Intracellular Hypoxia of Tumor Tissue Estimated by Noninvasive Electron Paramagnetic Resonance Oximetry Technique Using Paramagnetic Probes

Atsuko Matsumoto,[a,b] Ken-ichiro Matsumoto,*[,a,b] Shingo Matsumoto,[a] Fuminori Hyodo,[a,c] Anastasia L. Sowers,[a] Janusz W. Koscielniak,[a] Nallathamby Devasahayam,[a] Sankaran Subramanian,[a] James B. Mitchell,[a] and Murali C. Krishna[a]

[a] *Radiation Biology Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health; Bethesda, MD 20892–1002, U.S.A.:* [b] *Heavy-Ion Radiobiology Research Group, Research Center for Charged Particle Therapy, National Institute of Radiological Sciences; Chiba, Chiba 263–8555, Japan: and* [c] *Innovation Center for Medical Redox Navigation, Kyushu University; Fukuoka, Fukuoka 812–8582, Japan.*

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Electron paramagnetic resonance (EPR) oximetry at 700 MHz operating frequency employing a surface coil resonator is used to assess tissue partial pressure of oxygen (pO_2) using paramagnetic media whose linewidth and decay constant are related to oxygen concentration. Differences in extracellular and intracellular pO_2 in squamous cell carcinoma (SCC) tumor tissue were tested using several types of water-soluble paramagnetic media, which localize extracellularly or permeate through the cell membrane. The nitroxide carboxy-PROXYL (CxP) can only be distributed in blood plasma and extracellular fluids whereas the nitroxides carbamoyl-PROXYL (CmP) and TEMPOL (TPL) can permeate cell membranes and localize intracellularly. EPR signal decay constant and the linewidth of the intravenously administered nitroxides in SCC tumor tissues implanted in mouse thigh and the contralateral normal muscle of healthy mice breathing gases with different pO_2 were compared. The pO_2 in the blood can depend on the oxygen content in the breathing gas while tissue pO_2 was not directly influenced by pO_2 in the breathing gas. The decay constants of CmP and TPL in tumor tissue were significantly larger than in the normal muscles, and lower linewidths of CmP and TPL in tumor tissue was observed. The SCC tumor showed intracellular hypoxia even though the extracellular pO_2 is similar to normal tissue in the peripheral region.

Key words electron paramagnetic resonance oximetry; tissue oxygen concentration; nitroxide; tumor hypoxia; hyperoxia

The hypoxic environment in tumor tissue limits the efficacy of radiotherapy and/or chemotherapy for cancer and/or tumors.[1)] A variety of methods have been investigated to estimate the oxygen concentration and/or oxygenation level in tumor tissues non-invasively.[2,3)] Electron paramagnetic resonance (EPR) oximetry can quantify oxygen concentration in a sample using stable nitroxides that can physically interact with molecular oxygen.[4)]

The EPR linewidth of the paramagnetic probe and oxygen concentration surrounding the probe display a linear relation. When a paramagnetic probe is implanted or administered to a living animal, oxygen concentration in specific tissue can be obtained by EPR spectroscopy or imaging. There are two classes of stable spin probes for EPR oximetry: insoluble particulate materials and soluble materials. An insoluble particulate probe, lithium phthalocyanine (LiPc), has been used widely due to its sensitivity, reproducibility, and reliable quantifying capabilities and can be implanted at predetermined sites where partial pressure of oxygen (pO_2) can be monitored several times over days and weeks.[5—10)] On the other hand, two types of soluble paramagnetic oximetry probes, triarylmethyl radicals and nitroxides, can be infused.

Nitroxides have been widely used as redox probes to determine the biological tissue redox status in living experimental animals using low frequency *in vivo* EPR.[9,11—15)] When a nitroxide is administered to a living animal, *in vivo* EPR signal intensities of the nitroxide in the tissue show characteristic decay profiles as a function of time. The *in vivo* or *in vitro* reduction rates of the nitroxides depend on the oxygen level in the tissue or sample.[16)] A fast decay constant of nitroxide in hypoxic tumors is attributed to the rapid conversion of the nitroxide to hydroxylamine.[9,13,17)]

