Effect of Photodynamic Therapy with Photodithazine on Morphofunctional Parameters of M-1 Sarcoma

Yu. S. Romanko, A. F. Tsyb, M. A. Kaplan, and V. V. Popuchiev

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Efficiency and mechanisms of action of photodynamic therapy of M-1 sarcoma were studied experimentally on rats using photodithazine, a novel chlorine photosensitizer. The tissue and cellular targets for this agent are vascular wall, plasma membrane of tumor cells, and intracellular structures responsible for proliferation and biosynthesis.

Key Words: *photodynamic therapy; photodithazine; PCNA; AgNOR*

Elaboration and development of new methods of photodynamic therapy (PDT) in oncology attracted much recent attention [1-3,5]. The effect of PDT is based on selective accumulation of photosensitizer (PS) and subsequent irradiation of the tumor with visible light at the wavelength corresponding to light absorption peak of PS. In the presence of tissue oxygen, the induced photochemical reaction yields singlet oxygen and other biological oxidizers producing a cytotoxic effect [5,7,11]. Realization of the antitumor effect of PDT depends on activation of various mechanisms [8], and in many respects, is determined by the level of accumulation of PS in the tumor and its optical properties. The selectivity of available PS is poor, and the rates of their accumulation and excretion are low. Therefore, the search for novel PS and studies of their effects are of particular importance.

Our aim was to study the mechanisms of PDT on M-1 sarcoma, a connective tissue tumor, with the help of photodithazine, a new generation PS.

Photodithazine (N-methyl glucosamine chlorine e_6 salt) was synthesized in VETA-GRAND company on the basis of chlorophyll A derivatives. It has a powerful absorption band in long-wavelength red region of the light spectrum (λ_{max}=662 nm) and can deeply penetrate into biological tissues [1].

State Medical Radiological Research Centre, Russian Academy of Medical Sciences, Obninsk. ***Address for correspondence:*** y_romanko@hotmail.com. Yu. S. Romanko

MATERIALS AND METHODS

Experiments were carried out on random-bred albino male rats aging 3 months and weighing 160-180 g. Sarcoma M-1 was inoculated in the right thigh [1]. By day 10 postinoculation, the rats with tumor nodes of regular shape were randomized into three groups. The group 1 was control (n=15), group 2 rats were treated with photodithazine (n=18), and the rats of group 3 were subjected to PDT after injection of photodithazine (n=20). The volume of tumor located on the site of inoculation was calculated by the formula [3]: $V=\pi/6\times D_1\times D_2\times D_3$, where D_1, D_2, and D_3 are the length, width, and height of the tumor (cm), respectively. The tumor growth/regression rate (TGR) was calculated as $(V_4-V_0)/V_0$, where V_0 is tumor volume on the day of experimental treatment (day 12 after inoculation), V_4 is tumor volume on day 4 after inoculation. The overall TGR was calculated as the geometric mean: $\sqrt[4]{V_0\times V_4\times V_{12}\times V_{21}}$.

On day 12 after inoculation, the group 2 and 3 rats were injected intraperitoneally with photodithazine (2.5 mg/kg). After injection, the group 2 rats were kept under natural illumination regimen. PDT was carried out in group 3 rats with an Atkus-2 laser 1.5 h after administration of photodithazine. The parameters of light irradiation were as follows: λ=662 nm, power density 0.48 W/cm^2, and density of light irradiation 300 J/cm^2.

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The days of histological examination of sarcoma were chosen according to the rate of tumor growth. In control group, the specimens were examined on days 12, 16, 24, and 33 after inoculation. In the experimental groups 2 and 3, the specimens were analyzed on hour 3 and the days 4, 12, and 21 after injection of PS alone or in combination with PDT, respectively. The tumors were isolated under nembutal narcosis. Tumor tissue was cut into plates with a width of 3-4 mm, which were oriented along the long axis, fixed in acid Bouin fixative, dehydrated, and embedded in HISTOMIX™. The microtome sections (6 μ) were mounted onto poly-L-lysine-coated glass slides (Sigma).

