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CHRONOBIOLOGICAL APPROACHES TO ANTIANGIOGENIC PHOTODYNAMIC THERAPY OF TUMORS: THE FIRST EXPERIMENTAL EVALUATION

N.F. Gamaleia[1], I.A. Lisnyak[1], E.D. Shishko[1], A.A Mamchur[1], I.V. Prokopenko[1], V.V. Kholin[2]*
[1]Kavetsky Institute for Experimental Pathology, Oncology and Radiobiology NAS of Ukraine, Kiev 03022, Ukraine
[2]SME "Photonika Plus", Cherkassy 18023, Ukraine

In research of the last decade, rhythmic (circadian) variations of vascular endothelial growth factor (VEGF) production by tumors were discovered. The present paper authors have earlier synthesized and characterized a new derivative photosensitizer — an immunoconjugate of hematoporphyrin with antiVEGF antibodies. *Aim*: To elaborate and to test a novel modification of the photodynamic therapy of tumors (PDT) method, founding upon a timed introduction of the immunoconjugated photosensitizer to tumor-bearing animals, so that this coincides with a maximum content of VEGF in tumor tissues. *Methods*: Circadian variations of VEGF contents in murine transplanted tumors, Lewis lung carcinoma and sarcoma 180, were determined by ELISA method. Immunoconjugated photosensitizer concentrations in tumors were estimated by spectrofluorometry. Photoirradiation of the tumors was carried out with a red light (wavelength of 635 nm) from a semiconductor laser. Light doses were chosen, calculating on a partial inhibition of tumor growth, in order that a dependence of PDT efficiency on a daily time-moment (circadian rhythm phase) of the treatment could be observed distinctly. *Results*: Circadian variations of the VEGF levels in Lewis lung carcinoma and sarcoma 180 were demonstrated with the maximum at 14:00 h and the minimum at 02:00 h. Intra-abdominal introduction into tumor-bearing mice of the immunoconjugated photosensitizer resulted in a greater accumulation of the immunoconjugate in tumors at 14:00 h than at 02:00 h. Laser irradiation of carcinomas and sarcomas at 14:00 h or 02:00 h after introduction of the immunoconjugated photosensitizer to mice the day before at the same time points, induced a significantly enhanced inhibition of tumor growth in animals treated at day-time versus those treated at night-time. Conclusion: The obtained results justify further attempts to transfer principles of tumor chronochemotherapy onto photodynamic therapy.

***Key words*: circadian rhythm, circadian rhythm-guided PDT, chronotherapy, immunoconjugated hematoporphyrin, antibodies to vascular endothelial growth factor.**

Photodynamic therapy of tumors (PDT), a comparatively new, modern method of cancer treatment, has a remarkable set of merits such as a considerable selectivity of antitumor effects, a low invasiveness and an absence of serious side reactions. However, due to a poor penetration of light radiation into biological tissues, the method practical application is rather limited, and radical curative results with it are feasible only in early stages of cancer, in tumors with a superficial type of growth, and so on. To partly overcome the drawback, efforts are undertaken to synthesize combined photosensitizers containing antibodies or other biologically active agents (peptides, growth factors, cytokines, etc) more or less specific for malignant cells [1, 2]. Such photosensitizers are better accumulated in tumor tissues, making them responsive even to a scarce light that penetrates into a tumor depth.

Another way to obtain photosensitizers, targeted to a site of tumor growth, is to conjugate them with angiogenic elements of vessels that are actively formed in a growing tumor. In particular, the conjugates of photosensitizers with factors (inductors) of angiogenesis or antibodies to them can be created. In this context, our attention was drawn to the observations in which rhythmic (circadian) character of a secretion by tumors of a vascular endothelial growth factor (VEGF) was established [3]. Since earlier we synthesized and characterized a conjugate of hematoporphyrin with antiVEGF as the means to enhance transportation of the photosensitizer into tumors [4, 5], we now explored a possibility to raise efficiency of PDT with this immunoconjugated photosensitizer by timing its introduction in tumor-bearing animals relatively to the circadian rhythm of VEGF secretion by tumors.

