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***In vivo* Monitoring Response to Chemotherapy of Human Diffusion Large B-Cell Lymphoma Xenografts in SCID Mice by ^1H and ^{31}P MRS**

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Abstract

Rationale and Objectives— A reliable non-invasive method for *in vivo* detection of early therapeutic response of non-Hodgkin's lymphoma (NHL) patients would be of great clinical value. This study evaluates the feasibility of ^1H and ^{31}P magnetic resonance spectroscopy (MRS) for *in vivo* detection of response to combination chemotherapy of human diffuse large B-cell lymphoma (DLCL2) xenografts in SCID mice.

Materials and Methods. C—ombination chemotherapy with Cyclophosphamide, Hydroxydoxorubicin, Oncovin, Prednisone, and Bryostatin 1 (CHOPB) was administered to tumor-bearing SCID mice weekly for up to four cycles. Spectroscopic studies were performed before the initiation of treatment and after each cycle of the CHOPB. Proton MRS for detection of lactate and total choline was performed using a selective multiple-quantum-coherence-transfer (Sel-MQC) and a spin-echo-enhanced Sel-MQC (SEE-Sel-MQC) pulse sequence, respectively. Phosphorus-31 MRS utilizing a non-localized single-pulse sequence without proton decoupling was performed on these animals.

Results— Significant decreases in lactate and total choline were detected in the DLCL2 tumors after one cycle of CHOPB chemotherapy. The ratio of phosphomonoesters to β -nucleoside triphosphate (PME/ β NTP, measured by ^{31}P MRS) significantly decreased in the CHOPB treated tumors after two cycles of CHOPB. The control tumors did not exhibit any significant changes in either of these metabolites.

Conclusions— This study demonstrates that ^1H MRS and ^{31}P MRS can detect *in vivo* therapeutic response of NHL tumors and that lactate and choline offer a number of advantages over PMEs as markers of early therapeutic response.

Keywords

Non-Hodgkin's lymphoma; chemotherapy; therapeutic response; MRS; lactate; choline

Introduction

Non-Hodgkin's lymphoma (NHL) comprises a heterogeneous group of closely related malignancies of the lymphoid system in which the cells usually express either B-cell or T-cell markers, or both (1–3). This disease results from disruption of normal development of the hematopoietic system at a precursor stage, probably due to immune deficiency, chronic inflammation, and chronic infection (2,4). The incidence of NHL has been steeply rising (e.g., by 3% per annum in the USA), increasing by 90% in the past 50 years (5). An estimated 58,870

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new cases of NHL are diagnosed in the United States annually. This disease ranks fifth and sixth in prevalence among cancers, and seventh and eighth as a cause of cancer death among females and males, respectively (6); however, in terms of prevalence and numbers of cancer deaths, it affects males more than females and Caucasians more than other races. Furthermore, NHL affects the younger and middle aged population and is the leading cause of cancer-related death among people between 20 and 40 years old; it ranks fourth among all cancers in terms of total number of productive years lost (4).

Currently, only a third of NHL cases are curable by standard chemotherapy (7). The availability of noninvasive methods for prediction and/or early detection of therapeutic response of NHL tumors would be of considerable clinical value. Such methods would facilitate the rational design and individualization of therapy protocols. This would spare non-responsive patients the unnecessary toxicity and expense of ineffective therapy and would offer them opportunities to explore more effective alternative treatment at an earlier time. However, an effective method of detecting early response of NHL to the wide range of therapeutic agents available for treatment of this disease remains elusive since sensitive and specific markers of therapeutic response are still not available.

Positron emission tomography (PET) with ^{18}F -fluorodeoxyglucose (FDG) has become the standard of practice for the management of aggressive B-cell and indolent mantle cell lymphomas (8). PET images are routinely obtained before and after initiation of the first round of chemotherapy; a negative FDG PET scan is considered a strong predictor of prolonged disease-free survival. Although FDG PET offers higher sensitivity than anatomic imaging modalities, both MRI and CT offer higher spatial resolution and MR methods avoid ionizing radiation. Therefore, development of an MR-based method for early detection of therapeutic response is highly desirable.

