Tissue Redox Activity as a Hallmark of Carcinogenesis: From Early to Terminal Stages of Cancer

Rumiana Bakalova1,2, Zhivko Zhelev1,3, Ichio Aoki1, and Tsuneo Saga1

Abstract

Purpose: The study aimed to clarify the dynamics of tissue redox activity (TRA) in cancer progression and assess the importance of this parameter for therapeutic strategies.

Experimental Design: The experiments were carried out on brain tissues of neuroblastoma-bearing, glioma-bearing, and healthy mice. TRA was visualized in vivo by nitroxide-enhanced MRI on anesthetized animals or in vitro by electron paramagnetic resonance spectroscopy on isolated tissue specimens. Two biochemical parameters were analyzed in parallel: tissue total antioxidant capacity (TTAC) and plasma levels of matrix metalloproteinases (MMP).

Results: In the early stage of cancer, the brain tissues were characterized by a shorter-lived MRI signal than that from healthy brains (indicating a higher reducing activity for the nitroxide radical), which was accompanied by an enhancement of TTAC and MMP9 plasma levels. In the terminal stage of cancer, tissues in both hemispheres were characterized by a longer-lived MRI signal than in healthy brains (indicating a high-oxidative activity) that was accompanied by a decrease in TTAC and an increase in the MMP2/MMP9 plasma levels. Cancer progression also affected the redox potential of tissues distant from the primary tumor locus (liver and lung). Their oxidative status increased in both stages of cancer.

Conclusions: The study shows that tissue redox balance is very sensitive to the progression of cancer and can be used as a diagnostic marker of carcinogenesis. The study also suggests that the noncancerous tissues of a cancer-bearing organism are susceptible to oxidative damage and should be considered a therapeutic target.

Clin Cancer Res; 19(9); 2503–17. ©2013 AACR.

Introduction

Redox signaling plays a crucial role in carcinogenesis (1–4). The increase in cellular oxidants [e.g., reactive oxygen species (ROS) and reactive nitrogen species (RNS)] above a critical level triggers genomic instability and uncontrolled proliferation (1–7), which causes normal cells to become malignant.

Cancer cells are also characterized by an abnormal production of reducing equivalents as a result of accelerated glycolysis (Warburg effect) and the pentose phosphate cycle and by a rapid consumption of these reducers to maintain accelerated anabolism, which is necessary for cell proliferation and immortalization (1, 2, 7, 8). Cancer cells also require high amounts of antioxidants to maintain an ROS/RNS level that is below the threshold for the induction of apoptosis and cell death, but is sufficiently high to ensure genomic instability (1, 7, 9, 10). All of these processes provoke redox imbalance in cancer, which is a hallmark of carcinogenesis. The tissue redox status could be a diagnostic marker, a therapeutic target, and a marker for the evaluation and planning of a therapeutic strategy in real time.

The primary endogenous triggers of redox imbalance in cancer are defective mitochondria and NADPH oxidase complexes. These triggers are involved simultaneously in 2 processes that affect tissue redox status: (i) an excessive generation of ROS (in particular, superoxide and/or hydrogen peroxide) and RNS and (ii) an increased consumption of 3 of the major cellular reducers, as NADH, NADPH, and glutathione (1, 11–18). The high-oxidative activity of cancerous tissue is known despite the hypoxia that occurs in solid tumors. The oxidative capacity of cancer cells is due to abnormal ROS/RNS levels and is not necessarily associated with a high oxygen tension. It is widely accepted that cancer cells are characterized by increased ROS/RNS production compared with that of normal cells that ensure genomic instability (1–15). ROS and RNS are involved in hypoxic signaling pathways and have important implications for the adaptation of cancer to oxidative stress, the induction of uncontrolled proliferation, and immortalization.

Most likely, there is a significant difference between the redox activity of the tumor core and periphery in addition to...
tumors in different stages (i.e., early, intermediate, and terminal) of development. However, there are methodologic restrictions that hamper the visualization and evaluation of such a difference \textit{in vivo} on humans following the development of cell-penetrating nitroxide probes with high contrast, low toxicity, and minimal side effects.

The most important observations are that the oxidative status of noncancerous tissues (even those distant from the primary tumor locus) increases with cancer progression and that these tissues become susceptible to oxidative stress and damage.

**Translational Relevance**

This study directly relates to cancer diagnosis, the assessment of cancer progression (from early to terminal stage), and planning a therapeutic strategy. This study shows that tissue redox balance is very sensitive to cancer development and can be used as a diagnostic marker of carcinogenesis. The method is simple and applicable on isolated tissue and blood specimens. The method shows the potential for promising application in molecular imaging diagnostic \textit{in vivo} on humans following the development of cell-penetrating nitroxide probes with high contrast, low toxicity, and minimal side effects.

Recently, we reported a noninvasive methodology for the estimation of TRA on intact mammals, allowing a differentiation of cancer development from normal (healthy) conditions (19–21). The method is based on the redox cycle of cell-penetrating nitroxide derivatives and their MRI and electron paramagnetic resonance (EPR) contrast properties, which make them useful molecular sensors for TRA (Fig. 1).