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*Correspondence: Fax: 8 044 258 16 56
E-mail: gamaleia@onconet.kiev.ua

Abbreviations used: ELISA – enzyme-linked immunosorbent assay; PDT – photodynamic therapy of tumors; VEGF – vascular endothelial growth factor.

MATERIALS AND METHODS

Circadian variations of VEGF production were studied in mice with transplanted tumors — Lewis lung carcinoma and sarcoma 180. Two month old male mice, bred in the animal facility of R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, NAS of Ukraine, were utilized. All animal procedures were carried out according to the rules of local Ethic Committee and were approved by the Ethic Board of IEPOR NASU. The tumors were excised during 24 hours at four different time points: at 08:00 h, 14:00 h, 20:00 h and 02:00 h. VEGF from tumor samples was isolated and evaluated for the factor concentration and specifity by ELISA method using recombinant VEGF-165 (Sigma, USA) and monoclonal antiVEGF antibodies (Sigma, USA) as described in [6].

To synthesize the combined photosensitizers, antibodies to VEGF were conjugated to hematoporphyrin dihydrochloride (Fluka, Netherlands) emploing 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide-HCl as a linker [7]. Molar ratio of anti-VEGF antibodies to hematoporphyrin in the immonoconjugate was

estimated spectrophotometrically by absorption at 280 nm and 505 nm, respectively.

To ascertain that accumulation of the hematoporphyrin-antiVEGF in tumors obeys circadian rhythmicity, the immunoconjugated photosensitizer or free hematoporphyrin (in a parallel control group) were injected to mice intra-abdominally, 3 mg per mouse of the immunoconjugate and 0.05 mg per mouse of the free photosensitizer, so that doses of hematoporphyrin were in both cases equal. Photosensitizer contents in samples were determined by a hematoporphyrin fluorescence (λ_{max} 620, 680 nm, fluorospectrometer Nanodrop 3300, USA) in tumor tissue samples after their freezing in liquid nitrogen, mechanical homogenization and extraction with a methanol-water solution.

For circadian rhythm-guided PDT, two tumor models of different histological origin were used: metastasizing Lewis lung carcinoma, transplanted into a foot pad of C57Bl/6 mice, and non-metastasizing sarcoma 180, transplanted under the skin of white outbred mice. The therapy started when carcinomas reached a diameter of 5–7 mm and sarcomas — 10 mm. The conjugated photosensitizer (3.0 mg/mouse) or free hematoporphyrin (0.05 mg/mouse) were introduced intra-abdominally at 14:00 h or 02:00 h, that is at time points of a maximum and minimum accumulation of the immunoconjugated photosensitizer, respectively, as it was preliminarily established by experiments on determination of circadian variations in the VEGF production, described above. In 24 hours after the photosensitizer introduction, tumors were treated with red-light radiation (wavelength of 635 nm, power density of 26 mW/cm^2, dose of 30 J/cm^2) from a semiconductor laser (Photonika Plus, Ukraine). A treatment efficiency was estimated by excised tumor masses (Lewis carcinoma) or by tumor sizes on the second, ninth and 12th days after animal photoirradiation (sarcoma 180).

In all three series of animal experiments (on determination of a rhythmicity in the VEGF production /I/ and in the immunoconjugated photosensitizer accumulation /II/, as well as on the circadian rhythm-guided PDT /III/), before every experiment was started, mice were kept under continuous darkness for two weeks, so that a free-running circadian rhythm set in. Then, the regimen was switched to an alternation of light and darkness (light from 08:00 h to 20:00 h, darkness from 20:00 h to 08:00 h), and under these conditions animals remained until the end of the experiment.

All the experiments were performed in autumnal season (October — November).

For all data calculations the statistical t-test was used.