Proliferating cells were stained using mouse monoclonal antibodies to PCNA (clone PC10, Calbiochem; dilution 1:50) and biotin streptavidin peroxidase detection kit for mouse immunoglobulins (ICN). Functional activity of tumor cells was evaluated by quantification of silver-stained nucleolar organiser regions (AgNOR) [9]. Apoptosis of the cells was determined on preparations stained with hematoxylin and eosin and impregnated by the method of Moser [4,9].

Morphometry was performed with IMSTAR microscopic image analysis system. The following stereological parameters were analyzed: PCNA volume density (ρ_{PCNA}) calculated as the ratio of total PCNA immunostaining area to total area of tumor node section, numerical density of tumor cell nuclei (N_{TCN}) equal to the number of nuclei per unit section area, and volume density of NORs, which was the integral index of NOR content in nuclei. Proliferation activity was evaluated by the mitotic index and PCNA index (I_{PCNA}). The latter was calculated as the ratio of numerical density of PCNA-positive nuclei to N_{TCN} for AgNOR-positive nuclei. The relative fraction of proliferating cells was calculated by the formula: $F_{PCNA}=\rho_{PCNA}\times I_{PCNA}\times K_N$, where K_N=(experimental N_{TCN})/(control N_{TCN}).

In each animal, the examined structures were counted in 60 visual fields (total area of 1.2 mm^2) selected in three sections of the tumor in the regions with maximum reaction for PCNA. The fields were determined based on histological structure of M-1 sarcoma [1]. The mitotic and apoptotic indices of cells were obtained by routine procedure with immersion microscopy of fields containing no less than 3000 nuclei of the tumor cells. The nuclei with stuck NOR or disrupted karyolemma were not counted. The data were processed statistically using Mann—Whitney non-parametric *U* test.

RESULTS

In the control group, the growth of M-1 sarcoma was described by curve characteristic of the majority of rapidly developing experimental tumors. The most intensive (exponential) growth was observed during the first 24 days (Fig. 1).

The curve describing the tumor growth in photodithazine-treated rats lay below the corresponding control curve (Fig. 1). A significant decrease in tumor growth rate was observed only during the first 4 days, *i.e.* photodithazine produced only short-term inhibitory effect on the growth of neoplasm (TGR=1.96±0.27; n=18, p<0.05). The overall TGR decreased insignificantly to 1.32±0.52.

On day 4 after PDT in group 3 rats, the volume of tumor decreased, but then sarcoma progressed. During the regression period, TGR was negative (-0.68± 0.30), which indicates high antitumor efficiency of photodithazine-based PDT. The overall TGR decreased more than 2-fold compared to the control (to 0.68± 0.30, p<0.05).

In the control group, M-1 sarcoma presented as a tumor consisting of polymorphic cells with signs of tissue and cell atypia. In small nodes (<4 mm) PCNA-positive cells were homogenously distributed in the tumor node parenchyma. In larger nodes, the reaction for PCNA was more intensive at the periphery (Fig. 2, *a*), while the center of the tumor demonstrated islets of spontaneous necrosis. In solid tumor structures, the cells were tightly packed. Their nuclei had 1-2 large nucleoli and up to five NOR (Fig. 2, *b*). Numerous mitotic figures and solitary apoptotic cells were clearly differentiated on histological sections (Fig. 2, *c*). Heterogeneity of the parenchyma significantly increased on days 24 and 33 postinoculation. The solid structure of the sarcoma was preserved primarily at the periphery. On day 33, immunostaining for PCNA de-