\textit{In vitro} studies indicate that the contrast-enhancing nitroxide radical could be converted rapidly to the noncontrast-enhancing hydroxylamine and/or oxoammonium by different cellular compounds (e.g., free ions of transition metals, hydroxyl and hydroperoxyl radicals, ubiquinols, NAD(P)H, and ascorbate/dehydroascorbate; Fig. 1; refs. 22–28). In turn, hydroxylamine and oxoammonium are “superoxide dismutase mimetics” and could restore the nitroxide radical (22, 26, 29). The logarithmic acid dissociation constant value of 4.8 is reported for the equilibrium between superoxide anion and its protonated form, that is, the hydroperoxyl radical. The interaction of oxoammonium with superoxide occurs at pH $<4.5$, whereas under physiologic conditions (pH $\sim 7.4$) the oxoammonium is reduced to hydroxylamine by NAD(P)H (26). The interaction of hydroxylamine with superoxide occurs at approximately pH 7.4 with the release of hydrogen peroxide and restoration of the radical form of the nitroxide (26). Ui and colleagues have reported that the exposure to hydrogen peroxide inhibits the reduction of the nitroxide radical \textit{in vivo}, and hydrogen peroxide reoxidizes hydroxylamine to the original radical form (22, 30). It seems that \textit{in vivo}, nitroxide exists primarily in 2 forms: as a radical and as hydroxylamine. Various reducers and oxidizers are involved (directly or indirectly) in the formation of hydroxylamine, but only the interaction of hydroxylamine with superoxide and/or hydrogen peroxide seems to dominate \textit{in vivo} as the process that restores the nitroxide radical and its MRI/EPR contrast.

**Figure 1.** Nitroxide redox cycle as a sensing platform for the imaging of TRA: principle of the method. In vitro studies show that the nitroxide radical, which is characterized by MRI/EPR contrast enhancement, can be converted to the noncontrast hydroxylamine and/or oxoammonium by different compounds in cells and body fluids (e.g., free ions of transition metals, hydroxyl and hydroperoxyl radicals, ubiquinols, NAD(P)H, ascorbate, glutathione, etc.). The contrast-enhancing radical form can be recovered by interaction of hydroxylamine with the superoxide radical at physiologic, pH (7.4), or interaction of oxoammonium with the superoxide radical at pH $<4.5$. Thus, the nitroxide-enhanced MRI/EPR signal follows the reduction/oxidation of the nitroxide derivative and indicates the redox status of tissues and body fluids.
Briefly, the nitroxide radical participates in electron transfer reactions with oxidizers and reducers with the formation of contrast-enhancing or noncontrast intermediate products (31–33). The rate constants of these reactions determine the intensity of the nitroxide-enhanced MRI/EPR signal in living cells and tissues. In healthy mammals, the intensity, duration, and/or half-life of the nitroxide-enhanced MRI/EPR signal \( t_{1/2} \) in the selected region of interest (ROI; e.g., cells, tissues, bloodstream) can be considered a reference value for the redox activity of the respective specimen in normal conditions (healthy organism; ref. 19). Any significant deviation from this reference value indicates a redox imbalance, such as the high-oxidative or reducing activity of cells, tissues, or physiologic fluids. In previous studies, we established that in cancer-bearing nonhuman primate tissues, the intensity, duration, and \( t_{1/2} \) values were completely different from the respective reference values and that this parameter is a valuable diagnostic marker for carcinogenesis (19–21).

The EPR contrast of the nitroxide radical allows the determination of the exact concentration and redox status of the nitroxide derivative in the respective tissue and/or cells using EPR spectroscopy or imaging (EPR; refs. 31–35). The comparative analysis of the results, obtained using both imaging techniques, gives accurate information about the TRA in vivo.

The reduction/oxidation of nitroxide is spatially separated and occurs in (i) the bloodstream, (ii) the extracellular–extravascular space (EES) of the tissue, and (iii) the cells of the tissue. The MRI/EPR signal dynamics is a result of various factors and processes that occur simultaneously in the following 4 areas: (i) the lifetime of the nitroxide in the circulation, depending on its water solubility (35, 36), (ii) the reduction/oxidation of the nitroxide in the bloodstream, which is expected to be low in the plasma but sufficiently high in blood cells (if it penetrates cell membranes; refs. 35, 36), (iii) the penetration and accumulation of the nitroxide in the EES and its reduction/oxidation, which is expected to be negligible, and (iv) the penetration and accumulation of the nitroxide in the cells of the tissue and its intracellular reduction/oxidation, which is expected to be substantial in comparison with the bloodstream and EES (20, 37). TRA is a combination of the redox capacity of the extracellular (EES) and intracellular space of the tissue. The metabolism and/or clearance of nitroxide from the organism is not related to the redox activity of the tissues and physiologic fluids and should be negligible during short-term scanning.

The hydrophilic nitroxides are characterized by a long lifetime in the circulation and a very slow penetration or no penetration into the cells. In contrast, the hydrophobic (and amphiphilic) nitroxides are characterized by a short lifetime in the circulation and an easy penetration into the EES and cells. Therefore, only the hydrophobic cell-penetrating nitroxides are appropriate molecular sensors for MRI/EPR of TRA, especially in vivo.

