EFFECT OF PHOTODYNAMIC THERAPY ON TUMOR ANGIOGENESIS AND METASTASIS IN MICE BEARING LEWIS LUNG CARCINOMA

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Aim: To investigate the influence of photodynamic therapy (PDT) on the tumor angiogenesis and metastasis. Methods: The effect of PDT with a photosensitizer 5-aminolaevulinic acid (ALA) on tumor metastasis and angiogenesis was studied on the model of subcutaneously implanted Lewis lung carcinoma (3LL) in C57Bl/6 mice. VEGF level in blood serum of animals was evaluated by ELISA. Results: It has been shown that application of ALA-PDT resulted in the lowered rate of metastatic spreading and decreased VEGF level in blood serum of 3LL-bearing mice, and morphologic alterations of vascular system in tumor tissue. Conclusion: The antitumor action of PDT using ALA as a photosensitizer is caused partly by antimetastatic and photodamaging effect mediated by vascularization disturbances in tumor tissue. Key Words: photodynamic therapy, 5-aminolaevulinic acid, photosensitizer, metastases, neoangiogenesis, vascular endothelial growth factor (VEGF).

It is known that not only tumor cells but endothelial cells of vessels too may serve as primary targets upon photodynamic therapy (PDT) [1–3]. Photo-induced damage of endothelium causes an activation of the factors of blood coagulation and vasoactive intermediators, resulting in vessel occlusion disturbing blood supply of tumor and causing its death [4–8]. However, the action of PDT on the tumor vessels has been studied only in recent years [1, 8, 9].

Recently the first reports on the influence of PDT on the level of angiogenic factors in blood serum and ability of this method to suppress tumor angiogenesis have been published, and these few reports show alteration of the level of vascular endothelial growth factor (VEGF) in blood serum of tumor-bearing animals upon PDT [10, 11]. At the same time these studies are lacking trustworthy data on the presence or absence of correlation between VEGF levels in blood serum and metastasis after performed PDT. Basically, it remains unknown yet how PDT affects metastasis.

The aim of present study was to evaluate the influence of PDT using photosensitizer 5-aminolaevulinic acid (5-ALA) on vascularization and metastasis in mice bearing Lewis lung carcinoma (3LL). 5-ALA is a photosensitizer of a second generation, that in tumor cells is converted to protoporphyrin IX which causes an active photosensibilization of cells. The main advantage of 5-ALA compared to hematoporphyrin derivatives is its quick metabolism (the preparation is removed from the body in one day), as well as possibility to apply it locally, thus making it possible to reduce skin photosensitivity — the only relevant side effects of PDT [12, 13].

Experiments were carried out on C57Bl/6 mice bred in the vivarium of R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, NAS of Ukraine (Kyiv, Ukraine). Lewis lung carcinoma (3LL) served as an in vivo model of metastasizing tumor. The single-cell suspension of 3LL carcinoma was transplanted subcutaneously on the external surface of thigh (0.5 x 10^6 cells per animal). All animal procedures were carried out according to the rules of Institute’s Ethic committee. PDT was performed on the day 10 after tumor transplantation.

Hydrochloride of 5-ALA (NH₂CH₂COCH₂CH₂CO₂H) synthesized in the Institute of Organic Chemistry, NAS of Ukraine (Kyiv, Ukraine) was used as a photosensitizer. Preparation has been administered per os at the single dose of 500 mg/kg of body weight in the volume of 0.3 ml of physiologic solution. As a source of radiation, helium-neon lasers LGN-111 (Lviv, Ukraine) with a wavelength of 633 nm, power density of 150 mW/cm² and exposition of 20 min were used. Output power of a single laser is 30–40 mW. To achieve power required for irradiation of experimental tumors, the system of quartz light-guide fibers (“Photonika plus”, Kyiv, Ukraine) was used. Light-guide fiber from each laser was connected with a special device that allows to unite radiation from a few lasers in one light beam. The diameter of light spot made 1.2 cm. The power of radiation was controlled by IMO-2 photometer (Volgograd, Russia).

Tumor-bearing animals were divided into the following groups: 1) Control group (without 5-ALA administration and irradiation of tumor); 2) Laser irradiation of tumors (without preliminary administration of 5-ALA); 3) PDT with administration of 5-ALA (ALA-PDT).

Evaluation of antitumor effect of ALA-PDT was calculated by inhibition of tumor growth (ITG) by formula: ITG = Vc – Vf/Vc x 100%, where Vc and Vf — average tumor volumes (cm³) in the control and experimental groups respectively.

At the day 21 after tumor transplantation (i.e on the day 11 after ALA-PDT) animals were killed by ether narcosis; then the blood samples (to evaluate the VEGF levels) and lungs (for evaluation of metastasis levels — the rate of metastasis, average number and volume of metastasis) were taken.

The level of VEGF was determined by immunoenzyme method [14] using the earlier developed test-system with the use of anti-VEGF-polyclonal antibod-
ies and conjugates of the antibodies with horse-radish peroxidase [15].

In 20 h after ALA-PDT tumors were removed fixed in 10% neutral formaline, embedded in paraffin, sliced and stained by hematoxylin and eosin for light microscopy investigation.

Statistical analysis of the data was performed by Student’s t-test, p values < 0.05 were considered as significant.

PDT has been performed at the day 11 after tumor transplantation — that is at the initial stage of metastatic process.

The analysis of obtained data has shown that application of ALA-PDT resulted in pronounced inhibition of primary tumor growth (Table 1). At day 11 after applied ALA-PDT, average tumor volume was significantly lower than that in control group, and ITG value reached 92.9%. At the same time laser irradiation without ALA administration practically didn’t influence the growth of the tumors.

