Exp Oncol 2008
30, 4, 315–318

Experimental ONCOLOGY

STUDY OF PHOTODYNAMIC EFFICIENCY OF THE HEMATOPORPHYRIN CONJUGATED WITH ANTIBODY TO VEGF IN MOUSE LEWIS CARCINOMA

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Aim: **To examine photodynamic antitumor and anti-metastatic effect of hematoporphyrin, conjugated with antibodies to VEGF in comparison with native hematoporphyrin in experiments with high metastatic, angiogenesis-independent Lewis carcinoma (LLC) and its low metastatic angiogenesis-dependent variant — LLC/R9.** ***Methods:*** **Mice with LLC or with LLC/R9 were treated by photodynamic therapy (PDT) using new photosensitizer — hematoporphyrin conjugated with antibodies to VEGF, in comparison with hematoporphyrin or aminolevulinic acid. Tumor growth indices and metastasis incidence were calculated.** ***Results:*** **Strong antitumor effect of PDT with new conjugate was demonstrated — delay of tumor development by 70% whereas native hematoporphyrin with the same treatment regime did not show any significant effect. Most pronounced effect of conjugate was demonstrated on the growth and metastasing of LLC/R9, which was characterized by the resistance to chemotherapeutic drugs. Slowdown of the tumor growth — up to complete regression — was detected already at the third day after PDT and, on the average was about 70% of control values. Non-conjugated hematoporphyrin under parameters chosen did not cause antitumor effect. Metastases calculation demonstrated that total metastatic volume reduced by seven times in conjugate group and by 3.5 times in hematoporphyrin group.** ***Conclusion:*** **Hematoporhyrin conjugate studied had high photodynamic antitumor activity. Taking into consideration substantial prevalence in clinical practice of drug-resistant tumors and angiogenesis-dependent character of metastasis process, further study and application of photosensitizers, conjugated with antibodies to VEGF, may help to improve the clinical outcomes.**
Key Words: **photosensitizers, antibodies to VEGF, photodynamic therapy, Lewis lung carcinoma.**

Previously we have shown that photodynamic therapy (PDT) of Lewis lung carcinoma (LLC) with HP - antiVEGF conjugate, in contrast to application of native hematoporphyrin, results in 60% slowdown of tumor growth that, in accordance with provisions of State Pharmacological Center of Ukraine, is considered to be a significant antitumor activity of the agent [1]. These results necessitated subsequent studies of antitumor and antimetastatic activity in other tumor models. Thus, the purpose of present study was further investigation of anticancer action of HP-antiVEGF, in particular, the HP - antiVEGF effect on the growth of primary tumors and metastasis incidence on the model of LLC and its angiogenesis-dependent low-metastasis variant LLC/R9 (LLC/R9). Both tumors form metastasis in lungs after intramuscular or subcutaneous transplantation of tumors.

First, we had to estimate, whether antitumor action of HP-antiVEGF applies to LLC/R9, that was obtained as a result of selective action of cisDDP *in vivo*. Second, we had to clarify how HP-antiVEGF acts at the lifespan of mice with LLC, and also we wish to compare the action of HP-antiVEGF with a known photosensitizer — aminolevulinic acid (ALA). Third, we had to explore the action of HP-antiVEGF as antimetastastatic agent on angiogenesis-dependent LLC/R9.

Received: July 11, 2008.
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Abbreviations used: antiVEGF – antibodies to VEGF; cisDDP – cis-diamminedichloroplatinum; HP – hematoporphyrin; LLC – angiogen-dependent Lewis lung carcinoma; LLC/R9 – angiogen-independent cis-DDP-resistant variant of Lewis lung carcinoma; PDT – photodynamic therapy; VEGF – vascular endothelial growth factor.

MATERIALS AND METHODS

Tumor models. The experiments were done on the 16–20 g mice of CC57 Bl/6 strain at the age of 2 months bred in the vivarium of IEPOR NAS Ukraine. Experimental procedures were approved by the Ethical Committee of IEPOR. Experimental tumor models *in vivo* were Lewis lung carcinoma strains — high and low metastatic variants (LLC and LLC/R9, respectively).