This study aims to clarify the difference between TRAs in different stages of cancer development in vivo. For this purpose, we used 2 cancer models (neuroblastoma- and glioma-bearing mice), blood–brain barrier (BBB)-penetrating and cell-penetrating nitroxide derivatives, and nitroxide-enhanced MRI and EPR spectroscopy.

**Materials and Methods**

**Chemicals**

2,2,6,6-tetramethylpiperidine-1-oxyl–labeled nitrosourea (SLENU) was synthesized according to the procedure described by Gadjeva (38). SLENU is a spin-labeled analog of the conventional anticancer drug 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (Lomustine). 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPOL) was purchased from Sigma-Aldrich (cat. No. 176141).

**Cancer models and experimental design**

All experiments were carried out in accordance with the guidelines of the Physiological Society of Japan and were approved by the Animal Care and Use Committee of the National Institute of Radiological Sciences (NIRS; Chiba, Japan).

The mice (nude Balb/c; male) were separated into the following groups: healthy mice (controls) and cancer-bearing mice with brain neuroblastoma or glioma. In all groups, the mice were of identical age, nearly identical weight (23 ± 3 g), and maintained under identical conditions.

Two cancer models were developed using Neuro2a (neuroblastoma) or U87 (glioma) cells. Cancer cells \( (0.5 \times 10^5 \text{ cells in } 10 \mu L) \) were grafted in one hemisphere of the brain. Anatomically visualized brain neuroblastoma or glioma developed within approximately 9 or 20 days after inoculation, respectively.

The mice were subjected to MRI measurements on the third and ninth day after the inoculation of Neuro2a cells or on the seventh and 20th day after inoculation of U87 cells, when the cancer was in the early and intermediate/terminal stage of its development, respectively.

In preliminary experiments, we investigated the effect of “sham tumor inoculation” (using 10 μL of PBS) on the dynamics of the nitroxide-enhanced MRI signal. Because there was no difference between inoculated and noninoculated hemispheres, or between untreated healthy mice and mice with “sham tumor inoculation,” we used untreated healthy mice as controls in this study.

**In vivo MRI measurements**

MRI measurements were conducted on a 7.0 Tesla horizontal magnet (Kobelco and Jastec) interfaced to a Bruker Avance console (Bruker BioSpin) and controlled with the ParaVision 4.0.1 program (Bruker BioSpin).

The mice were anesthetized by isoflurane (1.2%) and placed in a head or body holder (RAPID Biomedical). A respiration sensor (SA Instruments Inc.) was placed on the back of the mice. A temperature probe (FOT-M and FTI-10, FISO Technologies Inc.) was used to monitor the rectal temperature. The tail vein was cannulated using a polyethylene tube (PE-10, Becton-Dickinson) for the drug injection. The mouse was placed in the ¹H-volume...
radio-frequency resonator (Bruker BioSpin) with surface radio-frequency receiver (RAPID Biomedical), which was prevarmed using a body temperature controller (RAPID Biomedical). The resonator units, including the mouse, were placed in the magnet bore. The body temperature was maintained at 37 ± 1°C during the MRI measurements.

Five control images of the mouse brain or body were taken before injection with the following parameters: T1-weighted incoherent gradient-echo sequence (fast low-angle shot), repetition time = 75 ms; echo time = 3.5 ms; flip angle = 45°; field of view = 3.2 × 3.2 cm; number of averages = 4; scan time = 19.6 seconds; matrix = 64 × 64; slice thickness = 1.0 mm; and number of slices = 4. A solution of nitroxide derivative [SLENU, dissolved in dimethyl sulfoxide (DMSO), or TEMPOL, dissolved in 10 mmol/L PBS, pH 7.4, and reduced by ascorbate; 1:1, mol: mol; 100 mmol/L stock solutions] was injected via the tail vein (100 μL/25 g mouse; 0.4 μmol/g body weight) 1 minute and 40 seconds after beginning the scan. T1-weighted images were acquired continuously within approximately 14 minutes, using the parameters described above. Mice injected with DMSO served as negative controls. DMSO did not act as a radical scavenger under the experimental conditions described above. TEMPOL, dissolved in DMSO, produced an identical nitroxide-enhanced MRI signal (as intensity and profile) in anesthetized mice as TEMPOL produced an identical nitroxide-enhanced MRI signal (normalized to the baseline) in neuroblastoma-bearing mice. ELISA was used to determine plasma total matrix metalloproteinases (MMP)2 and total MMP9 levels, according to the manufacturer’s instructions. The MMP2 ELISA Kit [Human/Mouse/Rat MMP2 (total), Quantikine; R&D Systems Inc.] detects pro-, active, and tissue inhibitor of metalloproteinase (TIMP)-complexed MMP2. The MMP9 ELISA Kit [Mouse MMP9 (total), Quantikine; R&D Systems Inc.] detects pro-, active, and TIMP-complexed MMP9.

Statistical analysis
The data were statistically analyzed by ANOVA using Student t test.