Upon morphologic examination of tumors after ALA-PDT, the signs of therapeutic pathomorphosis (disintegration of the cells, marked dystrophy alterations of different degree up to necrosis, large zones of hemorrhage) were registered. It has been noted also the significant blood-filling of vessels, in some vessels — hemolysis and thrombosis.

So, the mentioned pathomorphological data allow to conclude that ALA-PDT may result in direct damaging effect of tumor cells, and also in disturbance of tumor vascularization.

Data on influence of ALA-PDT on metastatic activity of 3LL in parallel with changes of the level of VEGF in blood serum of 3LL-bearing animals are presented in Table 1. As it is seen, the laser irradiation without preliminary administration of 5-ALA practically didn’t influence metastatic process: the number of animals with metastases was the same as in control group (100%), whilst average number of metastases and their volume were slightly (and insignificantly) lower than these indexes in control group (p > 0.05). Similarly, laser irradiation didn’t influence significantly nor the average volume of primary tumors, nor the level of VEGF in blood serum of tumor-bearing animals.

In the group 3 (ALA-PDT) the significant suppression of metastatic process has been observed: 80% of animals didn’t develop metastases and in remaining 20% of animals the number of metastases was 1.7-fold lower and their volume — 17-fold lower than these indexes in control group. Also, in the group 3, the substantial (2-fold) decrease of VEGF level compared that in control group has been registered.

As it is known, the tumor and metastases during the growth are undergoing avascular and vascular phases of the development: on a vascular phase the number of tumor cells is increasing, forming spheroid aggregates; on vascular phase, when tumor reaches 1–2 mm in diameter, tumor cells are stimulating generation of new vessels [16].

We have analyzed the influence of ALA-PDT on the growth of metastases assuming that metastases with diameter < 1.0 mm are on avascular phase of growth, whereas these of larger size — on the vascular phase. Proceeding from such assumption on avascular and vascular phases of development of metastases development [17], we have evaluated the relative intensity of vascularization in metastatic lesions (Table 2).

We have revealed that at day 21 after tumor transplantation in the group 3 (ALA-PDT) the part of metastases in “avascular” phase (< 1 mm) was significantly (1.5-fold) higher than in control group (Figure). Application of laser irradiation didn’t alter the distribution of metastases in avascular and vascular phases compared to the control (65.4 and 34.6% versus 64 and 36%, respectively). At the same time, the number of metastases in “vascular” phase in mice that received ALA-PDT was 10-fold lower than in control. Thus, ALA-PDT significantly suppresses vascularization in lung metastases in 3LL-bearing animals.

In conclusion, application of ALA-PDT results in significant inhibition of the growth of primary tumors and suppression of metastatic process along with the reduction of VEGF level.

** — р < 0.05 compared to the control.

** — p < 0.05 compared to the control.

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>Inhibition of tumor growth*</th>
<th>Number of animals without metastases</th>
<th>Average number of metastases per animal</th>
<th>Average volume of metastases per animal, mm³</th>
<th>VEGF content, ng/ml</th>
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<tbody>
<tr>
<td>Control (group 1)</td>
<td>10</td>
<td>–</td>
<td>0</td>
<td>13.2 ± 3.0</td>
<td>48.6 ± 19.6</td>
<td>183.9 ± 2.9</td>
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<tr>
<td>Laser irradiation (group 2)</td>
<td>9</td>
<td>12.4</td>
<td>0</td>
<td>16.3 ± 3.0</td>
<td>41.6 ± 23.9</td>
<td>178.1 ± 3.6</td>
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<tr>
<td>ALA-PDT (group 3)</td>
<td>10</td>
<td>92.9</td>
<td>8</td>
<td>8 ± 6</td>
<td>2.9 ± 2 ± 2 ± *</td>
<td>88.6 ± 5.6± *</td>
</tr>
</tbody>
</table>

Notes: * — day 11 after laser irradiation or ALA-PDT in groups 2 and 3 respectively; ** — p < 0.05 compared to the control.

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<table>
<thead>
<tr>
<th>Group</th>
<th>Diameter of metastases (mm)</th>
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<tr>
<td></td>
<td>0.5</td>
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<tr>
<td>Control (n = 10)</td>
<td>36.5 ± 7.0 27.5 ± 4.4 17.9 ± 3.5 8.6 ± 3.0 3.9 ± 1.9 2.0 ± 1.4 2.0 ± 1.3 1.2 ± 0.6 0.4 ± 0.4</td>
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<tr>
<td>Laser irradiation (n = 9)</td>
<td>37.6 ± 6.8 27.8 ± 4.1 12.8 ± 2.7 10.8 ± 2.8 6.4 ± 2.7 3.3 ± 2.0 0.5 ± 0.5 0.4 ± 0.4 0.4 ± 0.4</td>
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<tr>
<td>ALA-PDT (n = 10)</td>
<td>50.0 ± 0.0 46.5 ± 3.6 3.5 ± 5.5 0 0 0 0 0 0</td>
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** — р < 0.05 compared to the control.

** — p < 0.05 compared to the control.
significant decrease of the level of VEGF and vascularization in lung metastases. Using pathomorphological approach, we have detected that after ALA-PDT the dystrophy of endothelial cells, necrosis, and disturbed circulation of blood in tumors of experimental animals are occurring. The obtained data point on the existence of the vascular-targeted component in ALA-PDT antitumor therapy.

**REFERENCES**