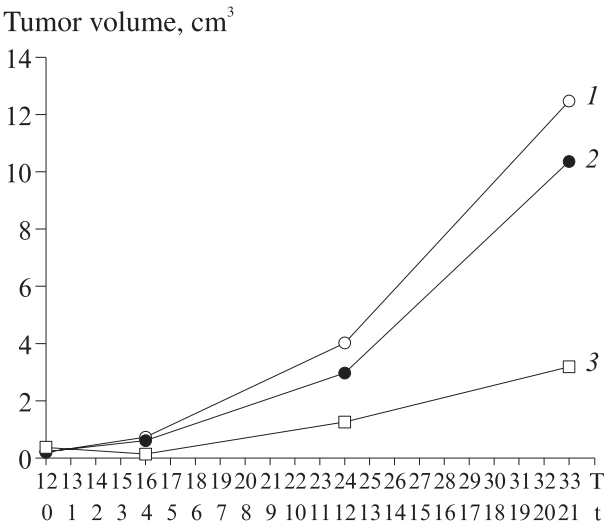

Fig. 1. The development of M-1 sarcoma in control rats (*1*), rats injected with photodithazine (*2*) and rats treated with photodithazine-based PDT (*3*). Abscissa: *T* is postinoculation time, and *t* is the period after injection of photosensitizer, days.