Results
Early stage of brain neuroblastoma
Figure 2 shows typical images of the extracted nitroxide-enhanced MRI signal (normalized to the baseline) in healthy (Fig. 2A) and neuroblastoma-bearing mice in the early stage of cancer (3 days after inoculation of cancer cells into the brain; Fig. 2B). In this stage, the tumor cannot be detected anatomically even with high-resolution MRI (spin-echo sequence). The images from both experimental groups were similar. The averaged kinetic curves, however, showed that there was a significant difference in the duration and half-life ($t_{1/2}$) of the nitroxide-enhanced MRI signal between control and neuroblastoma-bearing mice (Fig. 2C and D).

Two ROIs were selected: (i) the brain area (ROI1) and (ii) the surrounding (nonbrain) area (ROI2; Fig. 2A, a). In both ROIs, the signal increased after the injection of nitroxide followed by a rapid or slower decrease to the baseline. The enhancement of the MRI signal in the beginning is due to the presence of nitroxide in the blood and its penetration and accumulation in the subsequent tissue, whereas the decrease is due to its reduction to noncontrast hydroxylamine, which occurs predominantly in cells.

In the brain area (ROI1) of healthy mice, the half-life of the nitroxide-enhanced MRI signal ($t_{1/2}$) was approximately 80 seconds and the duration of the signal was approximately 5 minutes and 40 seconds, and these values can be considered a reference for the T1R of the normal brain (Fig. 2C, gray curve). The profile of the histograms indicates a high reducing activity of normal brain tissue for the nitroxide radical. In ROI1 of neuroblastoma-bearing mice, $t_{1/2}$ was approximately 56 seconds and the duration of the signal was approximately 3 minutes, which is significantly higher than that of healthy mice.
Figure 2. Healthy and neuroblastoma-bearing mice (early stage of cancer development). A and B, typical MR images of healthy brain (A) and neuroblastoma-bearing brain in the early stage of cancer (B): a, MR image of mouse brain; b, extracted nitroxide-enhanced MRI signal obtained before (baseline; b); and after injection of nitroxide (SLENU; c, d, e). C and D, kinetics of the nitroxide-enhanced MRI signal in the brain (ROI1; C) and surrounding (nonbrain) tissues (ROI2; D) obtained before and after injection of SLENU. Control kinetics (gray dotted lines) obtained in healthy mice are given for comparison. The data are the mean ± SD from 10 animals for the control group and 7 animals for the neuroblastoma-bearing group. ROIs are indicated by dotted lines on MR images. SLENU was in the oxidized (radical) form.
shorter than that of control mice \( (P < 0.05; \text{Fig. 2C, red curve}) \). This result indicates that the reducing capacity of the brain tissue in the early stage of brain neuroblastoma was higher than that of the control brain.

In the surrounding (nonbrain) tissues (ROI2), the opposite tendency was observed (Fig. 2D). In healthy mice, the half-life of the MRI signal was significantly shorter than that in the neuroblastoma-bearing mice; approximately 82 seconds versus approximately 140 seconds, respectively \( (P < 0.01) \). The duration of the signal in neuroblastoma-bearing mice was more than 14 minutes versus 5 minutes and 40 seconds in healthy mice. This result indicates that the reducing activity of nonbrain tissues in the early stage of neuroblastoma is lower than that in the control group. In neuroblastoma-bearing mice, the MRI signal did not reach the baseline within 14 minutes of continuous scanning (Fig. 2D, blue curve), which indicates that the oxidation of nitroxide dominates over reduction.

**Terminal stage of brain neuroblastoma**

In the terminal stage of brain neuroblastoma (9 days after inoculation), the kinetics of the nitroxide-enhanced MRI signal in the brain and nonbrain tissues were completely different from the reference profiles, recorded for the control group, and from the profiles of the early neuroblastoma group (Fig. 3B and C). Three ROIs were selected: (i) the cancerous hemisphere (ROI1), (ii) the noncancerous hemisphere (ROI2), and (iii) the surrounding (nonbrain) area (ROI3; Fig. 3A, a). The cancer was visualized anatomically by MRI.

In ROI1 and ROI2, the signal increased after injection and reached a plateau without a decrease within 14 minutes \( (\tau_{1/2} > 14 \text{ minutes}; P < 0.001 \text{ vs. control}) \). In ROI3, the signal increased after injection then decreased slowly without reaching the baseline within 14 minutes \( (\tau_{1/2} < 14 \text{ minutes}; P < 0.001 \text{ vs. control}) \). The histograms indicate a high-oxidative activity of the cancerous and noncancerous tissues of neuroblastoma-bearing mice for the nitroxide. In all ROIs of neuroblastoma-bearing mice, the duration of the signal was more than 14 minutes versus 5 minutes and 40 seconds in controls.

The kinetics of the MRI signal showed identical profiles in both hemispheres of the neuroblastoma-bearing brain (Fig. 3B); however, the signal intensity was significantly higher in the cancerous area than in the noncancerous hemisphere (Figs. 2B and 3A).

**Early stage of brain glioma**

Figure 4A shows a typical image of brain glioma in the early stage of development (9 days after the inoculation of cancer cells into the brain). The tumor is very small but can be visualized anatomically using high-resolution MRI (spin-echo sequence). Two ROIs were selected: (i) the brain area (ROI1) and (ii) the surrounding (nonbrain) area (ROI2; Fig. 4A).