RESULTS AND DISCUSSION

Circadian variations of the VEGF content in tumors. VEGF concentrations were determined for 24 h at four different time points in the samples of tumor tissues, obtained from mice with Lewis lung carcinoma or sarcoma 180. As a result, regular circadian fluctuations of the angiogenic factor production were observed: a maximum content of the factor in tumors fell on the middle of the day-time and a minimum — on the middle of night (Fig. 1).

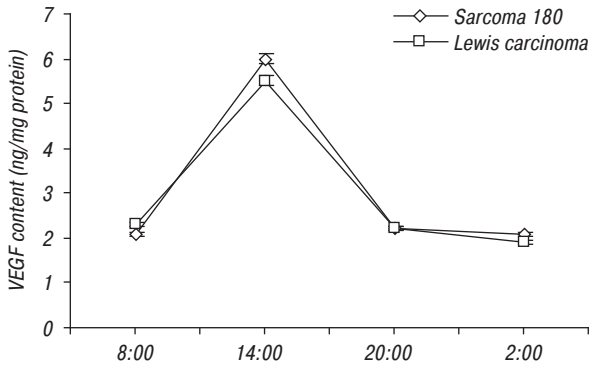

Fig. 1. Circadian variations of the VEGF content in sarcoma 180 and Lewis lung carcinoma. Data for every time point are presented as the mean ± SD of three animals

Circadian character of the immunoconjugated photosensitizer accumulation. The immunoconjugated photosensitizer hematoporphyrin-antiVEGF was introduced to mice intra-abdominally at two different time points: at 14:00 h or 02:00 h. Next day, at the same time points, tumor samples were obtained, and the accumulated photosensitizer fluorescence was measured by spectrofluorometry. Fluorograms, presented in Fig. 2 and 3, show higher fluorescence levels (and therefore, elevated photosensitizer accumulation) at the time of VEGF production maximum (at 14:00 h), in comparison with its minimum (at 02:00 h).

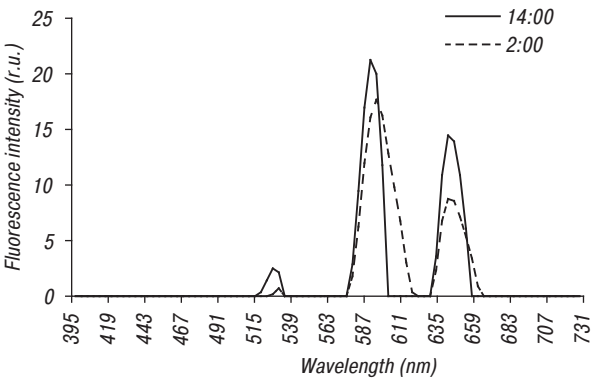

Fig. 2. Immunoconjugated photosensitizer fluorescence in Lewis carcinoma as a function of daily time-points (circadian rhythm phases) exploited for the photosensitizer introduction/analysis

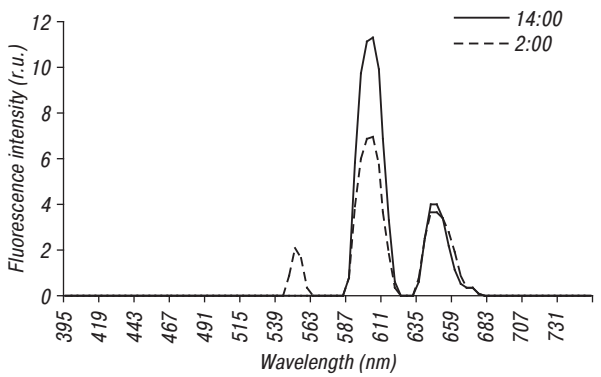

Fig. 3. Immunoconjugated photosensitizer fluorescence in sarcoma 180 as a function of daily time-points (circadian rhythm phases) exploited for the photosensitizer introduction/analysis

Circadian rhythm-guided photodynamic therapy. Proceeding from the data on circadian variations of VEGF production by tumors and on a synchronism of this

process with hematoporphyrin-antiVEGF accumulation in tumor tissues, we carried out experiments on a rhythm-guided PDT of Lewis carcinoma and sarcoma 180, using the immunoconjugated photosensitizer. Parameters of laser irradiation were chosen in anticipation of a partial inhibition of tumor growth, so that a dependence of PDT efficiency on a daily time-moment (circadian rhythm phase) of the treatment could be observed distinctly.