LLC/R9 — is a varient obtained from initial Lewis carcinoma strain after 9 consecutive courses of chemotherapy with cis-DDP [2], strain LLC was received from the National cell culture and tumor strain bank of IEPOR. Mice of CC57Bl line were inoculated into the foot pad with 0.3×10^6 cells either of LLC or of LLC/R9 in 0.1 ml of Hanks solution. Cells were obtained by routine method of cell disintegration with trypsin solution. Experiments were done on the animals with tumors of 0,5–0,7 cm diameter on the 21st day after inoculation.

Experimental treatment. In experimental group HP - antiVEGF was applied for exploration of photodynamic effect. Preparation was dissolved in the mixture of saline (0.9% NaCl) and DMSO in a 5 : 1 ratio. Dose per animal — 3 mg of conjugate in 0.1 ml of solution mixture. Conjugate was inoculated by intraperitoneal injection. Dose of hematoporphyrin in the conjugate was 0.04–0.05 mg. For comparison native hematoporphyrin or ALA (as additional control) were introduced in saline solution by intramuscular injection and per os, respectively, in the dose of 1 mcg/0.2 ml per animal. 24 h after conjugate or hematoporphyrin inoculation and 4 h after ALA treatment tumors were irradiated with laser radiation — initial capacity 25 mWatt, radiation dose —

50 watt-second/cm^2, time of the procedure — 5 min. Other animal groups received either photosensitizer or laser irradiation alone.

Therapy assessments. Mice were observed during 7–40 days after PDT: life span, body mass, tumor size and mass, lymphoid organs mass, size an amount of lung metastases were estimated. Tumor growth kinetics was studied by calculation of the following parameters: dynamics of tumor volume alteration and percentage of growth inhibition. Measurement of tumor diameter was performed with caliper every two days, beginning from the day of the sensitizer introduction. Tumor volume was calculated by the formula:

$$V = 4/3 \times \pi \times R^3,$$

where R — tumor radius.

Tumor growth inhibition was calculated from the ratio of tumor volumes in control and experimental groups by the formula:

$$\text{Tumor growth inhibition} = \frac{V_k - V_e}{V_k} \times 100\%,$$

where V_k — mean tumor volume in control group, V_e — mean tumor volume in experimental group.

Metastases in lungs were calculated by generally adopted method of Bouin fixative immersion on day 30–50 after cells inoculation. Volume of metastatic lesions and the ratio of metastases in vascular and avascular ($d \leqslant 1{,}5$ mm) phases were calculated [3].

Volume of a metastatic lesion was determined by the formula:

$$V = \sum_{i=1}^{N} n \times \frac{\pi \times d_i}{6},$$

where d_i — diameter of metastases, n_i — number of metastases with diameter d_i.

Statistics was calculated with generally adopted methods by Student's t-criterion and coefficient of correlation.

RESULTS AND DISCUSSION

Experiments were performed on three groups of mice: 1st group (control) — mice, that received tumor LLC/R9 cells only; 2nd group — mice that on day 21 after tumor cells transplantation, when the tumors achieved 0.5–0.7 cm in diameter, were injected with HP - antiVEGF; 3rd group — PDT of mice, whose tumors were treated by laser irradiation 24 h after photosensitizer injection. Mice from all groups were sacrificed at the same time 7 days after laser irradiation in the 3rd group. Tumor mass in parallel with body and lymphoid organs mass were estimated.

Results of PDT with conjugated photosensitiser are presented in Table 1. From the data presented in the table it is evident that, in spite of the substantial difference in tumor mass between control and PDT groups, statistically calculated difference is not significant. However, the presence of the animals — PDT responders in the groups of conjugate alone and PDT with conjugate is evident from the data obtained. For potential drugs with antitumor activity, one of the criteria in the assessment of antitumor action is tumor growth inhibition, and for Lewis lung carcinoma the minimal percentage of the growth inhibition should be equal or higher than 50% [1]. Under the conditions of our experiment in the group with conjugate this index is 70%. During this experiment the regimen of observation was adhered to those recommended by State Pharmacologic center of Ukraine — i. e. 7 days after final introduction of the drug (in our case, after performance of laser irradiation). Therefore new conjugated photosensitizer should be considered perspective for further approbation with prospective involvement into clinical practice.