In ROI1 of glioma-bearing mice, the half-life of the MRI signal decay was significantly shorter than that in control mice \( (~63 \text{ seconds vs. } 80 \text{ seconds, respectively}; P < 0.05) \); the duration of the signal was approximately 3 minutes versus approximately 5 minutes and 30 seconds in controls (Fig. 4B, red curve). This result indicates that the reducing activity of the brain tissue in the early stage of brain glioma is higher than that of the control brain.

In the surrounding tissues (ROI2), an opposite tendency was observed (Fig. 4C). In healthy mice, the half-life of the MRI signal was significantly shorter than that in the glioma-bearing mice; approximately 82 seconds versus approximately 180 seconds, respectively \( (P < 0.01) \). The duration of the signal in glioma-bearing mice was more than 14 minutes versus approximately 5 minutes and 40 seconds in control mice. This result indicates that the reducing activity of nonbrain tissues in the early stage of brain glioma is lower than that in the control group. In glioma-bearing mice, the MRI signal did not reach the baseline within 14 minutes of continuous scanning (Fig. 4C, blue curve), which indicates that the oxidation of nitroxide dominates over reduction.

**Terminal stage of brain glioma**

In the terminal stage of brain glioma (20 days after inoculation), the kinetics of the nitroxide-enhanced MRI signal in the brain and nonbrain tissues were completely different from the reference profiles that were recorded for the control group (Fig. 5B and C). Three ROIs were selected: (i) the cancerous hemisphere (ROI1), (ii) the noncancerous hemisphere (ROI2), and (iii) the surrounding (nonbrain) area (ROI3; Fig. 5A, a). The cancer was visualized anatomically by MRI.

In ROI2, the signal increased after injection and reached a plateau without a decrease within 14 minutes \( (\tau_{1/2} > 14 \text{ minutes}; P < 0.001 \text{ vs. control}) \). In ROI1 and ROI3, the signal increased after injection then decreased slowly without reaching the baseline within 14 minutes \( (\tau_{1/2} > 440 \text{ seconds}; P < 0.001 \text{ vs. control}) \). In ROI2: \( \tau_{1/2} < 330 \text{ seconds} \); \( P < 0.001 \text{ vs. control} \). The histograms indicate a high-oxidative activity of the cancerous and noncancerous tissues of glioma-bearing mice for the nitroxide.

The kinetics of the MRI signal showed identical profiles in both hemispheres of the glioma-bearing brain. However, the signal intensity was significantly higher in the cancerous area than in the noncancerous hemisphere (Figs. 5A, b).

**Tissue-oxidizing capacity in vivo and in vitro**

To verify the data from the nitroxide-enhanced MRI in vivo, we used a second experimental strategy. Nitroxide (TEMPOL) was reduced until there was a complete loss of the MRI and EPR contrast. The reduced noncontrast-enhancing form was injected in anesthetized mice (healthy and cancer bearing), and the dynamics of the nitroxide-enhanced MRI signal were detected under identical conditions, as in Figs. 2 and 3.

The data in Fig. 6A indicate that the nitroxide-enhanced MRI signal appeared and increased within 14 minutes of continuous scanning only in the brain of neuroblastoma-bearing mice in the terminal stage of cancer (red and yellow curves). The signal intensity was higher in the cancerous hemisphere than in the noncancerous hemisphere. In the
controls and early-stage neuroblastoma, the nitroxide-enhanced MRI signal was not detected (violet and gray curves). The kinetic measurements obtained with injection of oxidized TEMPOL are shown for comparison in Fig. 6B. The dynamics were characterized by an initial peak as a result of the accumulation of nitroxide in the brain. For healthy mice or mice with early neuroblastoma, the signal decreased rapidly to the baseline. For mice with terminal neuroblastoma, the signal remained high in both hemispheres.

The results suggest that the noncontrast reduced nitroxide was converted to its contrast-enhancing oxidized form only by the tissues of the neuroblastoma-bearing mice in the terminal stage of cancer. This experimental design proves...
that these tissues are characterized by high-oxidative activity, which is not typical for tissues of healthy mice or neuroblastoma-bearing mice in the early stage of cancer.

Similar data were obtained in vitro using tissue specimens (brain, liver, and lung) and EPR spectroscopy (Fig. 6C). The tissues were isolated from healthy or neuroblastoma-bearing mice in the early or terminal stage of cancer. A reduced noncontrast nitroxide was added to each sample, and the appearance of the EPR spectra was detected after 10 minutes of incubation at room temperature. The appearance of EPR spectra is a result of the conversion of nitroxide probe from the reduced to oxidized (radical) form and is indicative of high-oxidative activity of the respective tissue. The EPR triplet appeared in all tissues of neuroblastoma-bearing

Figure 4. Healthy and glioma-bearing mice (early stage of cancer development). A, typical MR image of glioma-bearing mouse brain in the early stage of cancer: a, MR image of mouse brain; b, extracted nitroxide-enhanced MRI signal obtained 3 minutes after injection of nitroxide (SLENU). B and C, kinetics of the nitroxide-enhanced MRI signal in the brain (ROI1; B) and surrounding (nonbrain) tissues (ROI2; C) obtained before and after injection of SLENU. Control kinetics (gray dotted lines) obtained in healthy mice are given for comparison. The data are the mean ± SD from 10 animals for the control group and 5 animals for the glioma-bearing group. ROIs are indicated by dotted lines on the MR image. SLENU was in the oxidized (radical) form.
mice in the terminal stage of cancer and in the liver of neuroblastoma-bearing mice in the early stage of cancer. This result indicates that all of these tissues are characterized by high-oxidative activity for the nitroxide probe. The cancerous hemisphere was characterized by the highest tissue-oxidative capacity.

