Time-resolved fluorescence imaging technique for rat brain tumors analysis

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Abstract. The paper presents a new approach to assessing the state of tissues that differ in phenotype and in the degree of immunocompetent cells activity using photosensitizers (PS) and time-resolved fluorescence analysis methods. The main attention is paid to the detection of differences between tumor cells and tumor-associated macrophages (TAM) using spectroscopic and microscopic methods by the fluorescent kinetics signal and the difference in the accumulation of PS (the accumulation is several times greater in macrophages). The results of the PS photoluminescence study were obtained using two different techniques: time-resolved spectroscopy and time-resolved fluorescence microscopy (FLIM). Time-resolved spectroscopic analysis of the PS fluorescence lifetime was performed on adult female rats with induced C6 glioma in vivo. 5-ALA-induced Pp IX, which is widely used in clinical practice for carrying out effective conduction photodiagnostics and PDT, was used as the PS.

1. Introduction

Today, the problem of diagnosis and therapy of glial brain tumors is acute. Due to the impossibility of their total surgical removal, the accompanying treatment methods are usually radiation- and chemotherapy [1], as well as immunocorrective therapy and specific antitumor immunotherapy developed in some clinics, which are not currently the standard and are at the stage of clinical trials [2]. Despite the continuous development of the technical equipment for clinics and the improvement of navigation methods during surgical removal, it is not possible to significantly improve the results of the combined treatment of malignant gliomas, and patient survival does not exceed 14 months. In this regard, the development of a fundamentally new approach to the therapy of deep-lying brain tumors is relevant. According to the latest data, the immune system plays the most significant role in the invasion and progression of the tumor, namely, immunocompetent cells, both resident (microglia) and newly arrived because of the disrupted blood-brain barrier (macrophages). Immune system agents, which, according to some sources account for up to 60% of the total tumor volume [3], are reprogrammed by the tumor, becoming tumor-associated and standing up for its defense. Thus, to increase the effectiveness of the treatment of brain tumors, there is acute problem of rapid and objective...
comprehensive assessment of the tissue state during surgery or a laser-induced therapy session. The comprehensive assessment should include several parameters that characterize not only the tissue state at the moment, but also a forecast of its possible evolutionary changes of pathological nature or normalization that may occur as a result of or in the absence of timely surgical or therapeutic treatment. The use of laser-spectroscopic methods provides a unique opportunity to non-invasively determine the most significant parameters identifying the state of tissues and to control, in particular, the dynamics of accumulation and elimination of photosensitizers, including nano-photosensitizers, the presence of immunocompetent cells in the tissue, that signals the severity of the disease and the likely direction of its development.

Most tumor cells are known to efficiently accumulate photosensitizers (PS), but it was also found out that immunocompetent cells can accumulate the PS to 10 times more, in particular, tumor-associated agents. Thus, with the systemic administration of PS, as a result of variety in the cellular metabolism and drug accumulation not only by tumor cells, but also by immunocompetent cells, it seems possible to evaluate the cellular composition of pathological tissue based on differences in the fluorescence lifetime of the PS. The main goal of this research was to develop a new approach directed on the assessment of the tissues state differing by the phenotype of the existing macrophages and by the level of their activity, using time-resolved laser spectroscopy with PS.

2. Materials and methods

2.1. Biological materials

To conduct this study, a series of experiments was performed on adult female Wistar rats weighing 200–220 g at the beginning of the experiment with simulated glioblastoma multiforme by stereotactic implantation of 5×10^6 C6 glioma cells into the striatum (4). Dynamic magnetic resonance imaging (MRI) of the rat brain to assess the dynamics of the intracranial tumor development was performed on BioSpec 70/30 tomograph (Bruker, Germany) with a constant magnetic field of 7 T. A photosensitizer was injected into the femoral vein of rats under ketamine anesthesia (100 mg/kg of body weight). For analysis of brain tissue at the microscopic level, 20 μm thick cryosections were prepared.

2.2. Studied photosensitizer

An effective endogenously formed photodynamic agent that has been widely used in clinical practice for the treatment and diagnosis of cancer (especially gliomas of high malignancy) is 5-aminolevulinic acid-induced protoporphyrin IX (5-ALA-induced Pp IX)(5, 6). Pp IX itself is an intermediate product of the chain of heme synthesis from 5-ALA. 5-ALA-induced PP IX is characterized by high fluorescence contrast and increased accumulation in some types of rapidly proliferating tissues, characterized by a lack of ferrochelatase. The lack of the ferrochelatase enzyme in tumor cells, compared to normal cells, results in the accumulation of Pp IX not only inside the cell, but also into the intercellular space. Pp IX accumulates and remains in significant quantities in the tumor for several hours, while in normal cells it quickly turns into a photoinactive heme under the action of ferrochelatase. In this work, preliminary studies of a neuroport with an internal fiber-optic structure in vivo were carried out using the Alasens® pharmaceutical (NIOPIK, Russian Federation) on a model of experimental animals with induced C6 glioma. A sterile aqueous solution of PS for application (concentration c = 100 mg/kg), was prepared an hour before use by dissolving the required amount of Alasens® powder in a 5% sterile solution of sodium bicarbonate.

2.3. Study of fluorescence lifetime using a streak camera

To study the photoluminescence kinetics of PS under in vivo conditions (not invasive in experimental animals), a measuring complex based on a Hamamatsu C10627-13 streak camera (15 ps time resolution) coupled with a fiber-optic spectrometer was used. A Hamamatsu semiconductor laser with a pulse duration of 67 ps and a wavelength of 637 nm was used for excitation. The measurement process incorporated a time-correlated single photon counting method. Photoluminescence excited by a laser source is collected in an optical fiber and is then resolved spectrally.