Assessment of absolute and relative lymphoid organs mass ratio between a lymphoid organ mass and a total animal mass, estimated by comparison of organ indices in different groups, did not reveal statistically significant differences. As Table 1 shows, dispersion (standard deviation) for each index inside each group was quite significant, therefore, we considered appropriate to carry out correlation analysis for such indices as spleen index, thymus index and tumor masses for each animal separately (Table 2).

Table 2. Ratio between relative masses of lymphoid organs and tumor masses during photodynamic therapy of LLC/R9 with HP-antiVEGF

Group	Correlation coefficient between:		
	Spleen index/ Thymus index	Spleen index/ tumor mass	Thymus index/ tumor mass
Control LLC/R9	–0.97 (–0.44)*	0.86 (–0.93)*	–0.96 (0.06)*
HP-antiVEGF	0.10 (–0.51)*	0.01 (–0.02)*	0.42 (–0.18)*
PDT with HP-antiVEGF	–0.38 (–0.37)*	0.78 (–0.06)*	–0.63 (–0.3)*

Note: in parenthesis data from LLC original strain.

Direct correlation was found between ratios of spleen index and tumor mass. Also, the strong inverse correlation was revealed between ratios of thymus index and tumor mass in untreated mice with tumors. As it is known, spleen is secondary immune organ, where sensitized lymphocytes migrate from lymph nodes. In spleen induction of T-dependent B-cell immune response, generation of B-lymphocytes, producing antibodies, and proliferation of CD8+ T-lymphocytes take place. Therefore, the direct correlation between spleen index and tumor mass may indicate the stimulation of these functions by the developing tumor.

It is known that two studied variants of Lewis carcinoma are different in their influence on immune system. One of differences between LLC and LLC/R9 is different impact of tumor process on lymphoid organs mass. In tumor mice with LLC carcinoma the most pronounced involution of thymus in comparison with intact control animals takes place. Those effects have considerable

Table 1. PDT with HP-antiVEGF effect on the development of resistant strain LLC/R9, transplanted into the foot pads of mice C57Bl – 7 days after PDT

Group	Body mass (g)	Spleen mass (mg)	Thymus mass (mg)	Spleen index	Thymus index	Tumor mass (mg)
Control R9 (n = 7)	18.2 ± 2.25	164.67 ± 81.02	41 ± 8.88	8.9 ± 3.94	2.26 ± 0.51	54 ± 43.03
HP-antiVEGF (n = 6)	18.18 ± 2.4	104.16 ± 26.32	39.16 ± 7.33	5.9 ± 1.97	2.17 ± 0.45	49.7 ± 46.9
PDT with HP-antiVEGF (n = 6)	21.26 ± 3.08	145.16 ± 60.25	51.5 ± 12.29	6.77 ± 2.46	2.4 ± 0.34	16 ± 15.18 Inhibition – 70% *p* contr. > 0.05

input on tumor-induced suppression. The last is caused by decrease of T-lymphocytes number due to thymus atrophy, which means inhibition of antitumor reactions [4]. In mice with LLC/R9 involution of thymus also takes place, however, in less extent than in mice with LLC. In animals with LLC/R9 tumor the highest size of spleen and spleen index in comparison with intact mice was detected. Augmentation of the named indices may be the result of the activation of humoral responce of tumor bearing host. Tables 1 and 2 show that photodynamic therapy with conjugate somewhat normalizes thymus indices, in other words, inhibits involution of this organ, while there was no noticeable influence on splenomegaly.

Thus, high antitumor activity of the conjugate (inhibition of tumor growth by 68%) was also confirmed with LLC/R9.

The other experiments demonstrated that photodynamic therapy with conjugated photosensitizer, in contrast to PDT with hematoporphyrin leads to more than 60 percent inhibition of tumor growth (Table 3). This index was obtained 7 days after the conducted therapy — the term officially defined for registration of antitumor activity of the investigated preparations. Further we studied whether the single application of conjugate could lead to longer survival of animals — up to complete recovery of tumor-bearing mice. It was also necessary to compare the effects of the conjugate with those of well-known photosensitizers — hematoporphyrin and ALA [5, 6].