The potential utility of *in vivo* magnetic resonance spectroscopy (MRS) for detection of therapeutic response of cancers has been explored by a number of investigators (9–17). *In vivo* ^1H MRS detects a number of tumor phospholipid and bioenergetic metabolites associated with cell proliferation or degradation (9,14,18,19). Phosphorus-31 MR spectra predominantly contain resonances of precursors and catabolites of membrane phospholipids, high-energy phosphates, and inorganic phosphate (17,20–22). Thus, ^{31}P -MRS has often been used to evaluate bioenergetic status and pH of tumors and to detect metabolic changes associated with changes in tumor perfusion and therapeutic response (21,23). A multi-institutional clinical trial has recently demonstrated that pre-treatment ^{31}P MRS of NHL tumors identified approximately two thirds of the NHL patients that subsequently failed to exhibit complete clinical response (i.e., disappearance of the local tumor) to a variety of therapeutic modalities (16,24). The study suggests that proton-decoupled ^{31}P MRS measurements of the phospholipid precursors phosphocholine (PC) and phosphoethanolamine (PE) normalized to total nucleoside triphosphates (NTP) serve as general predictors of therapeutic response failure for NHL. However, the low sensitivity and spatial resolution of ^{31}P MRS compared to ^1H MRS limits its clinical utility to relatively large superficial tumors ($\geq 27\text{ cm}^3$ at 1.5 T or $\geq 10\text{ cm}^3$ at 3 T) (16). In contrast, ^1H MRS can detect much smaller tumors ($1\text{--}2\text{ cm}^3$ at 1.5 T) with the same signal-to-noise ratio as ^{31}P MRS at the same acquisition time (25). Proton MRS has been demonstrated to be useful in differentiating between cancer, necrosis, and normal tissue of various organs such as brain (26–28), prostate (29), neck (28), breast (30,31), kidney (32) and liver (33).

Mouse xenograft models of human NHL facilitate development of non-invasive techniques for serial assessment of therapeutic response. These methods hold great promise for tailor-fitting of therapeutic protocols to the needs of individual patients, for evaluating new therapeutic

agents and for identification of therapeutic biomarkers. Studies of animal models serve as guides for clinical application of these methods.

In the present study, we have evaluated the utility of both ^1H and ^{31}P MRS for detecting response of mouse xenografts of human diffuse large B-cell lymphoma, the most common form of NHL, to combination chemotherapy with CHOPB (34). Bryostatin 1, an activator of protein kinase C, down-regulates the *mdr1* gene, improving therapeutic response to CHOP (34,35). This agent also exerts a modulatory effect on *Bcl2* and *p53* gene expression, thereby modifying drug sensitization (35).

Materials and Methods

Cell Line and Tumor Implantation

The Institutional Animal Care and Use Committee (IACUC) of the University of Pennsylvania approved all the procedures employed in these animal studies. The human WSU-DLCL2 cell line was initiated at Wayne State University and kindly supplied by Dr. Mohammad (34). WSU-DLCL2 cells were cultured in RPMI 1640 medium with 10% heat-inactivated fetal bovine serum (Hyclone, Rogan, UT), 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA) and 1% HEPES (Mediatech, Inc., Herndon, VA). The cells were passaged 2–3 times a week and maintained under humidified 5% CO_2 at 37°C . WSU-DLCL2 tumors were subcutaneously (s.c.) implanted in the upper thighs of 6–8 week old female SCID mice (NCI, Bethesda, MD) by inoculating 1.0×10^7 WSU-DLCL2 cells in 0.1 mL Hanks' Balanced Salt Solution (Invitrogen/Gibco, Carlsbad, CA). Palpable tumors developed within a month. Tumor volume was measured twice a week with calipers and calculated as $(\pi/6) \times a \times b \times c$, where a, b, and c are three orthogonal diameters. Magnetic resonance studies and chemotherapy were initiated when the tumor volume reached approximately 500 mm^3 . Animals were euthanized at the conclusion of the MR experiments or to avoid distress when the tumor burden reached $1,500 \text{ mm}^3$.

Tumor Treatment

Tumor-bearing mice were treated once a week with CHOPB over a period of four weeks. Each cycle of CHOPB consisted of Cyclophosphamide (Baxter Healthcare Corporation, Deerfield, IL), intravenous (i.v.) injection at a dose of 40 mg/kg in a 20 mg/ml solution on Day 1; Hydroxydoxorubicin (Bed Venue Laboratories, Inc., Bedford, OH), i.v. at 3.3 mg/kg in a 2 mg/ml solution (Day 1); Oncovin (SICOR Pharmaceuticals, Inc., Irvine, CA), i.v. at 0.5 mg/kg in a 1 mg/ml solution (Day 1); Prednisone (Mathew J. Ryan Veterinary Hospital Pharmacy, Philadelphia, PA), per os (p.o.) at a dose of 0.2 mg/kg in a 0.1 mg/ml saline solution (Days 1–5); and Bryostatin 1 (Biomol International LP., Plymouth Meeting, PA), intraperitoneal (i.p.) at a dose of 75 $\mu\text{g/kg}$, in a 38 $\mu\text{g/ml}$ saline solution (Day 1). The three i.v. drugs were mixed together and administered in a single tail vein injection. Control tumor bearing animals ($n=5$) were sham-treated with saline.