**Figure 5.** Healthy and glioma-bearing mice (terminal stage of cancer development). A, typical MR image of glioma-bearing mouse brain in the terminal stage of cancer: a, MR image of mouse brain; b, extracted nitroxide-enhanced MRI signal obtained 3 minutes after injection of nitroxide (SLENU). B and C, kinetics of the nitroxide-enhanced MRI signal in the brain, cancerous hemisphere (ROI1), and noncancerous hemisphere (ROI2; B), and surrounding (nonbrain) tissues (ROI3; C) obtained before and after injection of SLENU. Control kinetics (gray dotted lines) obtained in healthy mice are given for comparison. The data are the mean ± SD from 10 animals for the control group and 7 animals for the glioma-bearing group. ROIs are indicated by dotted lines on the MR image. SLENU was in the oxidized (radical) form.

**Total antioxidant capacity of brain tissue and plasma levels of matrix metalloproteinases**

To investigate the potential molecular mechanism(s) of redox imbalance in cancer-bearing mice, we analyzed 2 biochemical parameters: (i) the TAC of brain tissue and (ii) the plasma levels of the MMPs, MMP2 and MMP9.
It was established that the TAC of brain tissue slightly increased (~20%) in the early stage of cancer, whereas in the terminal stage, it markedly decreased (~40%) in comparison with the control (Fig. 7A). The plasma total MMP2 and MMP9 levels were significantly higher in neuroblastoma-bearing mice in the terminal stage of cancer than those in healthy mice (Fig. 7B). The plasma MMP9, but not MMP2, level also increased in neuroblastoma-bearing mice in the early stage of cancer.

Discussion

The data show 2 important trends in carcinogenesis: (i) the tissues of cancer-bearing mammals (cancerous and noncancerous, including areas distant from the primary tumor locus) are characterized by high-oxidative activity, whereas the tissues of a healthy organism are characterized by high-reducing activity for the nitroxide and (ii) the tissue redox balance is very sensitive to the progression of cancer: in the early stage, reduction dominates over oxidation, whereas in the terminal stage, oxidation dominates over reduction. Our additional experiments on colon cancer-grafted mice treated with camptothecin within 3 weeks showed a suppression of tumor growth and significant normalization of tissue reducing potential compared with the placebo group (data to be published elsewhere).

Similar results have been reported by Matsumoto and colleagues and Hyodo and colleagues (22, 23). The authors have investigated the dynamics of the nitroxide-enhanced
MRI signal in cancer-bearing mice injected with TEMPOL (partially water soluble, BBB-penetrating, and cell-penetrating), carbamoyl-PROXYL (water soluble, partially BBB-penetrating, and partially cell-penetrating), or carboxy-PROXYL (water soluble, BBB nonpenetrating, and cell nonpenetrating). The tumor was grafted in the right hind paw of C3H mice, and the left hind paw was used for comparison. The authors found that the intensity of the nitroxide-enhanced MRI/EPR signal in the noncancerous hind paw was significantly smaller than that in the cancer-bearing hind paw (22), which is similar to our observations (Fig. 3A). The authors do not provide data about the MRI/EPR signal decay in healthy mice. They have calculated that the rate of the MRI signal decay of TEMPOL and carbamoyl-PROXYL in cancerous tissue is more rapid than in noncancerous tissue of cancer-bearing mice. However, the ROI covers the central portion of the tumor (tumor core; ref. 22). It is very difficult for water-soluble and even partially hydrophobic substances to penetrate the tumor core, especially within a short time (within 15–20 minutes) and without a specific transport mechanism. In our studies, we observed a significant difference in the intensity and duration of the nitroxide-enhanced MRI signal between the tumor core and periphery in the terminal stage of cancer (data are not shown). In the tumor core, the signal enhancement was usually negligible even after the injection of cell-penetrating nitroxide (e.g., TEMPOL or SLENU). It is unclear whether the absence of a signal was a result of the rapid reduction of nitroxide or an inability to penetrate the tumor core. In this study, our ROI covers the entire cancer area, which minimizes the effect of different penetration and distribution of nitroxide in different parts of tumor (Figs. 2–5).

Most likely, for water-soluble nitroxides (such as carbamoyl-PROXYL and carboxy-PROXYL), the tumor is visualized predominantly on the basis of angiogenesis and the prolonged circulation of nitroxides in the bloodstream and/or prolonged retention in the EES of cancerous tissue. In this case, the MRI signal decay should be a result of nitroxide reduction in the bloodstream and/or EES and clearance from the organism through the kidneys. The same authors have shown that nitroxide-enhanced MRI signal appears first in kidney and after that in cancer area after injection of carboxy-PROXYL (23). Our previous report indicates that the penetration of nitroxide in cells and tissues is obligatory for MRI of the cancer based on the TRA (20).

