Evaluation of Treatment-Associated Inflammatory Response on Diffusion-Weighted Magnetic Resonance Imaging and 2-[18F]-Fluoro-2-Deoxy-D-Glucose-Positron Emission Tomography Imaging Biomarkers

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Abstract

Purpose: Functional imaging biomarkers of cancer treatment response offer the potential for early determination of outcome through the assessment of biochemical, physiologic, and microenvironmental readouts. Cell death may result in an immunologic response, thus complicating the interpretation of biomarker readouts. This study evaluated the temporal effect of treatment-associated inflammatory activity on diffusion magnetic resonance imaging and 2-[18F]-fluoro-2-deoxy-D-glucose-positron emission tomography imaging (FDG-PET) biomarkers to delineate the effects of the inflammatory response on imaging readouts.

Experimental Design: Rats with intracerebral 9L gliosarcomas were separated into four groups consisting of control, an immunosuppressive agent dexamethasone (Dex), 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), and BCNU+Dex. Animals were imaged using diffusion-weighted magnetic resonance imaging and FDG-PET at 0, 3, and 7 days posttreatment.

Results: In the BCNU- and BCNU+Dex-treated animal groups, diffusion values increased progressively over the 7-day study period to ∼23% over baseline. The FDG percentage change of standard uptake value decreased at day 3 (−30.9%) but increased over baseline levels at day 7 (+20.1%). FDG-PET of BCNU+Dex-treated animals were found to have percentage of standard uptake value reductions of −31.4% and −24.7% at days 3 and 7, respectively, following treatment. Activated macrophages were observed on day 7 in the BCNU treatment group with much fewer found in the BCNU+Dex group.

Conclusions: Results revealed that treatment-associated inflammatory response following tumor therapy resulted in the accentuation of tumor diffusion response along with a corresponding increase in tumor FDG uptake due to the presence of glucose-consuming activated macrophages. The dynamics and magnitude of potential inflammatory response should be considered when interpreting imaging biomarker results.

Promising approaches for the individualization of oncology patient care include the development of imaging-based biomarkers as early readouts of therapeutic efficacy (1, 2). Imaging readouts can be based on a pre-treatment-measured parameter or a parameter that is found to change early on following treatment initiation.

Molecular imaging biomarkers under active evaluation for early treatment assessment include diffusion magnetic resonance imaging (MRI; refs. 3–6) and 2-[18F]-fluoro-2-deoxy-D-glucose-positron emission tomography (FDG-PET; refs. 7–16).

Diffusion-weighted MRI (DW-MRI) can be used to quantify the Brownian motion of water molecules within the tumor tissue and is expressed as an apparent diffusion coefficient (ADC). The random motion of water molecules is influenced by the underlying tumor morphology (i.e., cellular density; refs. 17, 18); therefore, it is sensitive to early changes in the tumor cell death during successful treatment regimes. More specifically, as the tumor cells are killed during treatment, diffusion values have been shown to increase in those regions of positive response, thus providing an opportunity for the use of DW-MRI-determined changes in ADC as a surrogate for cellular death.
Inflammatory Response Effects on Imaging Biomarkers

Translational Relevance

Imaging biomarkers of cancer treatment response are under active investigation throughout the world. To provide clinically prognostic and useful information, changes in imaging biomarker readouts must provide consistent changes reflective of specific underlying alterations in tissue properties including cellular viability, biochemical, and molecular responses to therapeutic intervention. Data presented herein show the temporal effect of treatment-associated inflammatory response following chemotherapeutic treatment on 2-[18F]-fluoro-2-deoxy-D-glucose positron emission tomography and diffusion-weighted magnetic resonance imaging readouts. Findings reveal that imaging readouts may be altered significantly by temporal changes associated by the dynamic underlying immunologic response to tumor treatment. Interpretation of imaging biomarker readouts of tumor treatment response should take into consideration the potential for changes in cell type subpopulations within the tumor mass over time.

Because the removal of tumor macromolecular debris takes time for the overall tumor mass to shrink, changes in tumor diffusion is considered to be an early event in the successful treatment of a tumor, thereby allowing for early and noninvasive imaging of treatment response within an individual patient. Data supporting the application of DW-MRI for cancer treatment response monitoring were initially reported in the 9L glioma model (19) and have since been supported by numerous studies using a variety of tumor models (20–34) and in cancer patients (3, 35–46). Moreover, treatment-induced changes in ADC values of brain tumors are dose dependent in animal models (21, 29) and predictive of outcome in brain tumor patients (47).