The experiment included 4 groups of mice, bearing LLC: group 1 — treated with conjugate HP - antiVEGF; group 2 — treated with HP; group 3 — treated with ALA; untreated control with LLC.

The animals were observed for seven weeks after PDT — until death of the last mouse in control group. The Figure presents the dynamics of the survival in tumor bearing animals.

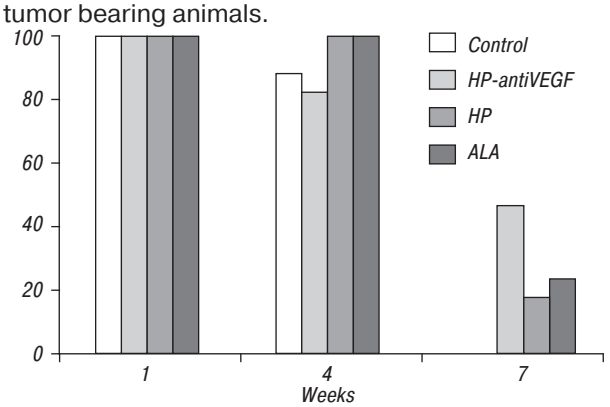

Figure. Survival (%) of mice with LLC after photodynamic therapy with different sensitizers

As the presented data show, up to four week after photodynamic therapy the survival of animals remains at about the same level in all groups. Seven weeks after treatment (9 weeks after tumor inoculation) all mice in control group and hematoporphyrin group died. Survival in group with conjugated hematoporphyrin was 40%, and in ALA group — 26%.

Therefore, it may be concluded that the conjugate of hematoporphyrin with VEGF antibodies was more effective antitumor agent in comparison both with native hematoporphyrin and with other widely used photosensitizer — aminolevulinic acid.

The next step in the study of antitumor activity of the conjugate was elucidation of its potential to induce long or suspended antitumor action in case of single application of PDT, as well as affecting metastases formation. Also, aiming at perspective clinical application we had to study regression/progression of tumor process in each separate mouse.

Mice with LLC/R9 tumors were divided into three groups with 10–12 animals in each group. The volume of primary tumor was measured. The inhibition of tumor growth on day 21 after inoculation (date of therapy start, in Table - day 0), on the 7th, 14th, 21st and 27th day (day of animals sacrificing) was calculated. The results of this study revealed that the conjugated preparation had pronounced antitumor activity towards LLC/R9 (Table 3).

Table 3. Dynamics of change of tumor volume in animals with transplanted LLC/R9 after photodynamic therapy

Day after PDT	Control	Hematoporphyrin		HP-antiVEGF	
	V of tumor, mm^3	V of tumor, mm^3	Percent of growth inhibition	V of tumor, mm^3	Percent of growth inhibition
0**	60.38 ± 23.3	51.94 ± 23.2	–	34.71 ± 20.4	42%
7	181.68 ± 4.2	104.27 ± 77.6	43%	70.6 ± 10.1	61%
14	866.46 ± 373.6	503.75 ± 205.5	–	245.67 ± 42.6	72%
21	1637.18 ± 345	948.5 ± 649.3	–	153.0* ± 27.3	90%*
28***	1970.0 ± 111.7	1136.02 ± 52.5	42%	402.36 ± 62.7	80%

*The marked indices statistically significant differ from analogous indices in control group. $p < 0.05$; **the day of laser treatment – 1 day after application of photosensitizer; ***term of sacrificing of animals.

In particular, it was shown that conjugate has not only immediate, but also long-term, suspended antitumor action. Thus, conjugated hematoporphyrin inhibits by 61% the growth of primary tumor by the 7th day after photodynamic therapy comparing to control and elicits the long-lasting therapeutic effect, as by day 21 after PDT with the conjugate the percent of tumor inhibition grew to 90% ($p < 0.05$).