A strict regimen of aseptic cleaning of the site of injection, placement of injection sites at different positions in the tail, and gentle and slow injection of the fluid, was used to avoid extravasation of caustic drugs by multiple tail vein injections that can cause infection and necrosis in the tail.

^1H and ^{31}P MRS Studies

MR experiments were performed before treatment and after completion of each cycle for three cycles (three weeks) following initiation of treatment. During all MRS studies, the mice were anesthetized with 1.0–1.5% isoflurane in oxygen administered at a flow rate of 1 L/min through a nose cone. An MR-compatible small animal monitoring system with a rectal fiber-optic

temperature probe (Luxtron, Mountain View, CA) was used to record the animal body temperature, which was maintained at 36.8°C by blowing warm air through the magnet bore.

In vivo MRS was performed on an Oxford 9.4 T/8.9 cm vertical bore NMR spectrometer equipped with a 25-gauss/cm and 55 mm ID gradient set (Resonance Research, Inc., Billerica, MA) and interfaced to a Varian Inova console (Palo Alto, CA). A home-built slotted-tube resonator (inner diameter of 14 mm and depth of 12 mm) dual-tuned for ^1H and ^{31}P was used for RF transmission and detection. Tumor-bearing mice were placed into the RF probe with the tumor taped to the center of the resonator. A selective multiple-quantum coherence (Sel-MQC) sequence (36) was used to measure global steady-state lactate of tumors and completely suppress lipid and water. A spin-echo-enhanced Sel-MQC (SEE-Sel-MQC) sequence was designed for simultaneous detection of lactate and choline (37). It is, however, difficult to optimize this sequence for both lactate and choline. Therefore, the SEE-Sel-MQC sequence was optimized for detection of choline only.

Typical parameters of the SEE-Sel-MQC sequence were: spectral width, 4 kHz; number of complex data points (np), 2048; repetition time, 4 s; number of averages, 64, and acquisition time, 4'55". The parameters used for the Sel-MQC sequence were: spectral width, 4 kHz; number of complex data points (np), 4096; repetition time, 1 s; number of averages, 128; and acquisition time, 3'33". The MR integral of lactate and total choline were first normalized to the unsaturated water signal measured with a single pulse-acquire sequence in the same study and then to the first data point from each group.

To evaluate tumor phosphorus metabolism, non-localized ^{31}P MRS was performed using a single-pulse sequence with spectral width, 7 kHz; complex data points (np), 4096; repetition time, 1 s; number of averages, 256; and acquisition time, 5'32Prime;

Data were processed with NUTS software (Acorn NMR Inc., Livermore, CA). An exponential filter with 5 Hz line broadening was employed to improve the apparent signal to noise ratio. Baseline correction was applied to MR spectra before calculating MR integral.

Statistical Analysis

Data from control and CHOPB treated tumors were compared by Student's T-test analysis. A P-value ≤ 0.03 was considered statistically significant. Data are presented as mean $\pm$ standard error.

Results

Tumor Growth Delay

Figure 1 displays the time course of average tumor volume (N=5) over a period starting three days before and during CHOPB chemotherapy for up to four cycles of treatment. For each cycle, there were three measurements, one before the cycle, one in the middle of the cycle and one after the cycle with a single measurement between two consecutive cycles. The average tumor volume of CHOPB treated animals increased from $\sim 600 \text{ mm}^3$ to 1000 mm^3 by the middle of the first cycle then decreased by about 40% from the peak value of tumor volume; tumor volume then monotonically decreased slightly over the remainder of the four week treatment period. Control tumors exhibited a monotonic increase over the whole study period. Differences between volumes of controls and treated tumors were significant ($p < 0.03$) after the first cycle of CHOPB (i.e., one week after initiation of therapy).