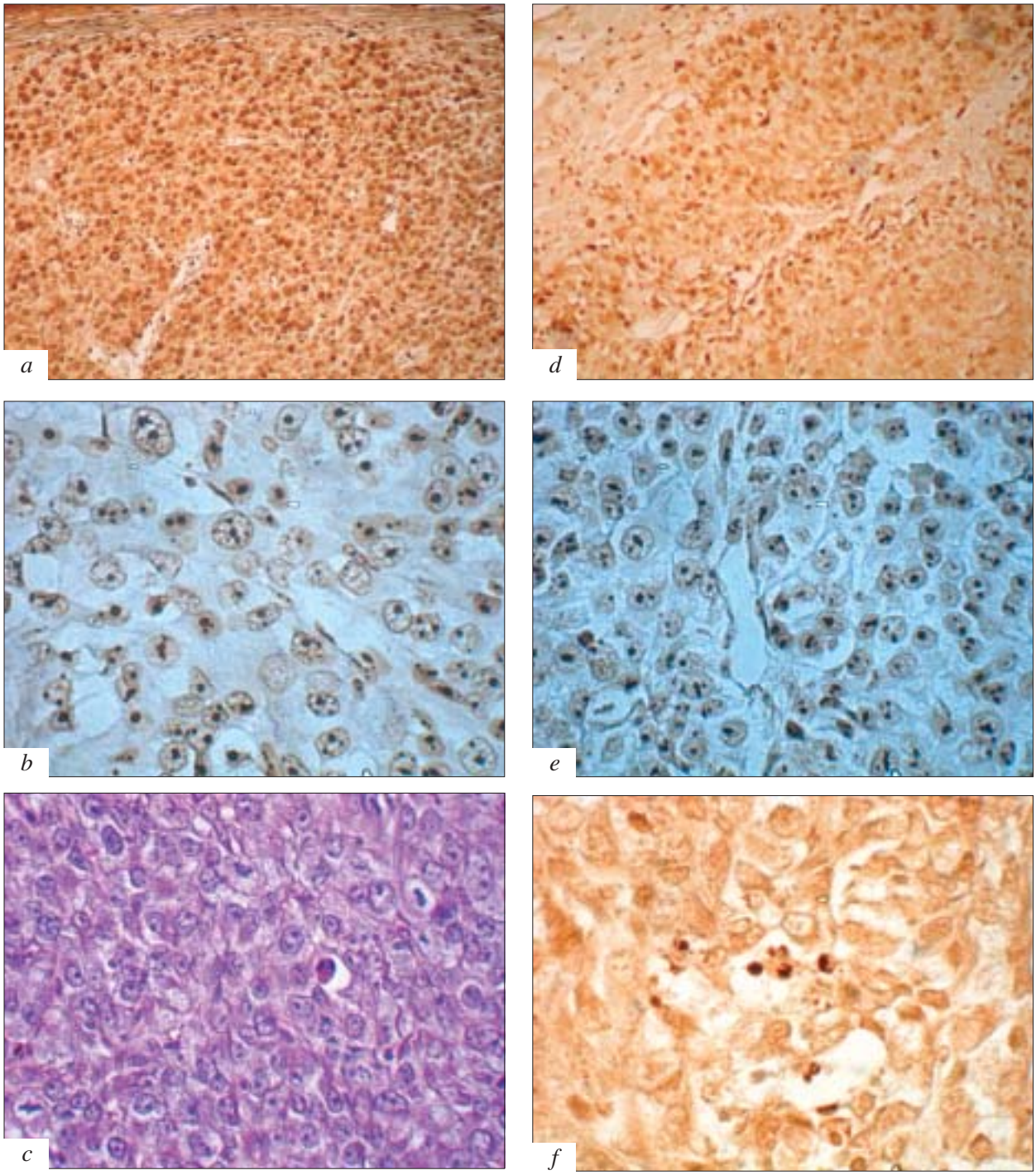

Fig. 2. Functional morphology of M-1 sarcoma in the control (*a-c*) and 3 h after administration of photodithazine (*d-g*). *a, c*: immunohistochemical reaction of cell nuclei with antibodies against PCNA, ×125; *b, e*: AgNOR staining, ×470; *c*: hematoxylin and eosin staining, ×370; *f*: Moser impregnation, ×470.

creased even at the tumor periphery. The increase in tumor volume was paralleled by moderation of PCNA reaction, decrease in I_{PCNA}, and elevation of mitotic and apoptotic indices. On day 33, the dystrophic alterations in tumor parenchyma were accompanied by an increase in the size of nuclei, volume density of NORs and their mean size.

Three hours after injection of photodithazine, immunostaining of the regions of positive reaction of nuclei for PCNA became heterogeneous, especially at the periphery of tumor nodes. The reaction was weaker near the blood vessels, where the characteristic changes in the gradient of immunostaining intensity were observed (Fig. 2, *d*). Pathomorphological analysis of

