Analysis of Ehrlich ascites carcinoma with electron paramagnetic resonance

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***Abstract*—The study was aimed to reveal the dependence of changes in the content of metal-containing proteins and free-radical forms in a suspension of the experimental tumor in different periods of tumor growth using electron paramagnetic resonance. The object of the study was Ehrlich's ascites carcinoma, an experimental undifferentiated tumor, which is used to study various aspects of tumor development under external influences. Ehrlich ascites carcinoma was inoculated into the peritoneal cavity of laboratory mice. We believe that the determination of the concentrations of metal-containing proteins and free-radical forms using this method may be an important prognostic parameter for assessing the state of the tumor. Changes in metabolism will inevitably affect the functionality of cells and, as a consequence, the dynamics of tumor growth. The results of the study showed that metal-containing proteins belonging to ascites plasma, such as transferrin, ferritin and ceruloplasmin, do not undergo significant changes. The signal from molybdenum-containing proteins also does not change at the stages of experimental tumor growth under consideration. However, in the course of this experiment, it was possible to record a slight decrease in the intensity of the formation of denitrosyl iron complexes with the development of a tumor in the body.**

Keywords— electron paramagnetic resonance, Ehrlich ascites carcinoma, dinitrosyl-iron complexes, nitrogen oxide, gem-containing proteins, molybdenum-containing proteins

I. Introduction

The electron paramagnetic resonance method (EPR) is widely used to investigate materials whose composition can include paramagnetic compounds. In recent years, EPR has been increasingly used to study tissues of living organisms; however, this method is reported to be a secondary one and is applied for obtaining additional information.

The EPR method consists in measuring the absorption of microwave radiation by an unpaired electron spin in the presence of an external magnetic field. The degeneracy of the unpaired electron is removed, which leads to the splitting of the energy state of the electron. The difference in the energies of these states can be described by the expression:

$$\Delta E = g_e \times \beta_e \times B_0 \quad (1)$$

where B_0 is the magnetic field, g_e is the g-factor of the electron, which varies depending on the electronic configuration, and βe is the Bohr electron magneton. Resonance occurs at the moment when the energy of the microwave field begins to coincide with the energy of splitting the energy state of the electron:

$$\Delta E = h\nu = g_e \times \beta_e \times B_0 \quad (1)$$

where h is Planck's constant and ν is the frequency of microwave radiation [1]. In this case, the measurement takes place in the "X band", at a constant microwave frequency of 9 GHz.

Electronic paramagnetic resonance is a promising method in research in the field of biophysics and medicine, in the study of the body's response when exposed to electromagnetic fields and magnetic nanoparticles, since these issues have not been fully studied. An equally important aspect is the biological electronic energy transfer in cells, which is responsible for cell growth and apoptosis - an important role in the development of pathological processes and the development of cancer cells [2].

Important indicators of the living organisms state are metal-containing proteins and free-radical forms participating in nutrient transport and many significant biochemical reactions, thus controlling exchange processes in mammal organisms.

Usually the content of free iron is not high, however, in some pathological processes, in particular tumor growth, the free pool of iron increases. This is due to the decompartmentalization of Fe^{2+} ions from ferritin and the destruction of metal-containing proteins. These events can be recorded using EPR. Free iron is defined as a broad signal in the region g = (2.2 - 2.4) [2]. When it comes to cancer cells, it

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is worth considering the increased release of free radicals that can react with cell macromolecules: proteins, lipids and DNA.

In our study, Ehrlich ascites carcinoma was taken as a model for estimating the biological activity of various substances. EAC is an experimental non-differentiated tumor used for studying various aspects of tumor metabolism due to it's high sensitivity to external impacts. [3].

At this stage, it isn't fully known how the changes found in EPR will occur at different stages of development of Ehrlich's ascites carcinoma cells. The present study was aimed to estimate changes in the EPR signals from metal-containing proteins and free-radical components in an EAC suspension at different stages of tumor growth.

II. Experimental Method

In this experiment, we used outbred laboratory ICR mice obtained in the State Scientific Center of Virology and Biotechnology "Vector" nursery in experiments A suspension of EAC was inoculated into the abdominal cavity of mice in the amount of $3x10^6$ cells per animal, diluted in 2 ml of saline solution.

To estimate the EPR signal from the tumor cells, the Ehrlich ascites carcinoma suspension was extracted from the peritoneal cavity of the animals on the 7th, 9th and 10th day after the inoculation. In the course of this work, the experiment was repeated three times. Samples were collected from 3-4 mice in each repetition, based on the calculation: one sample per one mouse, so that in all experimental groups the number of data was 10 or more.