Using several paramagnetic contrast agents with different lipophilicity, tissue cellular pO_2 in different environments, *i.e.* inside or outside of a cell, can be analyzed. In a study using cells in culture, Hu *et al.*[18)] demonstrated the existence of gradients in oxygen between extracellular and intracellular compartments using nitroxides that are membrane impermeable or nitroxides that accumulate in cells. In this study, three different types of nitroxides, carboxy-PROXYL (CxP), carbamoyl-PROXYL (CmP), and TEMPOL (TPL), were tested. Their hydrophilicity is as follows; CxP>CmP>TPL. CxP is expected to distribute only in blood plasma and extracellular fluids[19)] whereas CmP has limited membrane permeability.[20)] Amphiphilic TPL can easily permeate the cell membrane.[21)]

In this study, EPR signal decay constants and linewidths of the nitroxides in the femoral muscle of healthy mice breathing gases containing 21% (air) or 95% (caroben: 95% oxygen+5% carbon dioxide) were assessed. EPR signal decay constants and linewidths in the tumor tissue and contralateral normal tissues of squamous cell carcinoma (SCC)-bearing mice were also compared.

MATERIALS AND METHODS

Chemicals CxP (3-carboxy-2,2,5,5-tetramethylpyrrolidine-*N*-oxyl, also known as carboxy-PROXYL), CmP (3-carbamoyl-2,2,5,5-tetramethylpyrrolidine-*N*-oxyl, also known as

* To whom correspondence should be addressed. e-mail: matsumok@nirs.go.jp

carbamoyl-PROXYL), TPL (4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl, also known as TEMPOL) were purchased from Sigma-Aldrich Chem. Co. (St. Louis, MO, U.S.A.). CxP was prepared as a 100 mM solution. TPL and CmP were prepared as 300 mM solutions. All solutions were prepared as isotonic and adjusted to pH 7. Deionized water (deionization by the Milli-Q system) was used for all preparations. All other materials used were analytical grade.

Animals Female C3H mice were supplied by the Frederick Cancer Research Center, Animal Production (Frederick, MD, U.S.A.). Animals, received at six weeks of age, were housed five per cage in climate controlled circadian rhythm-adjusted rooms and were allowed food and water *ad libitum*. Experiments are carried out in compliance with the Guide for the Care and Use of Laboratory Animal Resources (1996), National Research Council, and approved by the National Cancer Institute Animal Care and Use Committee. Their body weight before the experiments was 22—27 g. A squamous cell carcinoma (SCCVII) tumor was implanted and grown on the right hind leg for a week. The approximate volume of the tumor was around 450 mm^3.

***In Vivo* EPR Measurements** Each mouse was anesthetized by 1.5—2% isoflurane in air or carbogen gas flow (700 ml/min) during the EPR measurement. The mouse was fixed on a special mouse holder using adhesive tape and a surface coil resonator (7.3 mm inside diameter (i.d.)) was set on the thigh. Body temperature was measured by a nonmagnetic temperature probe (FOT-C-PEEK; FISO, Quebec, Canada) introduced 2 cm in the mouse rectally and monitored by the corresponding conditioner (FTI-10, FISO). Body temperature was maintained between 36—37 °C using an air heater. CxP, CmP, or TPL was administered to the mouse through the cannula placed in the tail vein 15 min after starting the gas challenge. The doses were 0.75 μmol/g body weight (b.w.) for CxP, and 1.5 μmol/g b.w. for CmP and TPL. EPR spectra were measured repeatedly and saved automatically. EPR conditions were as follows; microwave frequency, 700 MHz; scan rate, 0.125—0.0625 mT/s; time constant, 0.003—0.3 s; field modulation frequency, 13.5 kHz. For EPR oximetry, the microwave power (0.04—10 mW) and field modulation width (0.06—0.8 mT) were carefully adjusted depending on the EPR linewidth of each paramagnetic probe to minimize microwave power saturation.

