

Electron Paramagnetic Resonance Study of Tumor Affected Bone Marrow

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Abstract To study the mechanism of interaction of tumor cells with bone marrow cells continuous wave electron paramagnetic resonance (EPR) experiments at 9 GHz including a spin trapping of superoxide were carried out. The common features of the EPR spectra in healthy and tumor affected tissues of donors and rats as well as their difference are presented and discussed. It is proposed that labile iron pool plays a significant role in mechanisms of tumor invasion. We hope that the observed EPR features could be used to study the mechanisms of invasion and progression of tumor in different organs.

Keywords Electron spin resonance spectroscopy · Medical oncology · Bone marrow · Iron metabolism disorders

Introduction

Though the evolution of cancer is unique in each individual patient, all cancers have certain similar features and mechanisms that control growth and progression. A lot of efforts are done to investigate them by different analytical methods. Electron paramagnetic/spin resonance (EPR/ESR) is widely used for the biomedical applications including cancer related research since 1950s [1–8]. EPR is well known as the most sensitive tool for the detection, identification and quantification of the naturally existing or artificially created radicals (intentionally incorporated,

generated during the chemical reactions, ageing, under the light, UV, X-ray or gamma irradiation, etc.). All the modern advanced EPR approaches such as an EPR imaging, high-field EPR, double resonance techniques and their pulsed variations [6, 9, 10] are still based on the conventional 9 GHz EPR measurements. Different companies all over the world produce low-cost table EPR spectrometers for their using in the bio-chemical and clinical laboratories.

Concerning cancer related research EPR was mainly focused on the investigations of tumor itself. In our opinion, the capabilities of the method for the scientific purposes and for its clinical applications are still not fully exploited. No EPR investigations of bone marrow (BM) tissues are done so far to our knowledge. We present the main output of our EPR research of the tumor affected BM in rats and humans.

BM is considered as an essential lymphoid organ with substantial impact on tumor cell dissemination and tumor-immune responses. Stromal cells of BM are widely recognized to collaborate with cancerous cells to create a tumor-permissive microenvironment capable of providing continuous support for tumor growth, progression, angiogenesis, invasion, and metastasis. Reactive oxygen species (including superoxide) generated by cancer cells and cells in cancer microenvironments can guide and maintain the growth and differentiation of local and distant cancers and their interactions with host microenvironments [7, 11].

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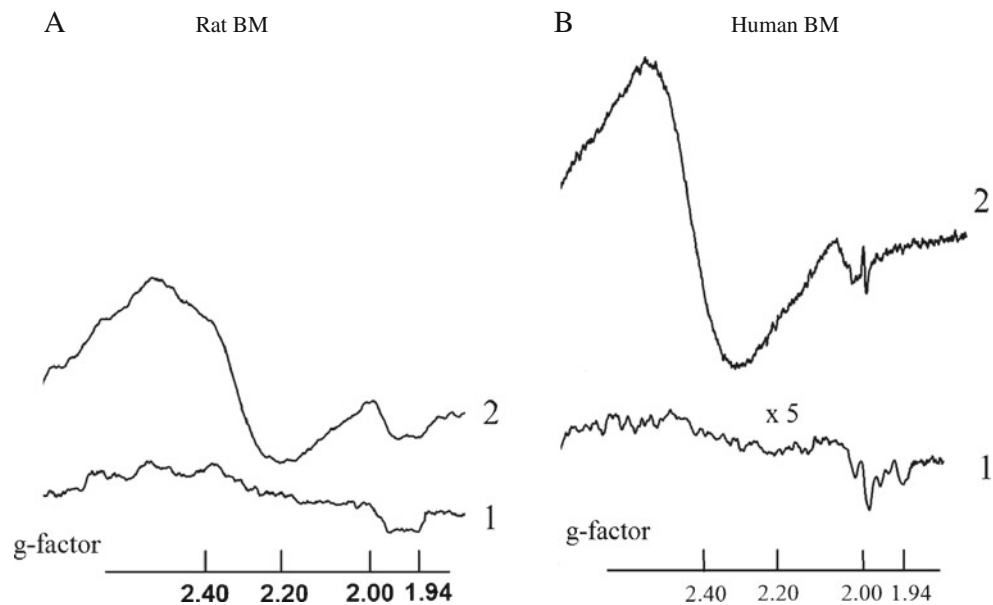
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Materials and Methods