¹H MRS of Total Choline and Lactate

Proton MRS of lactate and total choline were performed to longitudinally track the therapeutic response of tumors to CHOPB treatment. Figure 2a displays representative MR spectra of the CHOPB treated tumors before treatment, and after three cycles of CHOPB, in which the peaks of choline and lactate are presented. The two spectra show that the total choline peak decreased dramatically but the lactate peak decreased only slightly, probably due to the sub-optimal settings of the sequence for detection of lactate. Figure 2b depicts the time course of the MR signal intensity of the total choline peak of the control (dashed line, n=4) and CHOPB treated tumors (solid line, n=4). Signal intensity of total choline in the treated tumors significantly decreased after the first cycle of CHOPB (1.0 ± 0.17 in the beginning of the first CHOPB cycle vs. 0.35 ± 0.04 after the first CHOPB cycle, $p < 0.03$) and remained stable at a low level thereafter. Representative lactate spectra (from Sel-MQC sequence) of the CHOPB treated tumors before the treatment, and after three cycles of CHOPB are shown in Figure 3a. The peak of lactate diminished significantly in the spectrum after three cycles of CHOPB. Figure 3b displays the time course of the MR signal intensity of lactate of control and CHOPB treated tumors (n=4). Signal intensity of lactate in the treated tumors significantly decreased within a week after the initiation of CHOPB (1.0 ± 0.02 pretreatment vs. 0.19 ± 0.04 after the first cycle of CHOPB, $p < 0.03$). However, MR signal intensity of lactate in the control tumors did not significantly change as indicated by the dashed line in Figure 3b.

³¹P MRS of DLCL2 Tumors

Figure 4a presents a set of representative *in vivo* ³¹P MR spectra of the CHOPB treated tumors before treatment and after three cycles of CHOPB. A number of metabolites were observed in the ³¹P MR spectra of the tumors. The PME resonances originating from phosphoethanolamine (PE) and phosphocholine (PC), and the phosphodiester (PDE) resonances originating from glycerophosphoethanolamine (GPE) and glycerophosphocholine (GPC) were not well resolved due to proton coupling. Figure 4b depicts the time courses of the ratio of PME/ β NTP of the control tumors (dashed line) and CHOPB treated tumors (solid line, n=4). The ratio of PME/ β NTP exhibited statistically significant decreases after two cycles of CHOPB chemotherapy (from 1.0 ± 0.17 pre-CHOPB to 0.54 ± 0.10 after two cycles of CHOPB, $p < 0.03$). In the control tumors no statistically significant change was observed over the entire period of the treatment.

Discussions

Tumors generally exhibit much higher levels of total choline than normal tissues (38). Though the SEE-Sel-MQC is a non-localized sequence, contamination from choline originating from normal tissue was probably negligible because the tumors were positioned in the middle of the slotted tube resonator used for MRS study. Decreased total choline has been observed to accompany therapeutic response in models of human prostate cancer after radiation therapy (39), human breast cancer after neoadjuvant chemotherapy (40), and a radiation-induced fibrosarcoma after administration of a vascular-disrupting agent or nicotinamide (41,42). We have detected a significant decrease in total choline of the CHOPB treated tumors after the first cycle of CHOPB. The level of this resonance remained low during the rest of the treatment. These data reflect the partial response of this tumor to chemotherapy, which is consistent with the tumor volume data in Fig. 1.

The MR signal intensity of lactate exhibited a significant decrease in the CHOPB treated DLCL2 tumors relative to control tumors within a week after the first cycle of CHOPB treatment. Lactate is generally elevated in cancer cells (43). Decreases in lactate have been reported in different tumors (e.g., radiation-induced fibrosarcoma (RIF-1) and a breast tumor model (EMT6)) after treatment with chemo- or radiation- therapy (44–47). As the end-product

of anaerobic glycolysis, lactate probably decreases after treatment due to decreased glycolysis and/or increased lactate clearance. Using ^{13}C MRS and the two-compartment model of Artemov *et al.* (48), Poptani *et al.* have analyzed the rate of glycolysis, and lactate washout *in vivo* of RIF-1 tumors, following i.v. administration of $[1-^{13}\text{C}]$ glucose (49). These authors detected a decrease in glycolytic rate and no change in lactate washout in those tumors after treatment with cyclophosphamide. In contrast, Rivenzon-Segal *et al.* using a physiological-metabolic model have shown that both the rates of glycolysis and lactate clearance decreased in MCF7 breast tumors after tamoxifen treatment (50). Other investigators have suggested that the decrease in lactate level may result from decreased glycolysis, cell death, improved perfusion and/or tumor reoxygenation (45,51). The decrease in tumor cell density of DLCL2 tumors observed in H & E stained sections that we report may reflect decreased tumor cell proliferation as indicated by Ki67 staining (M. Q. Huang *et al.*, submitted) and could also be associated with increased perfusion due to decreases in interstitial pressure that usually accompany tumor cell death. Hence, elucidation of the mechanism underlying the decrease in tumor lactate that has been observed following CHOPB chemotherapy requires further study.