2.4. Study of fluorescence lifetime using a laser scanning microscope
We used a method for recording PS fluorescence lifetime using a FLIM attachment for a confocal laser scanning microscope, which makes it possible to estimate the distribution of lifetime at different points of the test sample based on cryosections of the brain of the experimental animals obtained directly after in vivo streak camera measurements. The actual localization (accumulation region) and the fluorescence spectrum of PS or endogenous fluorophores is estimated by analyzing the obtained images using fluorescence microscopy. Studies of the fluorescence kinetic characteristics were carried out using LSM-710-NLO laser scanning microscope (Carl Zeiss, Germany). The samples were excited using Chameleon Ultra II femtosecond pulsed laser (80 MHz, pulse duration 140 fs, wavelength range 690–1060 nm, Coherent Inc., USA) at 980 nm. Images were obtained in the following scan modes: 20× lens, scan area 400×400 μm, resolution 1024×1024 pixels, scan speed 1.2–3.2 μs/pixel. The total image acquisition time was 18 s. The average power density measured using a Coherent power meter (USA) at the sample position was 5.4 mW; a scanning spot of 10 μm in diameter had a power density of 7 kW/cm².

A study of photosensitizer accumulation in the cellular structures of the brain was carried out by cryosections with a thickness of 20 μm using confocal microscopy (LSM-710-NLO Carl Zeiss, Germany). Photodynamic therapy using 5-ALA-induced protoporphyrin IX (c = 100 mg/kg) was carried out by laser radiation at λ = 635 nm, a dose of 200 J/cm².

3. Results

3.1. FLIM analysis of the PS fluorescence lifetime

Time-resolved spectroscopic analysis of the PS fluorescence lifetime was performed on adult female rats with induced C6 glioma in vivo. 5-ALA was administered to experimental animals in the tail vein at a dose of 100 mg/kg under general anesthesia. Pp IX accumulation level control in normal and in vivo tumors was carried out using LESA-01-Biospec (Biospec, Russian Federation) fiber (7) spectrometer. Photodynamic treatment was performed at the experimentally determined moment of highest PS accumulation (t = 3 h). The control of the fluorescence lifetime of PS before and after PDT (after a repeated systemic administration of PS) was carried out using a measuring complex based on a streak camera with a picosecond time resolution coupled to a fiber-optic spectrometer. The presence of 3 different components characteristic of the lifetime of PPIX fluorescence was determined, but the contribution (the number of fluorescent photons) of each component depended strongly on the biological microenvironment and on the phenotype of the cells with which the PS interacts. In addition, it was found that, as a result of PDT and the change in the metabolic processes inside the cells, the contribution to the fluorescence kinetics of various time components changes, while the fluorescence lifetimes themselves remain similar (fig. 1.2) (8, 9). Based on the obtained results, it was concluded that there are different components in the tumor that actively accumulate PS, but interact differently with it. The role of such agents can be primarily attributed to immunocompetent cells, namely macrophages, due to their increased ability to accumulate PS.

This assumption was verified by analyzing tumor cryosections by confocal and FLIM (fig. 3, 4). As a result, fluorescence centers with lifetimes corresponding to the values obtained in vivo (fig. 3, 4) were detected. The localization and morphology of cells suggested their immune nature. Their close proximity to the tumor, in particular, their localization at the tumor edge, gives reason to consider them tumor-associated macrophages. Thus, the assessment of the tissue cellular composition in the non-invasive monitoring mode makes it possible to selectively deactivate pathological cells (including immune cells), which can significantly increase the effectiveness of antitumor therapy (10-14).
Figure 1. a) Pp IX fluorescence spectrum, obtained using the streak camera, b) Pp IX fluorescence decay kinetics.

Figure 2. Distribution of photons by each of the components of the fluorescence decay before and after PDT. The numbers signify the lifetime of each decay component in nanoseconds.
Figure 3. a) Fluorescent image of the Pp IX accumulation centers obtained from cryosections of rat brain at the border of tumor and normal tissue containing TAMs by confocal microscopy (20× magnification), b) Image of the spatial distribution of fluorescence lifetimes obtained from cryosections of rat brain at the same border of tumor and normal tissue using FLIM (20× magnification) in the range of lifetimes 528-2884 ps.

Figure 4. a) Fluorescent image of the Pp IX accumulation centers obtained from cryosections of rat brain after PDT at the border of tumor and normal tissue by confocal microscopy (20× magnification), b) Image of the spatial distribution of fluorescence lifetimes obtained from cryosections of rat brain after PDT at the border of tumor and normal tissue using FLIM (20× magnification).
4. Discussion
In the course of the study, it was shown that based on measurements of the fluorescence lifetime, both its integral and local characteristics, we can evaluate the metabolic activity of individual cells and the composition of the whole tissue site. Based on this approach, spectral and temporal fluorescence characteristics of fluorophores (both endogenous and PS) interacting with tumor-associated macrophages (TAM) were obtained. In this regard, a promising technique is the analysis of the cellular composition of the tumor in vivo, in particular, the evaluation of such an important parameter as the percentage of TAM in the tumor tissue, which in turn, actively influence the progression of the tumor. It was shown that the photodynamic effect changes the metabolic activity of tissues, probably leading to the destruction of TAMs that have accumulated PS, which is an important factor in the new approaches to antitumor therapy.

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