The concentration of Ehrlich ascitic carcinoma cells was calculated in each sample. Next, the samples were placed in special cone-shaped tubes of 0.8 ml each. The test tubes were then placed in a Dewar vessel with liquid nitrogen for storage and subsequent measurements.

EPR spectra were recorded on an EPR Fourier spectrometer Elexsys E580 (Bruker) of the Krasnoyarsk Regional Center for Collective Use of the Federal Research Center, Siberian Branch of RAS. First, we analysed the range from 650 to 4350 G (resolution 2043) and then chose the area of interest from 3150 to 3650 G (resolution 2048). During all measurements, the sample temperature did not exceed 85K.

Figure 1 shows the components observed during the experiment. In the course of this experiment, calculations were made for the intensities of each of the components of interest to us using the Xepr and MagicPlot software.

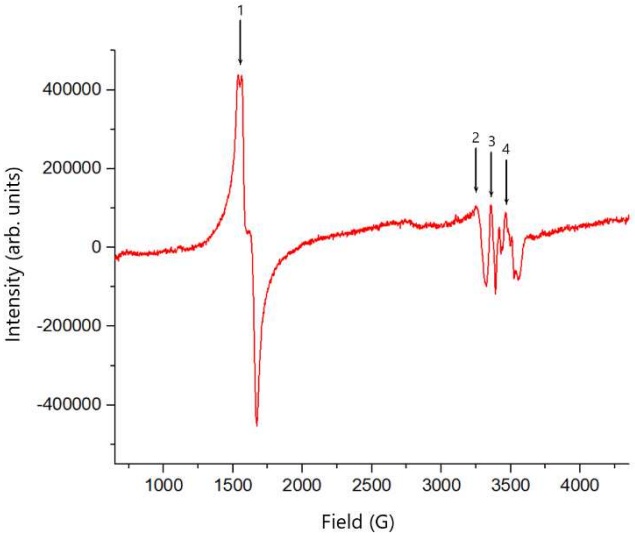

Fig. 1. Resonance absorption curve of the EAC suspension. Peaks: 1- transferrin; 2 - ceruloplasmin; 3 - DNIC -; 4- Mo^{2+}-containing molecules.

The main parameter determining the concentration of a particular component in the analysis of EPR spectra is the signal intensity - I. Intensity is defined as the area under the integral absorption curve. ΔH is the line width. H_n is the resonant field.

III. Results and Discussion

The Ehrlich ascites carcinoma suspension consists of EAC cells, and the biochemical composition of ascites plasma corresponds to blood plasma. The tumor development in the mice organism after the inoculation involves three stages: the first one is the organism's adaptation after the inoculation, the second stage includes the logarithmic cell growth, and the third is the stationary one, resulting in the death of the organism.

The analysis of the EPR spectra revealed the presence of a signal from metal-containing proteins as well as dinitrosyl iron complexes (DNIC) and free radical forms in the Ehrlich ascites carcinoma suspension at all the stages of the tumor growth.

Signal values from transferrin, ceruloplasmin, and ferritin were stable indicators, independent of the phase of tumor growth since they are found exclusively in plasma, and their functional activity applies to the entire organism as a whole. Transferrin is a blood plasma protein whose main role is to deliver iron to all the tissues. Ferritin protein molecules are a reservoir of iron in living organisms, performing the main functions of storing excessive iron and protecting cells from the toxic effects of Fe^{2+} ions. Ceruloplasmin is a copper-containing glycoprotein, which is mainly found in the blood. The EPR analysis is usually resolved as a signal of copper ions with a g factor equal to 2.05. Ceruloplasmin plays a key role in anti- and prooxidant reactions. It catalyzes the oxidation of Fe^{2+} , facilitating the incorporation of Fe^{3+} into apotransferrin. Ceruloplasmin participates in transferring four electrons to oxygen, preventing the non-enzymatic oxidation of iron with the formation of free radicals [4].

The presence of molybdenum-containing proteins is natural for a biological system. This important microelement is a co-factor of four enzymes (mitochondrial amidoxime-

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reducing component (mARC), sulfite oxidase, xanthine oxidase and aldehyde oxidase) that catalyze chemical exchange reactions of carbon, nitrogen and sulfur in the animal organism [5, 6]. Insignificant changes in the content of molybdenum-containing proteins in the EAC suspension are associated with changes in the homeostasis of the animal organism during the period of tumor development. The concentration of free iron, its various forms, and its availability has a strong effect on the state of Ehrlich ascites carcinoma cells. It is shown that with active production of nitric oxide, there occurs the formation of dinitrosyl iron complexes, which reduces the concentration of free iron in tumor cells, disrupting ATP synthesis. [7]. This results in decreased cell growth, proliferation, and apoptosis. NO in high concentrations is known to exert a cytotoxic effect on cancer cells; however, in low concentrations, it causes a vasodilating effect, increasing the supply of nutrients and provoking metastasis [8, 9]. It is also known that the active synthesis of nitric oxide leads to the formation of DNIC (Fig. 2), which is detected by EPR [7, 10].