Even when the BBB is disrupted (as in brain cancer), the water-soluble nitroxides are accumulated and retained in the EES despite penetrating the cells. This is a major contrast mechanism for the visualization of tumors using water-soluble gadolinium contrast agents.

In our study, we used SLENU, which is a strongly hydrophobic drug [octanol/PBS partition coefficient \((\log P_{Oct/PBS}) \) was 1.000 vs. 0.575 for TEMPOL, −0.158 for carbamoyl-PROXYL, and −2.000 for carboxy-PROXYL]. Nitrosoarenes are well-known DNA-annealing agents, penetrating cellular and even nuclear membranes (35, 40). Thus, the dynamics of the nitroxide-enhanced MRI signal using SLENU (Figs. 2–5) could be attributed to its penetration into the cells and subsequent intracellular reduction/oxidation.

Recently, Davis and colleagues have reported that the dynamic of nitroxide-enhanced MRI is very heterogeneous in different tissues of the same cancer-bearing organism (24). We also established that the intensity, half-life, and duration of nitroxide enhancement were different in brain tissue, tissues around the brain, and muscle tissue of the hind paw (Figs. 3–5; refs. 21, 39). Moreover, different cancerous tissues (e.g., glioma and neuroblastoma) showed heterogeneous dynamic of nitroxide-enhanced MRI (Fig. 3A vs. 3B). Heterogeneity of the signal existed in the same cancerous tissue using different cell-penetrating nitroxide probes: SLENU or TEMPOL (Figs. 5 and 6). Therefore, the different rates of MRI signal decay could be a result of overlapping of several processes occurring simultaneously in tissues: different penetration rates, different reduction rates, different retention time, and different excretion rates of nitroxide from different tissues.

Tissue Redox Activity as a Hallmark of Carcinogenesis

---

**Figure 7.** A, TAC of brain tissue in healthy and neuroblastoma-bearing mice. The data are the mean ± SD from 4 animals for each group. In the control group, TAC was considered 100%, which corresponded to 12 µmol/L uric acid equivalents per mg protein (~26 µmol/L copper reducing equivalents per mg protein). B, plasma levels of MMPs (MMP2 and MMP9) in healthy and neuroblastoma-bearing mice. The data are the mean ± SD from 4 animals for each group: *, \(P < 0.05\); **, \(P < 0.01\); ***, \(P < 0.001\).
The different interpretations of the experimental data from the nitroxide-enhanced MRI studies that are published in the literature can be explained by several reasons: (i) the use of nitroxides with different cell-penetrating ability, place of retention, and excretion rate, (ii) the heterogeneity of the signal in different tissues (as a result of their different structure and metabolism), (iii) the heterogeneity of the signal in same tissue for different cell-penetrating nitroxide probes (as a result of their different pharmacokinetics), and (iv) the different analytic approaches (conclusions based on: rate of MRI signal decay, half-life of nitroxide-enhanced MRI signal, and intensity of nitroxide-enhanced MRI signal; refs. 19–24).

Each of the previous interpretations is correct, but none of them takes into account all factors that may affect the intensity and dynamics of nitroxide-enhanced MRI signal in living organism. To assess whether oxidation dominates over reduction in carcinogenesis or vice versa, it is necessary to compare the intensity and dynamics of nitroxide-enhanced MRI signal (rate of decay or half-life) in the same tissue between 2 animal species—healthy and cancer bearing. Only in this case, the penetration, retention, and excretion of nitroxide will occur at approximately same speed in the selected ROI, allowing us to ignore the impact of these factors on the dynamics of the signal. Because the cancerous tissue is completely different (structurally and metabolically) from noncancerous, presumably, the most indicative parameter for its redox activity is the duration of nitroxide-enhanced MRI signal. The presence of long-lived signal in the cancerous tissue, whereas in healthy tissues it is on baseline (Fig. 8), is an indicator for the presence of nitroxide in oxidized form, respectively, for the higher tissue-oxidative activity in cancer. The results with reduced TEMPOL support this assumption (Fig. 6). In the case of reduced TEMPOL, the nitroxide-enhanced MRI signal can appear only if noncontrast hydroxylamine is oxidized to contrast-enhancing nitroxide radical. The data showed that nitroxide enhancement appeared only in neuroblastoma-bearing brain in terminal stage, but not in healthy brain or neuroblastoma-bearing brain in early stage of cancer.

Our study also shows that the tissue redox balance is very sensitive to the progression of cancer and can be used as a diagnostic marker of carcinogenesis. In the early stage of cancer, the target tissue is characterized by a high-reducing activity, whereas it is characterized by a high-oxidative activity in the terminal stage. Carcinogenesis correlates with the redox potential of nontarget surrounding tissues and tissues distant from the tumor. In both stages of cancer development, the oxidative status of noncancerous tissues increases and they become susceptible to oxidative stress and damage.