FDG-PET is a nuclear imaging–based biomarker that is under active evaluation in part as an early cancer treatment response indicator. This approach uses a radiotracer, [18F]-FDG, for the detection of treatment efficacy as alterations in tumor glucose uptake following treatment are anticipated due to tumor cell death. The standard uptake values (SUV) of FDG-PET are sensitive to early changes in gliomas following treatment (7, 48–50). A decrease in tumor FDG uptake using scintillation counting on excised tumors following radiotherapy was reported in mouse mammary carcinomas and a rat hepatoma (48). More recently, several reports have shown that chemotherapy (10, 49, 51) and radiotherapy (13, 51, 52) result in early increases in FDG uptake of brain tumors in humans. This contradiction has yet to be adequately explained.

Diffusion MRI and FDG-PET imaging biomarkers have been identified to be sensitive to early changes in the tumor microenvironment following cytotoxic intervention, thus it is important to understand the relationship/correlation of these two biomarkers for assessing treatment response. Moreover, because tumor inflammatory response can occur following treatment and because [18F]-FDG cannot differentiate tumor from inflammation (53), it would be prudent to investigate the relative sensitivities of each approach to inflammation. To our knowledge, there have been no studies investigating the relationship between diffusion MRI and FDG-PET simultaneously in an experimental tumor model undergoing therapy. In this study, longitudinal diffusion MRI and FDG-PET images of the 9L orthotopic glioma model during chemotherapy (with or without immunosuppressive therapy) was accomplished allowing for the quantification of temporal changes for each biomarker and evaluation of the relative influence of the tumor inflammatory response, namely macrophage infiltration and activation, on the individual imaging biomarker readouts.

Materials and Methods

Intracranial tumor implantation. The rat 9L gliosarcoma cell line (University of California, San Francisco, CA) was cultured as a monolayer in DMEM with glutamine, supplemented with 10% inactivated FCS in an incubator at 37°C with 5% CO2 and 95% air. Cells were harvested and resuspended in serum-free media at the time of tumor implantation.

All animal work was carried out in the animal facility at University of Michigan in accordance with federal, local, and institutional guidelines. Male Fischer 344 rats (Charles River Breeding Laboratories) weighing between 125 and 150 g were used for cell implantation. Animals were anesthetized using a xylazine (13 mg/kg)/ketamine (87 mg/kg) mixture dosed i.p. A small skin incision was made over the right hemisphere and a 1-mm diameter burr hole was made through the skull using a high-speed drill. The 9L cell suspension was prepared to consist of a suspension of 1 × 10^5 cells in 5 μL serum-free medium, which was introduced through a 27-gauge needle into the depth of 2.5 mm. Following implantation, the site was cleaned and the burr hole was filled with bone wax. The incision was then closed and the animals were allowed to recover under the care of an animal technician.

Treatment protocols. Animals with 9L tumors were entered into the imaging study. When in vivo tumor volumes reached 20 to 60 μL, animals were divided into four groups. Group one received 0.1 mL of drug vehicle (10% ethanol) and was used as a control group (n = 6). Group two (n = 4) was treated daily with 10 mg/kg dexamethasone (Dex), which was initiated 3 d before the sham 1,3-bis(2-chloroethyl)-1-nitosourea (BCNU) treatment (10% ethanol) and continued for the 7-d imaging study period. Thus, day 0 for this group had 3 d of Dex treatment before baseline imaging. Group three (n = 4) received 13.3 mg/kg BCNU diluted in 10% ethanol. Group four (n = 3) received the Dex treatment in an identical
Fourier rebinning. using OSEM (54), 16 subsets, and six iterations with 30-min average PET image. Images were reconstructed acquired serially and images were summed to form a injection and FDG was allowed to uptake for 40 min was injected as a bolus through an indwelling tail vein Animals were anesthetized with isoflurane and placed instrument was used for all subsequent time points. An animal began a study on a particular instrument, that time points both pretreatment and posttreatment. Once accomplished using either an R4 or P4 micro-PET scanner (Siemens) immediately after each diffusion MRI scan at all time points both pretreatment and posttreatment. Once an animal began a study on a particular instrument, that instrument was used for all subsequent time points. Animals were anesthetized with isoflurane and placed on the bed of the instrument, and FDG (0.9-1.2 mCi) was injected as a bolus through an indwelling tail vein catheter. Animals were fasted for at least 4 h before FDG injection and FDG was allowed to uptake for 40 min before data acquisition. Six 5-min images were then acquired serially and images were summed to form a 30-min average PET image. Images were reconstructed using OSEM (54), 16 subsets, and six iterations with Fourier rebinning.

