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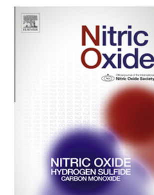

Nitrosylhemoglobin in photodynamically stressed human tumors growing in nude mice

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A B S T R A C T

The role of nitric oxide in human tumor biology and therapy has been the subject of extensive studies. However, there is only limited knowledge about the mechanisms of NO production and its metabolism, and about the role NO can play in modern therapeutic procedures, such as photodynamic therapy. Here, for the first time, we report the presence of nitrosylhemoglobin, a stable complex of NO, in human lung adenocarcinoma A549 tumors growing *in situ* in nude mice. Using electron paramagnetic resonance spectroscopy we show that the level of nitrosylhemoglobin increases in the course of photodynamic therapy and that the phenomenon is local. Even the destruction of strongly vascularized normal liver tissue did not induce the paramagnetic signal, despite bringing about tissue necrosis. We conclude that photodynamic stress substantiates NO production and blood extravasation *in situ*, both processes on-going even in non-treated tumors, although at a lower intensity.

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Introduction

NO and HbNO

Nitrosylhemoglobin (HbNO) is a stable complex of nitric(II) oxide and hemoglobin, which may be detected by electron paramagnetic (spin) resonance (EPR, ESR) spectroscopy [1,2]. There is a long record of studies on this complex, and they concern various aspects of the physiology and pathology of humans and animals. Initially, its presence was associated with neoplastic transformation [3,4], later on with tissue necrotizing [5,6], and since the discovery of the enzymatic biosynthesis of NO [7,8] it has been associated with biology of this molecule [9].

NO complexes in tumors

The role of NO in animal and human tumors has been thoroughly studied, and NO has turned out to show ambivalent activity, not only as a cytotoxic (including its metabolites) but also as a pro-angiogenic agent [10–12]. HbNO has been detected in many animal tumors [13], and its level correlated with the intensity of NO production [14], and (indirectly) with the intensity of the defense reaction, mainly non-specific, against a tumor [15,16]. The level of HbNO might be therefore seen as a marker of hemorrhagic necrotization and tumor tissue destruction. The same complex of NO has also been detected in blood and some organs of endotoxemic animals [17,18] and in rejected transplants [19–21], thereby becoming a universal and quite specific indicator of tissue destruction and defense reactions [9]. NO has been found to be an important factor in positive prognoses and, *via* the gene of inducible NO synthase, even a factor in gene therapy against tumor [12,22].

Despite the high levels of HbNO found in many animal tumors [13], it is difficult to detect it in human tumor tissues. In contrast to mice and rats [23], the promoter region of the human *iNOS* gene is one of the largest, and remains under the control of numerous transcription factors [24]. Consequently, only relatively weak HbNO signals have been detected in samples of human tumor tissue, as reported by various authors [25–28]. While searching for new *in vivo* models of human tumors, it is important to note that

Abbreviations: DPPH, 1,1-diphenyl-2-picrylhydrazyl; EPR (ESR), electron paramagnetic (spin) resonance; FCS, fetal calf serum; HbNO, nitrosylhemoglobin; HSCs, human hemopoietic stem cells; HTM, humanized tumor mouse model; *ip (iv)*, injection into peritoneum (vain); NOS, nitric oxide synthase; PD, photodiagnosis; PDT, photodynamic therapy; PS, photosensitizer; RNS (ROS), reactive nitrogen (oxygen) species; RTV, relative tumor volume; *sc*, subcutaneously; Zn-Pheide, zinc pheophorbide *a*.

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so far human tumors growing in nude mice (depleted of thymus-dependent immunity [29,30]) have not been examined for the presence of HbNO and for the intensity of the EPR signal. Such a study would deliver important information on the contribution of host immunity and its character (specific or non-specific) in the production of HbNO (and indirectly NO) in tumor tissue, and on the character of the background physiological processes.

NO in photodynamic therapy

Photodynamic therapy (PDT) is becoming a very promising alternative or supplementary strategy for the eradication and detection (photodiagnosis, PD) of tumors. This therapeutic function involves the systemic/topical application of a light-inducible pro-drug (photosensitizer, PS), its excitation with light in the appropriate wavelength range, and the generation of cytotoxic molecules as a result of interaction between excited PS and molecular oxygen stored in the tissue [31]. Among the toxic factors in PDT-related cellular/vascular damage to tumor, reactive oxygen species (ROS) play an important role [31], causing oxidative stress in the tissue [32]. They may act *via* (i) direct cell killing due to apoptotic/necrotic/autophagic cell death [33,34], (ii) damage to the vasculature, resulting in a limited delivery of oxygen and nutrients [31,33], and (iii) by affecting the immune system, e.g. by activating an immunological response against cancerous cells, and also by killing lymphocytes, which is taken advantage of in the PDT of some leukemias [35]. PDT may be accompanied by relatively minor side effects, such as prolonged skin/eye photosensitivity, which can in part be avoided by reducing exposure to light after the treatment [36]. This is in contrast to chemotherapy, radiotherapy and surgery, which can all be associated with severe side effects, often difficult to avoid.