Since lymphoma cells usually have negligible PCr, and since muscle is the major source of PCr, the appearance of the phosphocreatine (PCr) peak in the tumor spectra can be attributed to muscle contamination (16,24). The ratio of PME/ β NTP in ^{31}P MR spectra often decreases in responsive tumors following effective therapy (52). For example, the PME/ β NTP ratio of responsive NHL human tumors decreased post-treatment (16). In the present study, the average PME/ β NTP of the CHOPB treated tumors significantly decreased after two cycles of CHOPB, which indicates therapeutic response of the treated tumors. Phosphorus-31 MRS appears to detect response later than ^1H MRS in these NHL tumors. Though the acquisition time of each ^{31}P MR spectrum was one and a half times as long as that required for ^1H MRS, the signal to noise ratio of ^1H MR spectra is much higher than that of ^{31}P spectra. Therefore, the utility of PME/ β NTP as a response marker is limited by the relatively low sensitivity of ^{31}P MRS. Our study suggests that ^1H MRS of lactate and total choline may provide a more robust method for response detection.

Decrease in tumor volume is a direct index of therapeutic response. However, ^1H MRS provides other indirect indices of tumor response (e.g., lactate, total choline). Figure 5 evaluates the correlation between the changes of tumor volume and the changes in lactate, total choline and PME/ β NTP of the CHOPB treated tumors. The correlation coefficients (and p-values) between the tumor volume change and the changes of lactate, total choline and PME/ β NTP are 0.314 (0.54), 0.530 (0.36), and 0.528 (0.28), respectively, indicating that no correlation exists between the tumor volume change and the changes in MR metabolic indices of therapeutic response. However, ^1H MRS of lactate and total choline can detect tumor response to CHOPB therapy approximately three days earlier than tumor volume measurement. This may be because metabolic changes may occur much earlier than morphological changes in the tumors receiving chemotherapy (53). Therefore, ^1H MRS is capable of detecting tumor response to chemotherapy prior to volume changes.

In conclusion, this study has assessed the utility of ^1H and ^{31}P MRS for detecting *in vivo* response to CHOPB chemotherapy of DLCL2 xenografts in SCID mice. We have demonstrated that ^1H and ^{31}P MRS can detect therapeutic response of the DLCL2 tumors after one or two cycles of CHOPB, respectively. Thus, in this instance ^1H MRS of lactate and choline detects response earlier than ^{31}P MRS.

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Figure 1.

Time dependence of DLCL2 tumor volume of the control tumors (dashed line) and CHOPB treated tumors (solid line).

Figure 2.

(a) Representative ^1H MR spectra of total choline and lactate of the CHOPB treated tumors before the treatment, and after three cycles of CHOPB using the SEE-Seq-MQC sequence. (b) Time course of MR signal intensity of total choline (normalized to water signal then to the first data) in the control tumors (dashed line) and CHOPB treated tumors (solid line).

Figure 3.

(a) Representative *in vivo* lactate-edited ¹H MR spectra of the CHOPB treated tumors before treatment, after two cycles of CHOPB using the Sel-MQC pulse sequence. (b) Time course of MR signal intensity of lactate (normalized to water signal then to the first data) in the control tumors (dashed line) and CHOPB treated tumors (solid line).

Figure 4.

(a) Representative *in vivo* ^{31}P MR spectra of the CHOPB treated tumors before treatment, and after three cycles of CHOPB using non-decoupled one-pulse sequence. (b) Time courses of PME/ βNTP of the control tumors (dashed line) and CHOPB treated tumors (solid line).

Figure 5.

Plots of the volumes of the CHOPB treated tumors compared with the MR signal ratio of lactate/water (top), total choline/water (middle) and PME/βNTP (bottom) of the tumors. Each plot displays a correlation line between the tumor volume and the MR signal intensity ratio of lactate/water, total choline/water and PME/βNTP. The square of correlation coefficients (R^2) is displayed in each legend.