**Diffusion-weighted MRI.** Maps of tumor ADC values were acquired using our previously described method (6). Briefly, a trace diffusion weighted multislice spin echo sequence (with motion compensation gradient waveforms and a navigator echo) was used to acquire 13 slices with two different diffusion weightings ($b_1 = 100$ and $b_2 = 1,248$ s/mm$^2$). The $b$ values represent the sensitivity to diffusion-based contrast in MRI. At least two ($b$ values) are required ($b_1$ and $b_2$) to calculate an ADC as described in the ADC equation below. The image slice thickness was 1 mm, image matrix was 128 × 128 (zero filled to 256), field of view was 30 × 30 mm, and an echo time was 60 ms. During all MRI procedures, animals were anaesthetized using ∼1.5% isoflurane and body temperature was maintained at 37°C using a heated water recirculating pad. The images acquired with $b_1$ were essentially T2 weighted images and these were used to segment the tumor from normal brain for volumetric analysis using an "in house" region drawing tool developed in Matlab.

**FDG-PET imaging.** PET imaging of animals was accomplished using either an R4 or P4 micro-PET scanner (Siemens) immediately after each diffusion MRI scan at all time points both pretreatment and posttreatment. Once an animal began a study on a particular instrument, that instrument was used for all subsequent time points. Animals were anesthetized with isoflurane and placed on the bed of the instrument, and FDG (0.9-1.2 mCi) was injected as a bolus through an indwelling tail vein catheter. Animals were fasted for at least 4 h before FDG injection and FDG was allowed to uptake for 40 min before data acquisition. Six 5-min images were then acquired serially and images were summed to form a 30-min average PET image. Images were reconstructed using OSEM (54), 16 subsets, and six iterations with Fourier rebinning.

**Image registration and analysis.** Isotropic ADC maps were calculated for each MR image set using the following expression

$$ADC = \ln(S_1/S_2)/(b_2-b_1)$$

in which $S_1$ and $S_2$ are diffusion-weighted images acquired using $b_1$ and $b_2$, respectively. ADC pixel value histograms were generated from tumor regions of interest combined across slices. The tumor boundary was manually defined on each slice using a region-of-interest tool, and then integrated across slices to determine volume (21). Micro-PET images of FDG uptake were then spatially coregistered to their corresponding MR data sets by using a "mutual information for automatic multimodality image fusion" (MIAMI Fuse) algorithm (55). The region-of-interest masks that were generated to define each tumor volume at each time point were then applied to the corresponding PET data set and SUVs were calculated for each tumor at each time point. SUVs were calculated according to

$$SUV = FDG_{region}/(FDG_{dose}/WT),$$

in which $FDG_{region}$ is the decay-corrected regional radiotracer concentration in becquerels per milliliter, $FDG_{dose}$ is the injected radiotracer dose in becquerels, and the $WT$ is the body weight in kilograms. All data were analyzed as mean percentage change from pretreatment values [%$X=(X_{post-Tx}-X_{pre-Tx})/X_{pre-Tx}$], in which $X$ is ADC or SUV. Data are presented as the mean ± SEM.

**Histologic analysis.** In a parallel study, 16 animals with 91 tumors were divided into control, BCNU, and BCNU+Dex treatment groups as described above. Anatomical and diffusion MRI was done on the day of treatment and repeated on days 3 and 7 posttreatment to ensure that the tumors responded similarly to the imaging co-hort in terms of diffusion changes over these time intervals. Tissue was removed for control, BCNU-, and BCNU +Dex-treated animals and fixed in buffered formaldehyde for 3 d. Sections were cut on a microtome and paraffin embedded. H&E staining was accomplished on 8-μm-thick sections for quantification of cells within the tumors. Nuclei were counted in six randomly selected fields from sections that provided for the quantification of the number of nuclei for each treatment group. Data were quantified as the number of nuclei ± SEM.