Experimental group of 20 of the mongrel female rats with a body mass of 100 ± 9 g rats was implanted in-vivo by the Guérin carcinoma (GC or T8) cells under the control of the R.E. Kavetsky Institute Animal Ethics Committee. Ten animals served as a control group.

Fig. 1 **a** *left panel* EPR spectra (re-calculated to the values of g-factors) of samples of (1) BM of the intact animals; (2) BM of the Guérin carcinoma grafted rats. **b**, *right panel* EPR spectra the samples obtained from the human donors: (1) BM of patients with polytraumas; (2) BM of patients with breast cancer. The curve (1) is detected at five times higher receiver gain. All spectra are registered at $T=77$ K



Stromal and parenchyma cells of BM from the femur and tibia were acquired by flushing their diaphysis with the phosphate buffer solution followed by the disaggregation of the obtained suspension [12].

Human material was gathered from 18 female patients (45–71 y.o.) with the breast cancer at the disease stages T2–3, N1, N2 and M0 and from 11 female patients (41–68 y.o.) with polytraumas. The last group served as a control group. The patients gave their consent to use the tissues obtained by surgery and biopsy for the research purposes. BM samples were acquired by the puncture of the various sections of the breastbone (sternum) and the iliac crest. The obtained samples were frozen and stored in the special 0.5 ml containers in liquid nitrogen (77 K).

EPR measurements were conducted on RE-1307 EPR spectrometer (USSR) operating in continuous-wave (cw) mode at frequency of 9.03 GHz (X-band) by using conventional modulation technique. The obtained results were statistically compared using non-parametric Mann–Whitney U test. The values presented as Mean(SD) were considered to be statistically significant at the level of $P < 0.05$.

Oxidation of the 1-hydroxy-2,2,6,6-tetramethyl-4-oxo-piperidine hydrochloride (TEMPONE-H) to the stable nitroxyl radical TEMPONE was used to trap superoxide [13]. For this 200 μ L of the BM cells suspension containing of about 10^3 BM cells was mixed at room temperature with 20 μ L of the spin-trapping solution (2 mg of the powder of TEMPONE-H dissolved in

10 mL of the phosphate buffer with pH=7.4). Then EPR spectra of the nitroxyl radical in the resulting sample were acquired at room temperature with the magnetic field sweep rate of 100 G/120 s and modulation amplitude of 0.2 G. Concentration rate of growth of the nitroxyl radical in the EPR mesh which corresponds to the generation rate of superoxide anion was measured with a 2-min intervals via the change of the amplitude of the second hyperfine component of the EPR spectrum during 6 min after the introduction of the spin trap into the cell suspension. The measured relative values were re-calculated to the total number of the viable cells counted in a Goryaev chamber (hemocytometer) to extract the absolute quantities.

Results and Discussion

Figure 1 presents the typical EPR spectra of animal and human BMs detected at $T=77$ K. Due to the fast spin–lattice relaxation times these EPR spectra are not detected at room temperature (RT).

The response of the BM cells to the invasion of tumor cells and their metabolites manifests in the increased production of superoxide radicals. Superoxide generation rates for the rats and human BMs measured in the spin-trap containing mixture at RT are presented in Table 1. In the tumor affected tissues it is measured to be about eight times higher than that in a norm.