Considerable effort is being made to develop novel improved PSs, focusing on chemical modifications that may affect e.g. their selectivity, solubility in aqueous milieu, and increased yields of *in situ* ROS generation [37–39]. A good example of such chemically tailored PSs are (bacterio)chlorophylls, derivatives of natural photosynthetic pigments [40,41]. Also in the present study we utilized a transmetalated water-soluble derivative of chlorophyll *a*, zinc pheophorbide *a* (Zn-Pheide), with known pharmacokinetics [42]. Recently, Zn-Pheide was applied as a PS in the PDT of the human lung solid adenocarcinoma A549 tumor growing in nude mice [34].

While the contribution of ROS to the mechanisms of PDT is unquestionable, the actual involvement of NO, and the nature of its participation (tumoricidal or tumorigenic), remains an open question and is a matter for intensive studies [35,43–46], mainly *in vitro*[43–45]. The putative roles of NO and its metabolites (reactive nitrogen species, RNS) include the possibility of them being pro- or antioxidants, immunosuppressors or the effectors of inflammation, as well as that of their ability to modulate tumor angiogenesis [22,47]. It is therefore very important to investigate the influence of NO *in situ* in human tumors, in order to judge whether NO donation or NO trapping (NOS inhibition) would properly supplement therapeutic procedures, pushing the process of tumor involution in the desired direction.

Aim

We attempted, by applying EPR spectroscopy, to detect HbNO in human lung adenocarcinoma A549 solid tumors growing subcutaneously (*sc*) in BALB/cA nude mice. We also tried to correlate the parameters (intensity and shape) of the HbNO EPR signals with both the progress of PDT (expressed as inhibition of tumor growth) and with the presence of leukocytes infiltrating the region of necrosis. We checked for the presence of similar signals in the blood, liver, and other organs of photodynamically treated and control (untreated) animals. Also, we wanted to discuss the role of NO and non-specific immunity in the progress of PDT in a tumor.

Materials and methods

Materials

Animals

BALB/cA nude mice. The immunodeficient BALB/cA nude mice (C.Cg/AgBomTac-Foxn1^{nu}N20; male, 7 weeks old) were obtained from Taconic (Bomholtvej, Denmark). The mice were fed on a sterile laboratory rodent chow M-Z provided by Ssniff (Soest, Germany).

C57BL/6J black mice. Fully immunocompetent C57BL/6 J mice (7 weeks old, males) were purchased from the Mossakowski Medical Research Centre of the Polish Academy of Sciences (Warsaw, Poland). The mice were fed on a standard laboratory rodent chow Labofeed B provided by Morawski (Kcynia, Poland).

Pigment preparation

Zinc pheophorbide *a* (see the structure in Fig. 1) was synthesized, purified and used in the photodynamic experiments according to the previously described methods [34,42,48]. Briefly, chlorophyll *a* was extracted from cyanobacterium *Spirulina laporte*, then depleted of the hydrophobic phytyl moiety *via* an enzymatic reaction with plant enzyme chlorophyllase (EC No. 3.1.1.14). The pigment was demetalated using glacial acetic acid, and then metalated directly with zinc acetate. After the purification, Zn-Pheide was stored dried at −30 °C under Ar atmosphere.

Chemicals, cell culture media, and drugs

1,1-Diphenyl-2-picrylhydrazyl (DPPH), DMEM high glucose, eosin Y, human hemoglobin, sodium dithionite and sodium nitrite were purchased from Sigma–Aldrich (St. Louis, USA); fetal calf serum (FCS) from Gibco (Grand Island, USA); penicillin and streptomycin from Polfa Tarchomin (Warsaw, Poland); ethanol, formaldehyde and xylene from POCh (Gliwice, Poland); ketamine (Bioketan) and xylazine (Sedazin) from Vetoquinol Biowet (Pulawy, Poland); Polysine® Slides from Gerhard Menzel GmbH (Brunswick, Germany), and hematoxylin from Aqua-Med (Lodz, Poland).

Equipment

A ventilated cabin equipped with HEPA filters was obtained from Tecniplast (Buguggiate, Italy); a 655 nm diode laser from Eurotek (Warsaw, Poland); an EPR X-band (ca. 9.4 GHz) spectrometer EMX from Bruker BioSpin GmbH (Rheinstetten, Germany), a light microscope Nikon Eclipse Ti and a D7000 digital camera from Nikon (Tokyo, Japan).

Software

WinEPR program was purchased from Bruker BioSpin GmbH (Rheinstetten, Germany). Eleana 0.9.8 software was kindly provided by Dr. Marcin Sarewicz, (Department of Molecular Biophysics, The Jagiellonian University, Krakow, Poland; http://www.wbbib.uj.edu.pl/web/gbm/eleana).

Methods

Models

Tumor model. The human lung adenocarcinoma A549 cells were cultivated *in vitro* under standard conditions (37°C, 5% CO_2 in humid atmosphere). The cells were cultured in DMEM high glucose supplemented with 10% heat-inactivated FCS, penicillin and streptomycin.

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