at the expense of shrinkage of cell nuclei and NOR enlargement. Destruction of cell nuclei and NOR with the appearance of nuclear detritus was detected in the central zones of the tumors (Fig. 1, *j*). Apoptotic index was 0.82%, which was virtually the same as after PDT with a dose density of 300 J/cm^2.

Total or subtotal ischemic necrosis of sarcoma (Fig. 1, *k*) with virtually complete loss of the reaction to PCNA was observed 1 day after PDT with a dose density of 600 J/cm^2. Dead tissues were surrounded with inflammatory infiltration; fibroblast proliferation and active elimination of dead cells by macrophages started in the less damaged tissues; loops of newly formed vessels appeared. On day 4 small groups of survived tumor cells surrounded by capillaries and granulation tissue were detected in 2 animals. These tumor cells were small, with reduced slightly basophilic cytoplasm. Their nuclei contained small nucleoli and 1-2 NOR. After 12 days similar streaks of tumor cells in a state of pathomorphosis were detected. On day 21 small areas with groups of dead and/or dying cells infiltrated with lymphoid cells and macrophages were seen in sclerosed tissue. No signs of active tumor cell proliferation were detected.

According to our findings, decreased number of proliferating cells in the tumor is an indicator of the destructive effect of PDT with photoditasine on M-1 sarcoma. The dose-effect relationship was nonlinear (Fig. 2). Decreased reaction of sarcoma cell nuclei to PCNA 3 h after PDT seemed to be due to the direct action of the light on photoditasine-sensitized elements of the tumor parenchyma. One day after PDT the dose dependence of reduction of the proliferating

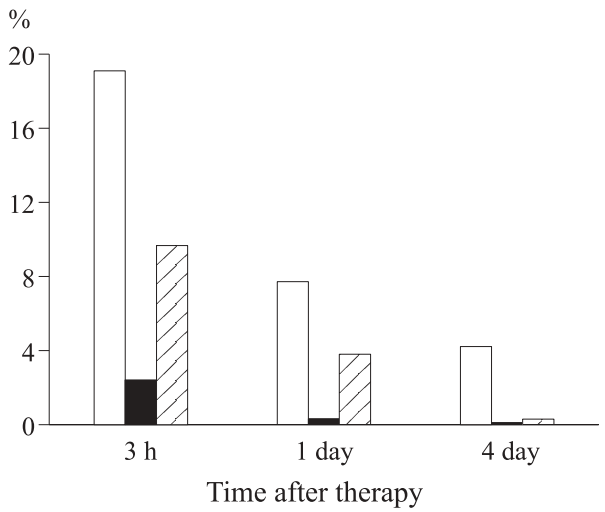

Fig. 2. Relationship between photoenergy density and proliferative activity of M-1 sarcoma cells after photodynamic therapy with photoditasine. Ordinate: fraction of $PCNA^+$ cells. Light bars: 150; cross-hatched bars: 300: dark bars: 600 J/cm^2.

cell fraction in comparison with the control [1] was exponential: F_{PCNA} decreased 10-fold after exposure at 150 J/cm^2, 21 times at 300 J/cm^2, and 257 times at 600 J/cm^2. Evidently, decreased proliferation of tumor cells during this period was caused by indirect effects and correlated with destruction of the vascular network, formation of clots, and development of ischemic necrosis in the tumors. The dose dependence on day 4 presumably reflects the fraction of surviving tumor cells with retained proliferative potential.

Hence, necrosis is the main type of tumor cell death at the early terms after PDT with photoditasine: direct photocoagulation necrosis caused by destruction of sensitized cell structures and ischemic necrosis developing as a result of tumor capillary damage and destruction. Antitumor effect of PDT with photoditasine depends on the delivered dose of photoenergy and is determined by the sequence of development of destructive and inflammatory changes in the parenchyma of M-1 sarcoma. Intensive inflammatory reaction is a central event in the mechanism of tumor destruction under the effect of PDT [7,9]. In addition, PDT-activated immune defense causes a series of interacting specific and nonspecific reactions of various cell types effectively eliminating tumor cells by the cytolytic and apoptotic mechanisms [10]. The maximum phototherapeutic effect (regression of M-1 sarcoma, decrease of cell proliferation, and destruction of the tumor in general) was attained after exposure to photoenergy of 600 J/cm^2.

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