EPR Data Manipulation EPR spectral data were manipulated using our own software, which can fit a simulated lineshape (Lorentzian, Gaussian, or a mixture) and give the linewidth and signal height used for the simulation as a function of the time. In this paper, all spectra were considered as 100% Gaussian lineshape, and the peak-to-peak linewidth was recorded. The logarithmic values of signal heights were plotted with time. The decay rate was obtained from the slope of least squares fitting. Periods used to calculate decay rates were from 15 min after administration of the probe to the end of the experiment (30 min after administration) for CxP, from 10 min after administration to the end of the experiment (20 min after administration) for CmP, and from 2 min after administration to the end of the experiment (5 min after administration) for TPL. The linewidth in each experiment was obtained as an average value during the same time period used for calculation of the decay rate.

pO_2 Calibration Curves An aliquot (100 μl) of 2 mM standard solution of a paramagnetic probe was placed in a 30 cm piece of gas-permeable PTFE tube (i.d. 0.032±0.001 inch, wall thickness 0.002±0.0005 inch; Zeus Industrial Products Inc., Raritan, NJ, U.S.A.). The PTFE tube containing the sample solution was placed on the surface coil resonator with the nonmagnetic temperature probe. The surface coil resonator carrying the sample and temperature probe was covered with plastic tube. Gas flowed inside the plastic tube at a flow rate of 700 ml/min. Whole resonator assemble was warmed to 36—37 °C using the combination of an IR lamp and hot air flowing outside the plastic tube. The instrumental details for the calibration curves were described elsewhere.[22] The EPR linewidths of the sample solution were measured under several gas flow conditions, 0, 5, 10, and 21% oxygen. The EPR conditions were as above. The *in vivo* linewidth of each pramagnetic probe was replaced with the pO_2 value according to the corresponding calibration curve.

Statistical Analyses Statistical differences were estimated using the TTEST function in Microsoft Excel XP. Suitable 'tail' and 'type' for the TTEST function were selected as follows. The 'tail' was 1 (one-tailed distribution), which was selected because changes are expected depending on the task. The 'type' was 2 (equal variance) or 3 (unequal variance), which was selected according to data variances, and Student's or Welch's *t*-test was performed according to the 'type,' respectively. Grades of significance were estimated by $p<0.05$, $p<0.01$, and $p<0.001$.

RESULTS AND DISCUSSION

Pharmacokinetic data, such as signal decay rates and spectral information (O_2-dependent linewidth information), were obtained from the muscle region of non-tumor-bearing mice at different levels of oxygen in inspired gases. Similar data were recorded from tumor-bearing mice from the tumor region and the muscle region of the contralateral leg.

Figure 1A shows comparisons of the decay constants of nitroxides obtained from the femoral muscle of healthy mice under air or Carbogen challenges. No significant difference in EPR signal decay rates of CxP was obtained between air- and Carbogen-breathing groups. Decay constants of CmP in the normal muscle of healthy mice did not display changes with pO_2 in the breathing gas. Decay constants of TPL in the normal muscle of healthy mice did not show changes with changes in pO_2 of the breathing gas.

Figure 1B shows comparisons of pO_2 values estimated from the EPR linewidth of nitroxides in the femoral muscle of healthy mice when the mice were breathing air or Carbogen. A significant increase of the linewidth was observed with Carbogen breathing in the muscle of normal (non-tumor bearing) mice. The linewidths under Carbogen were significantly greater than for air breathing. The linewidth of TPL in mice breathing Carbogen was significantly greater than under the air breathing condition.

Figure 1C shows comparisons of the decay constants of nitroxides obtained in the tumor tissue and the normal muscle in the contralateral normal muscle of tumor-bearing mice. No significant difference in the rate of signal loss between the tumor tissue and the contralateral normal muscle was noted. The decay constant of CmP in tumor tissue was significantly higher than that in the contralateral normal muscle.

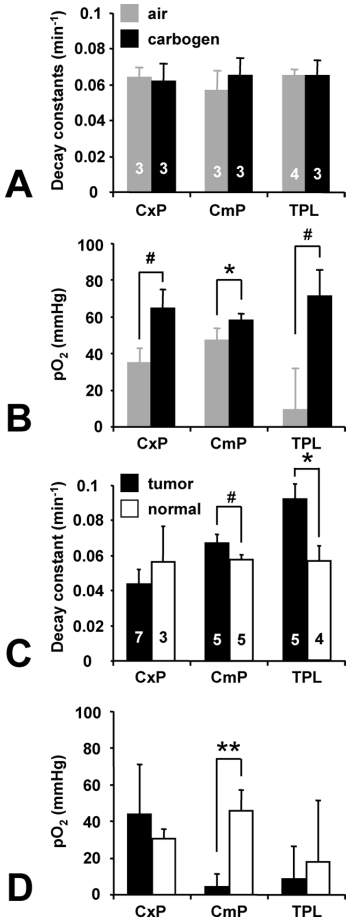

Fig. 1. Comparison of Decay Rates and Linewidths of Nitroxides under Various Conditions