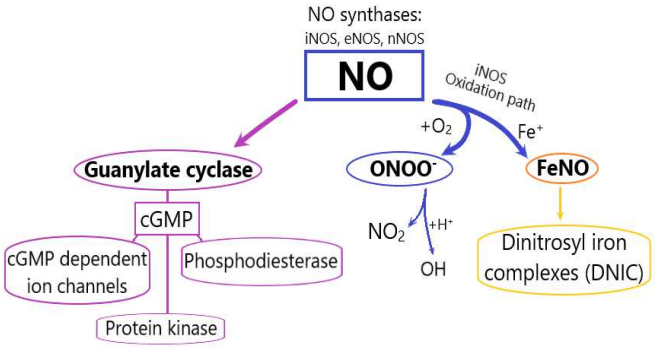

Fig. 2. DNIC formation in the organism.

Figure 3 shows high-field sections of three resonance curves of the tumor on days 7, 9, and 10 of tumor growth. Denitrosyl iron complexes are represented by segments between the apex and the bend, which are indicated by pointers in this image.

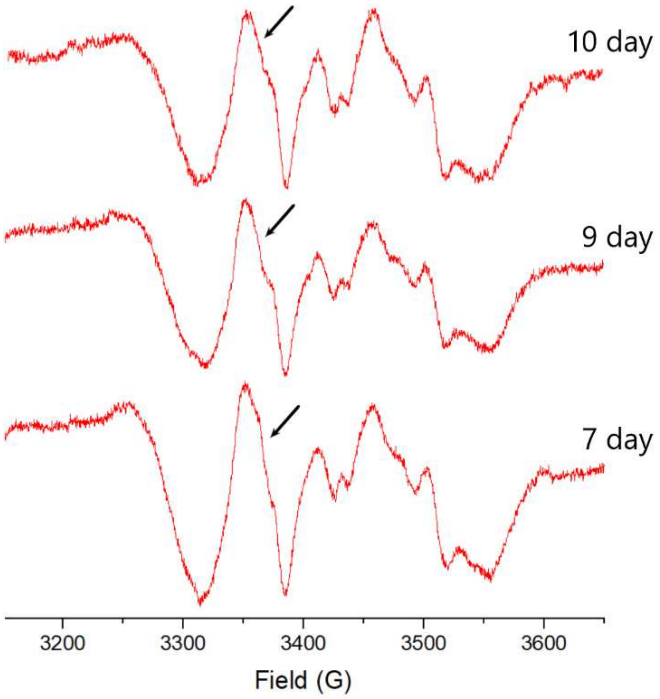

Fig. 3. EPR spectrum of Ehrlich ascites carcinoma on the 7th, 9th, 10th days of tumor growth.

Using the MagicPlot software, data were obtained for the area under the peak of interest, which corresponds to the signal intensity of denitrosyl iron complexes in the suspension. Table 1 summarizes our receipt of the data..

TABLE I. DATA OBTAINED FOR DNIC

Day	*I*	*ΔH*	*H_n*
7	348479	24,82	3364
9	290568	25,85	3360
10	239156	16,5	3360

As seen in the figure 2 and table 1, there is a tendency to decrease the DNIC content in the EAC suspension with the tumor growth. This may be due to a change in the concentration of NO, an essential component, which stimulates the synthesis of IL-10, TGF p and TGF pi growth factors and prostaglandin E2 (PGE 2), which suppress the protective activity of macrophages and improve the permeability of the capillaries of the abdominal cavity for the transport of nutrients, which has a positive effect on the tumor growth [5].

Thus, using the method of electron paramagnetic resonance, it is possible to observe the change in the intensity of DNIC formation. It is seen that with an increase in EAC, the concentration of DNIC, relative to free radicals, decreases. This may indicate that the NO concentration in the EAC suspension does not increase in the required volume for the formation of new DNICs. This can be important when simulating the conditions for changing the rate of tumor growth. This trend has been observed over the course of three experiments, but the difference is not always large enough for the method we use. Therefore, further research is needed in order to increase the database and refine our result.

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