Table 1 Generation rates of superoxide radicals at RT in healthy and tumor affected BMs (nmol/ 10^3 cells·min)

BM of the Guérin carcinoma grafted rats	3.50(7)	BM of patients with breast cancer	2.95(16)
BM of intact animals	0.45(3)	BM of patients with polytraumas	0.38(9)

Correlation between the integral intensities of the EPR spectra and superoxide generation rates confirms that the obtained EPR signals in the materials studied are mainly originated from the superoxide-generating cells in the bone marrow: fibroblasts, osteoblasts, endothelial cells, adipose tissue as well as from neutrophils and macrophages. The both human and rat derived BMs have very similar features in the obtained EPR spectra.

The EPR signal with $g \approx 2.00$ is usually ascribed to the free radical centers practically completely localized in mitochondria—semiquinones of flavoproteins found in the inner membrane of mitochondria and coenzyme Q semiquinones (ubisemiquinones) [1–5].

Signal at $g \approx 1.94$ is related to the activity of the iron-sulfur cluster N2 of NADH: ubiquinone oxidoreductase (complex I) which is responsible for the proton pumping in this largest and is the most complicated enzyme in the electron transport chain (ETC) of mitochondria [1–5]. In the tumor affected human BM this signal practically disappears while no great changes are observed in the rat tissues. It might be a manifestation of a difference between the rat and human stromal cells observed in [12] which nature is not yet explained. We hope the obtained EPR feature could help to unravel the origin(s) of that difference.

The most pronounced and the most intriguing in nature is an intensive broad signal at $g \approx 2.20$ – 2.60 that appears in the tumor affected BMs. The signal with the similar spectroscopic parameters is to be reported arising “sometimes” in “a part” of the pathologically changed tissues [1, 4, 8]. The last trend is to connect the origin of at least part of this broad signal with the ferritin molecules [8]. But how does this signal arise in the tumor microenvironment is still an open question.

Pulatova with her co-workers connected this signal obtained in some samples of the mouse and dogs blood at the latest stages of the acute radiation syndrome (in both in-vivo and in-vitro experiments) with the activity of a “free” iron pool (pp.101–106 and Fig. 2.31 in Ref. [4]). Speaking present-day language—with a pool of loosely bound iron, which is termed the labile iron pool (LIP) [14, 15]. Released due to the destructions of different proteins and iron-containing compartments, a “free” iron binds with the diverse low-molecular weight substances as phosphate, nucleotides, hydroxyl, amino and sulfhydryl groups, etc. which are in excess are existing in tumor and tumor affected tissues. A “free” iron concentration is increasing at the earlier and at the latest disease stages. Superposition of EPR signals from all these iron complexes results in a broad intensive signal. Therefore, this accumulated signal could probably serve to measure LIP level and to estimate a grade of tumor invasion.

Cells with higher LIP levels invariably exhibit higher levels of reactive oxygen species. It leads in its turn to the damage of other microenvironment cells. We hope, therefore, that the obtained EPR features could be further used to track the spatial tumor progression.

Conclusion

Tumor iron metabolism and mitochondrial dysfunction are considered as the major players in tumorigenesis [15]. Tumor cells undergoing profound changes in their own intrinsic metabolism affect the microenvironment. The main impact on the BM microenvironment is through the metabolic disorder and increased production of superoxide radicals in stromal cells. In our opinion, based on the presented EPR measurements, a “free” iron could serve as a tool for such kind of influence. These tumor metabolites can reprogram the mitochondrial metabolism and mitochondrial activity of NADPH oxidase of normal cells towards to the increased generation of superoxide radicals.

We hope that the observed EPR features could be fruitfully used to study the mechanisms of invasion and progression of tumor in different organs and motivate the scientific community for their advanced studies by using more elaborated EPR and other magnetic resonance techniques. Study of the mechanisms of tumor invasion and progression in BM by EPR is a matter of the ongoing research. Concerning clinical applications, we propose that the method could be used to measure a “free” iron level (or at least the paramagnetic part of that which thought to be the most reactive and cannot be measured by other analytical tools) and, therefore, to estimate the tumor malignant potential in lesions formation.

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