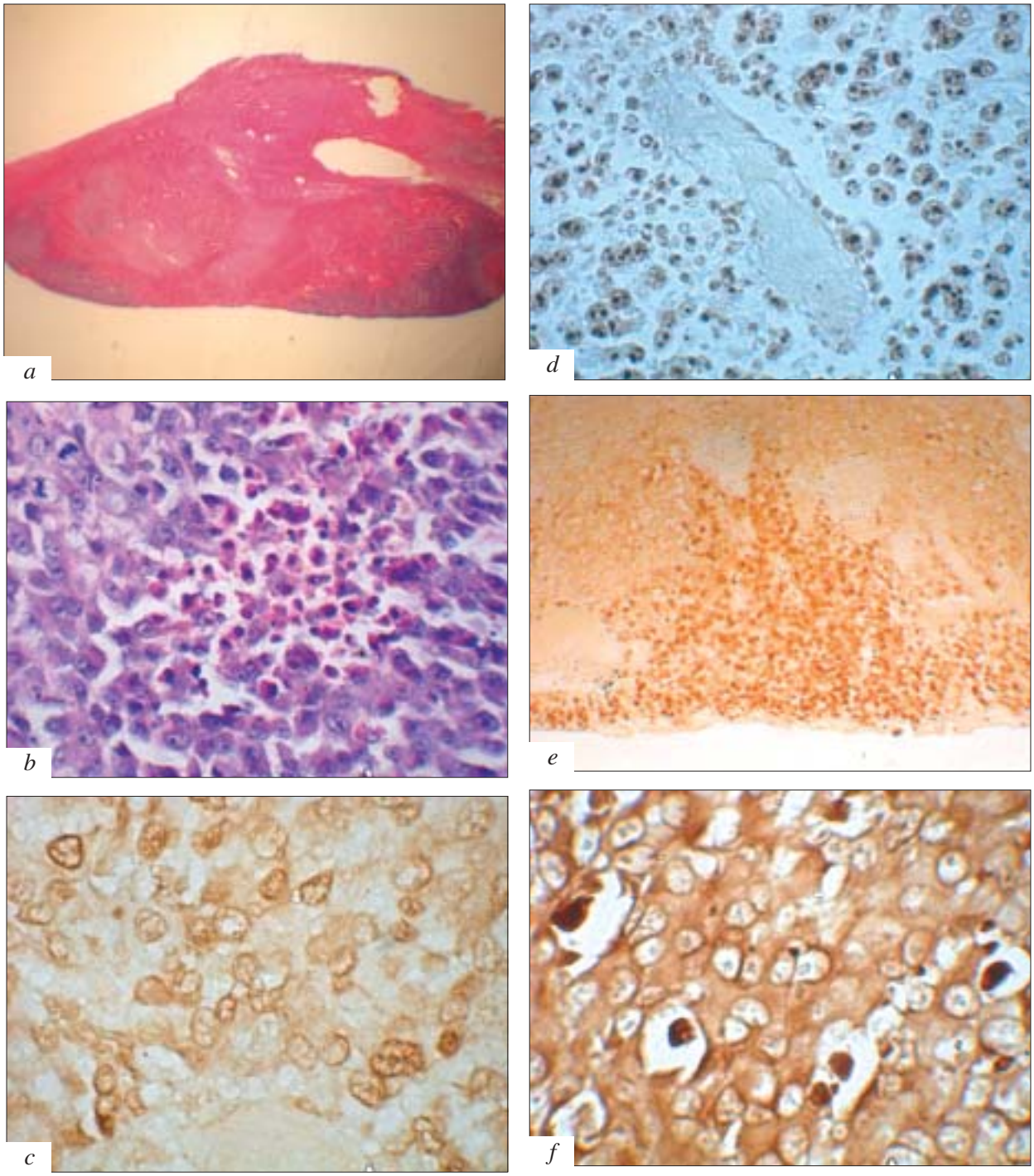

Fig. 3. Effect of photodynamic therapy with photodithazine on M-1 sarcoma 1 h after light dose 300 J/cm^2. *a, b*: hematoxylin and eosin staining, ×7.5 (*a*), ×370 (*b*); *c, e*: immunostaining of tumor cell nuclei with antibodies against PCNA; *d*: AgNOR staining; *f*: Moser impregnation, ×125 (*e*) ×470 (*c, d, f*).

serial sections of these perivascular regions revealed abnormalities in the integrity of vascular wall, degradation and desquamation of the endothelium. These alterations were accompanied by edema with poorly visible plasmatic impregnations and release of the blood cells into the intercellular space. Visually, NORs of tumor cells did not differ markedly from those in the control (Fig. 2, *e*). Some tumor cells clearly degraded, ruptures of the plasmalemma and even karyolemma were seen, which attested to their necrotic death. In addition, small clusters of apoptotic cells appeared in the subcapsular and perivascular area (Fig. 2, *f*). Morphometry showed that 3 h after photodithazine injection, ρ_{PCNA}, intensity of the reaction for PCNA,

and F_{PCNA} decreased by 12, 20 ($p<0.05$) and 15%, respectively. The mitotic index increased to 2.16±0.26%, but taking into consideration the decrease in PCNA reaction, this reflects cytostatic action of the drug, but not stimulation of proliferative activity of tumor cells. The apoptotic index increased 2-fold ($p<0.05$). The volume density of NORs and their size tended to increase.

On day 4 after injection of photodithazine, dystrophy of tumor cells was stimulated in the central parts of the tumors. Necrotic belts appeared at the tumor periphery in the areas of previously destroyed blood capillaries. At the same time, the subcapsular sites demonstrated activation of reproduction of the tumor cells indicated by increased I_{PCNA} and mitotic index. After 12 and 21 days, the parameters of M-1 sarcoma were within the data range observed in the control animals during the corresponding periods.

Light irradiation in a dose of 300 J/cm^2 induced skin erythema above the tumor. After 3 h, the boundaries of the tumors became unclear due to tissue edema, the skin above the tumor became grayish-cyanotic, and parenchyma demonstrated destructive alterations. Destruction foci in the parenchyma with hemorrhages and plasmatic impregnations caused by ruptures of vascular walls were seen in the upper part of the tumor. In specimens stained with hematoxylin and eosin, the destruction areas looked bright eosinophilic. As a rule, the destruction foci were located under the skin and had a crater shape. In some cases, the area of direct photodestruction protruded into the tumor and reached the basement of the tumor node (Fig. 3, *a*).