What is the potential molecular mechanism(s) of these observations? Our hypothesis assumes that the inoculation of cancer cells in the brain can be considered an "inflammatory signal" (Fig. 9; refs. 41, 42). This inoculation leads to a local migration and an activation of a wide variety of immune cells in the target tissue, especially in the microenvironment of the primary tumor locus. This activation may trigger redox imbalance due to the "oxidative burst" of the immune cells and the production and release of ROS/RNS in the grafted area. In turn, this process will oxidize the antioxidant defense systems in the "inflamed" area as a compensatory mechanism to prevent oxidative stress in the microenvironment of the primary tumor locus (1, 41, 42). Our study shows that the TAC of the cancer-grafted brain slightly increased in the early stage of cancer development (Fig. 7A). Because the experiments were carried out on immunodeficient mice (Balb/c nude), the early phase was comparatively short (within 3–4 or 7–9 days for brain neuroblastoma or glioma, respectively). Shortly after inoculation, cell proliferation accelerated after overcoming the immune response, and the solid tumor grew.

The initial redox imbalance and subsequent signal transduction in the grafted area could be a critical regulator of cancer progression. ROS/RNS, produced by the immune cells in the primary tumor locus, could provoke signal transduction in 3 targets with equal probability: (i) the grafted cancer cells, (ii) the surrounding normal cells, and (iii) the surrounding extracellular matrix.

![Figure 8. Typical nitroxide-enhanced MRI images of healthy and cancer-bearing mice in the early or terminal stage of cancer (brain neuroblastoma or brain glioma). A, MRI images of the brain with arrows indicating the tumor. B and C, extracted nitroxide-enhanced MRI signal obtained 6 and 14 minutes after injection of SLENU.](image-url)
ROS/RNS have emerged as important mediators of signal transduction that are associated with the activation of the integrin pathway and modulation of integrin function through conformational changes (43). The cross talk between cancer cells and the extracellular matrix also activates integrin-related signal cascades. The oncogenic miRNAs, secreted by cancer cells into the environment, are considered a primary mediator of this process (44, 45). The activation of integrins is linked to additional ROS/RNS production by NADPH oxidases, lipoxygenases, mitochondria, etc., leading to a vicious cycle (46). As a result, the tissue redox balance shifts toward oxidation. Cancer cells are adapted to high levels of ROS/RNS and survive. However, the normal surrounding cells and extracellular matrix are not adapted to this abnormal free radical attack and can undergo irreversible changes.

Integrin signaling also facilitates cell proliferation and migration, which is intimately linked to the degradation of the extracellular matrix, and activated MMPs are a prerequisite for cancer cell invasion (46). We established that the plasma level of MMP9 increases approximately 2 times even in the early stage of cancer development and approximately 3.5 times in the terminal stage (Fig. 7B). The plasma level of MMP2 also increases significantly in the terminal stage of cancer.

Cancer cells are adapted to the high levels of ROS/RNS and survive. However, the normal surrounding cells and extracellular matrix are not adapted to the abnormal free radical attack and can undergo irreversible changes. Our study also indicates that an antioxidant deficiency develops in tissues distant from the cancer locus of a cancer-bearing organism in the terminal stage. These normal tissues become highly sensitive to oxidative damage.

The data suggest that the cancerous and noncancerous tissues of a cancer-bearing organism are equally important therapeutic targets. Combining anticancer therapy with the protection of noncancerous tissues against oxidative stress may be essential for the survival and recovery of the organism.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: R. Bakalova, Z. Zhelev
Development of methodology: R. Bakalova, Z. Zhelev
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R. Bakalova, I. Aoki
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R. Bakalova, Z. Zhelev, I. Aoki
Writing, review, and/or revision of the manuscript: R. Bakalova, Z. Zhelev, T. Saga
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): I. Aoki
Study supervision: T. Saga

ROS/RNS have emerged as important mediators of signal transduction that are associated with the activation of the integrin pathway and modulation of integrin function through conformational changes (43). The cross talk between cancer cells and the extracellular matrix also activates integrin-related signal cascades. The oncogenic miRNAs, secreted by cancer cells into the environment, are considered a primary mediator of this process (44, 45). The activation of integrins is linked to additional ROS/RNS production by NADPH oxidases, lipoxygenases, mitochondria, etc., leading to a vicious cycle (46). As a result, the tissue redox balance shifts toward oxidation. Cancer cells are adapted to high levels of ROS/RNS and survive. However, the normal surrounding cells and extracellular matrix are not adapted to this abnormal free radical attack and can undergo irreversible changes.
Acknowledgments
The authors thank Ms. Sayaka Shibata and Mr. Nobuhiro Nitta (Molecular Imaging Center, National Institute of Radiological Sciences, Japan) for their participation in the MRI measurements.

Grant Support
The study was partially supported by a grant-in-aid (Kakenhi) from the Ministry of Education, Science and Technology of Japan.

References

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Tissue Redox Activity as a Hallmark of Carcinogenesis: From Early to Terminal Stages of Cancer

Rumiana Bakalova, Zhivko Zhelev, Ichio Aoki, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-12-3726

Cited articles
This article cites 41 articles, 3 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/19/9/2503.full.html#ref-list-1

Citing articles
This article has been cited by 3 HighWire-hosted articles. Access the articles at:
/content/19/9/2503.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.