(A) Decay constants of nitroxides in the femoral muscle of healthy mice obtained under air or Carbogen challenge. (B) pO_2 values estimated from the EPR linewidth of nitroxides in the femoral muscle of healthy mice when the mice were breathing air or Carbogen. (C) Decay constants of nitroxides in tumor tissue and normal muscle in the contralateral leg of SCC tumor bearing mice obtained under air breathing. (D) pO_2 values estimated from the EPR linewidth of nitroxides in tumor tissue and contralateral normal muscle of SCC tumor-bearing mice. Average±S.D. is indicated as a column and error bar. Numbers indicated in the columns are the number of mice used. *, #, and ** Indicate significance at $p<0.05$, $p<0.01$, and $p<0.005$ respectively.

The decay constant of TPL in tumor tissues was significantly larger than in the contralateral normal muscle.

Figure 1D shows comparisons of pO_2 values estimated from the EPR linewidth of nitroxides in the tumor tissue and the contralateral normal muscle of tumor bearing mice. There was no significance between the linewidths in tumor tissues and the contralateral normal muscles. The linewidth of CmP decreased significantly in tumor tissues. The linewidth of TPL in the tumor tissues was narrower than that of normal muscles, although there was no significance.

This study employed a surface coil resonator, which was applied to the skin surface of the leg of a mouse; therefore, only the peripheral region of the tumor (expected, 3—4 mm depth) can be detected, but most of thee diameter of the normal leg was covered. The peripheral region of the tumor may still have a blood supply and have active tumor cell proliferation rather than the necrotic core. Whether tumor cells in the peripheral region of tumor tissue can be radio-resistant is quite important to know the tumor physiology and to administer effective radiation therapy.

In this study, Carbogen was employed to achieve hyperoxic conditions in healthy tissue. Carbogen is widely utilized to achieve tissue oxygenation in various *in vivo* experimental models.[8,9,22—25] Consistent with the results from these previous reports, all spin probes in this study showed increasing linewidth, *i.e.* higher pO_2, in the femoral muscle under Carbogen challenge compared to when the mice breathed air. These results suggest that the Carbogen challenge can increase extra- and intracellular pO_2. No changes in the decay rate of nitroxides, *i.e.* CxP, CmP and TPL, under hyperoxic conditions were noted, suggesting that the increasing extra- and/or intracellular pO_2 itself did not influence the apparent decay rates of nitroxides in normal tissue.

CxP, with the carboxylate substituent at the 3-position in the pyrrolidine ring, is negatively charged and hence is membrane impermeable[19] and must be restricted to extracellular/plasma regions. CxP did not show a significant change of the linewidth and decay constant in tumor tissue compared to contralateral normal muscles. Extracellular pO_2 in the peripheral region of tumor tissues therefore may be the same level in contralateral normal tissue. CmP, which has limited membrane permeability, is mostly localized in the intercellular space but is partly localized in cellular membranes. Amphiphilic TPL, which can easily permeate the cell membrane, can localize in both intra- and extracellular space. The linewidths of CmP in tumor tissues were significantly decreased compared to contralateral normal muscles. The linewidths of TPL in tumor tissues also showed a lower value, but this was not significant compared to contralateral normal muscles. Although the relatively broad linewidth of TPL is not very sensitive to pO_2, these results suggest that intracellular pO_2 in tumor tissue is lower than in normal tissue, even in the peripheral region of the tumor. The decay constants of TPL and CmP in SCC tumor tissue were significantly increased compared to contralateral normal muscles.

In conclusion, breathing Carbogen can increase the extracellular pO_2 level higher than the normal air breathing condition. Breathing Carbogen can also increase the intracellular pO_2 level. The SCC tumor showed intracellular hypoxia even though extracellular pO_2 was similar to normal tissue in the peripheral region.

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