The subsequent data (Table 4) show that conjugated hematoporphyrin decreases both general number of metastases in a group (2.5 times), and volume of metastases (4 times) due to domination as a rule of metastases in avascular phase (data not presented). Thus, the conjugate may suppress vascularization of metastases, and PDT with this photosensitizer gains the characteristics of antiangiogenic therapy [7].

Table 4. Influence of photodynamic therapy on growth and metastases of LLC/R9

	Control	Hemato-porphyrin	HP-antiVEGF
Primary tumor volume, mm^3	1970.0 ± 1115.7	1136.02 ± 852.5	402.36 ± 223.7
Average number of metastases in lungs	10 ± 5.3	2.56 ± 2.9	1.72 ± 0.6
Average volume of metastases, mm^3	8.66 ± 5.3	2.36 ± 2.7	0.96 ± 0.5
General number of metastases in lungs	50	23	19
General volume of metastases, mm^3	43.3	21.21	10.45

Therefore, high antitumor activity of conjugated hematoporphyrin-antiVEGF (inhibition of growth of epidermoid Lewis carcinoma by 68%) was also confirmed for angiogenesis-dependent variant of Lewis carcinoma — LLC/R9. We observed that this therapeutic effect was sustained, as by the day 21 after PDT inhibition of tumor growth increased to 90%. The

conjugated hematoporphyrin decreased both general number of metastases in a group (2.5 times) and the volume of metastases (4 times).

The conjugate of hematoporphyrin with antibodies to VEGF was more effective antitumor agent in respect of survival prolongation in animals with Lewis carcinoma in comparison with both native hematoporphyrin and other widely spread photosensitizer — aminolevulinic acid.

Taking into consideration substantial prevalence in clinical practice of drug-resistant tumors and angiogenesis-dependent character of metastasis process, further study and application of photosensitizers, conjugated with antibodies to VEGF, may help to improve clinical outcomes.

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ИЗУЧЕНИЕ ФОТОДИНАМИЧЕСКОЙ ЭФФЕКТИВНОСТИ КОНЪЮГАТА ГЕМАТОПОРФИРИНА С АНТИТЕЛАМИ К VEGF НА МЫШАХ С КАРЦИНОМОЙ ЛЕГКОГО ЛЬЮИС

Цель: **исследование возможного противоопухолевого и антиметастатического действия гематопорфирина, конъюгированного с антителами к VEGF, по сравнению с исходным гематопорфирином при фотодинамической терапии высокометастатической зависимой от ангиогенеза карциномы Льюис (LLC) и ее низкометастатического независимого от ангиогенеза варианта — LLC/R9.** ***Методы:*** **мышам с LLC или с LLC/R9 проводили фотодинамическую терапию (ФДТ) с новым фотосенсибилизатором — гематопорфирином, конъюгированным с антителами к VEGF, при сравнении с исходным гематопорфирином или аминолевулиновой кислотой. Учитывали размеры опухоли и развитие метастазов.** ***Результаты:*** **при ФДТ с конъюгатом отмечен выраженный противоопухолевый эффект — задержка роста опухоли на 60–63%, в то время как исходный гематопорфирин в выбранных условиях значимого эффекта не оказывал. Наибольшее действие конъюгат оказывал на рост и метастазирование варианта опухоли LLC/R9, характеризующегося резистентностью к химиопрепаратам. Задержку опухолевого роста — вплоть до полной регрессии — отмечали уже на 3-и сутки после ФДТ и в общем она составляла около 70% по сравнению с контролем. Подсчет количества метастазов показал, что общий объем метастазов снизился в 7 раз в группе с конъюгатом и в 3,5 раза в группе с гематопорфирином.** ***Выводы:*** **принимая во внимание значительное превалирование в клинической практике химиорезистентных опухолей, а также зависимого от ангиогенеза характер метастазирования, дальнейшее изучение и применение фотосенсибилизаторов, конъюгированных с антителами к VEGF, может улучшить результаты лечения.**

Ключевые слова: **фотосенсибилизаторы, антитела к VEGF, фотодинамическая терапия, карцинома легкого Льюис.**