Most tumor cells located outside the photodestruction area were in a state of cytotoxic shock with characteristic consolidation of karyoplasm and cytoplasm accompanied by the appearance of intracellular clefts (phenomenon of "compression" of tumor cells). Dead endothelial cells were seen in blood vessels wall, while islets and even fields of coagulated necrotic cells were observed along the vessels (Fig. 3, *b*). In the area of cytotoxic shock, the reaction of tumor cell nuclei for PCNA virtually disappeared (Fig. 3, *c*). In addition, these areas were characterized by structural alterations of NOR with the loss of their argyrophilia and degradation or formation of the conglomerate lumps (Fig. 3, *d*). Taking into consideration the fact that the state of NOR in interphasic cells reflects biosynthetic potential of the nucleoli [11], one can hypothesize that photodithazine-based PDT rapidly inhibited functional activity of tumor cells.

Only small fragments of the parenchyma with relatively mild alterations and with PCNA-positive nuclei of tumor cells were preserved in the area of tumor basement (Fig. 3, *e*). The cells with morphological signs of apoptosis were concentrated in the same regions (Fig. 3, *f*). The specimens demonstrated either complete absence of mitotic figures or their zonal accumulation at the metaphasic stage, which attested to the effect of photodithazine-based PDT on cell division processes and on mitotic apparatus. The latter effect indicates the possibility of localizing photodithazine in the cytoplasm and availability of subcellular targets for this agent. The vessels of subcutaneous fat, tumor bed, and those of muscle and connective tissues surrounding the tumor were pronouncedly dilated and filled with stagnated erythrocytes. Three hours after light irradiation, the tumor periphery was filled with numerous cells of inflammatory infiltrate.

Three hours after light irradiation, ρ_{PCNA} and I_{PCNA} decreased to 0.36±0.08 and 54.1±4.3%, respectively, while F_{PCNA} decreased 3.5-fold compared to the control. Increased volume density of NORs (to 16.7±0.78%) probably reflected the reaction of the repair biosynthetic processes in cell systems damaged during oxidative stress. The apoptotic index increased to 0.61±0.07%, which 3.0-3.6 times surpassed the level of spontaneous apoptosis in tumors of control rats on days 12-16 after inoculation. In addition, this value by more than 2 times surpassed the apoptotic index of tumor cells in the terminal period of sarcoma growth. We assume that photodithazine-based PDT can initiate cell death via the apoptotic mechanism.

On day 4 after PDT, subtotal destruction of sarcoma was observed. Only small layers of tumor cells in the state of pathomorphosis were detected. In 2 cases, small solid fragments with signs of resumed cell proliferation were preserved in the tumor bed. The necrotic tissues were surrounded by inflammatory bank consisting of polymorphonuclear leukocytes, lymphocytes, and macrophages eliminating dead cells. Replacement of damaged areas with granulation tissue and intensive angiogenesis were noted. On day 4 after PDT, F_{PCNA} decreased to 12.5±8.9%. The increase in I_{PCNA} (to 76.8±5.4%) and intensification of nuclear staining for PCNA indicated reoxygenation of the sarcoma probably caused by the growth of capillary loops towards tumor cells that survived PDT and restoration of microcirculation.

From histological view, tumor growth resumption observed on days 12 and 21 after PDT was multifocal. At this term, sarcoma M-1 little differed from untreated tumors by morphofunctional parameters. The distinctive features were intensive lymphocyte infiltration of the connective tissue surrounding the tumor nodes, where some tumor cells were lysed. In these zones, tumor cells died via apoptosis were frequently detected.

Thus, administration of photodithazine without PDT delayed the growth of M-1 sarcoma at the early period postinjection. Probably, this background effect of the drug resulted from its natural phototoxicity caused by interaction of the daylight with the photosen-

sitizer accumulated in the tumor. Our data showed that the targets for photosensitizing action of photodithazine are vascular wall, plasmalemma of tumor cells, and intracellular structures and mechanisms responsible for proliferation and biosynthesis. Pronounced antitumor effect of photodithazine-based PDT on the growth and morphofunctional parameters of M-1 sarcoma results from destruction of microcirculatory bed, inhibition of proliferative and functional activities of tumor cells, induction of apoptosis, and development of necrosis.

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