As it is shown in Fig. 4, PDT treatment of mice with carcinomas on a middle of the day-time (at 14:00 h), that is at the time of the maximum in VEGF content, resulted in significantly ($p < 0.05$) greater tumor growth inhibition than the same treatment executed at 02:00 h of night which corresponded to the time of the minimum in VEGF content of tumor tissues. In the group of animals, treated at the day-time, growth inhibition made 60.4% comparing to control (untreated) animals, and in the group with the night-time treatment it amounted to 45%.

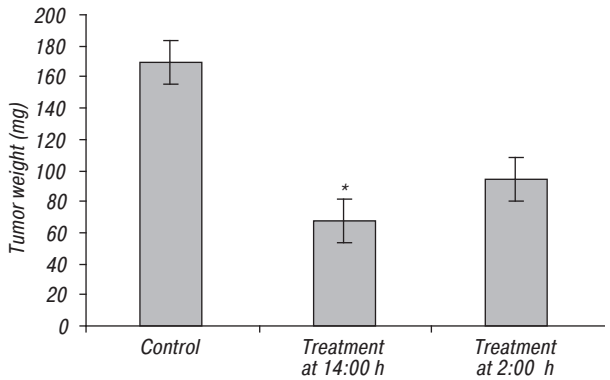

Fig. 4. Circadian rhythm-guided PDT of Lewis carcinoma with application of the immunoconjugated photosensitizer.

*The difference is significant as compared to both control and 02:00 h groups ($p < 0.05$)

Since in studies on the circadian rhythm-guided PDT with another experimental tumor — sarcoma 180 treatment efficiency was evaluated by a tumor size, there was a possibility to follow the tumor growth dynamics. In Fig. 5 the data obtained in these experiments are presented. As it is seen from the data, tumor growth inhibition was again considerably more pronounced in the group of mice, treated at 14:00 h. Virtually, tumors in this group were not growing for whole the observation period.

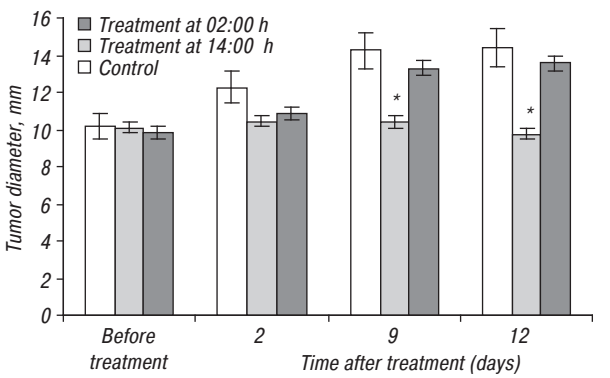

Fig. 5. Circadian rhythm-guided PDT of sarcoma 180 with application of the immunoconjugated photosensitizer.

*The difference is significant as compared to both control and 02:00 h groups ($p < 0.05$).

Thus, presented results apparently corroborate our assumption that PDT application of the immunoconjugated photosensitizer hematoporphyrin-antiVEGF, timed with the maximum in circadian variations of the VEGF content in tumors, may open a fresh opportunity for enhancing the therapy effectiveness. Although chronobiological approaches to the chemotherapy of tumors were already recognized for quite a long time [8–10], such chronotherapeutic modifications of PDT have not been, to our knowledge, proposed before. Only very recently we have formulated basic principles of the method which may be designated as a chronophotodynamic therapy of tumors [11]. The promising results obtained in our study justify further attempts to extend the principles of antitumor chronochemotherapy to PDT. Moreover, so far as the conjugated photosensitizers development is on the rise, it probably makes sense to expand the research scope, not confining it to the VEGF or angiogenic factors in general.

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