Paraffin sections were deparaffinized in xylene with three changes of 2 min each, then rehydrated through an alcohol gradient of 2 min each (100%, 90%, 70%, 50%, and 30%). Antigen retrieval was done by heating the slides in citrate buffer (pH 6.0) for 10 min in a microwave oven. Tissues were then blocked in PBS-T supplemented with 5% normal donkey serum for 1 h. The tissue sections were then incubated with Glut-1 antibody (Abcam) at a dilution of 1:100 and followed by cy3-coupled anti-rabbit secondary antibody (1:200, Jackson Immunoresearch). The slides were then counter stained with 1 μg/mL 4',6-diamidino-2-phenylindole, mounted, and visualized under a fluorescence confocal microscope Nikon Eclipse TE2000-U (Nikon). In case of MAC-1 staining, CD11b/C antibody coupled to FITC was used at a dilution of 1:100 and followed by cy3-coupled anti-rabbit secondary antibody (1:200, Jackson Immunoresearch). The slides were then counter stained with 1 μg/mL 4',6-diamidino-2-phenylindole, mounted, and visualized under a fluorescence confocal microscope Nikon Eclipse TE2000-U (Nikon). In case of MAC-1 staining, CD11b/C antibody coupled to FITC was used at a dilution of 1:100 and followed by cy3-coupled anti-rabbit secondary antibody (1:200, Jackson Immunoresearch). The slides were then counter stained with 1 μg/mL 4',6-diamidino-2-phenylindole, mounted, and visualized under a fluorescence confocal microscope Nikon Eclipse TE2000-U (Nikon). In case of MAC-1 staining, CD11b/C antibody coupled to FITC was used at a dilution of 1:100 and followed by cy3-coupled anti-rabbit secondary antibody (1:200, Jackson Immunoresearch). The slides were then counter stained with 1 μg/mL 4',6-diamidino-2-phenylindole, mounted, and visualized under a fluorescence confocal microscope Nikon Eclipse TE2000-U (Nikon). In case of MAC-1 staining, CD11b/C antibody coupled to FITC was used at a dilution of 1:100 and followed by cy3-coupled anti-rabbit secondary antibody (1:200, Jackson Immunoresearch). The slides were then counter stained with 1 μg/mL 4',6-diamidino-2-phenylindole, mounted, and visualized under a fluorescence confocal microscope Nikon Eclipse TE2000-U (Nikon). In case of MAC-1 staining, CD11b/C antibody coupled to FITC was used at a dilution of 1:100 and followed by cy3-coupled anti-rabbit secondary antibody (1:200, Jackson Immunoresearch). The slides were then counter stained with 1 μg/mL 4',6-diamidino-2-phenylindole, mounted, and visualized under a fluorescence confocal microscope Nikon Eclipse TE2000-U (Nikon). In case of MAC-1 staining, CD11b/C antibody coupled to FITC was used at a dilution of 1:100 and followed by cy3-coupled anti-rabbit secondary antibody (1:200, Jackson Immunoresearch). The slides were then counter stained with 1 μg/mL 4',6-diamidino-2-phenylindole, mounted, and visualized under a fluorescence confocal microscope Nikon Eclipse TE2000-U (Nikon). In case of MAC-1 staining, CD11b/C antibody coupled to FITC was used at a dilution of 1:100 and followed by cy3-coupled anti-rabbit secondary antibody (1:200, Jackson Immunoresearch). The slides were then counter stained with 1 μg/mL 4',6-diamidino-2-phenylindole, mounted, and visualized under a fluorescence confocal microscope Nikon Eclipse TE2000-U (Nikon). In case of MAC-1 staining, CD11b/C antibody coupled to FITC was used at a dilution of 1:100 and followed by cy3-coupled anti-rabbit secondary antibody (1:200, Jackson Immunoresearch). The slides were then counter stained with 1 μg/mL 4',6-diamidino-2-phenylindole, mounted, and visualized under a fluorescence confocal microscope Nikon Eclipse TE2000-U (Nikon). In case of MAC-1 staining, CD11b/C antibody coupled to FITC was used at a dilution of 1:100 and followed by cy3-coupled anti-rabbit secondary antibody (1:200, Jackson Immunoresearch). The slides were then counter stained with 1 μg/mL 4',6-diamidino-2-phenylindole, mounted, and visualized under a fluorescence confocal microscope Nikon Eclipse TE2000-U (Nikon). In case of MAC-1 staining, CD11b/C antibody coupled to FITC was used at a dilution of 1:100 and followed by cy3-coupled anti-rabbit secondary antibody (1:200, Jackson Immunoresearch). The slides were then counter stained with 1 μg/mL 4',6-diamidino-2-phenylindole, mounted, and visualized under a fluorescence confocal microscope Nikon Eclipse TE2000-U (Nikon). In case of MAC-1 staining, CD11b/C antibody coupled to FITC was used at a dilution of 1:100 and followed by cy3-coupled anti-rabbit secondary antibody (1:200, Jackson Immunoresearch). The slides were then counter stained with 1 μg/mL 4',6-diamidino-2-phenylindole, mounted, and visualized under a fluorescence confocal microscope Nikon Eclipse TE2000-U (Nikon). In case of MAC-1 staining, CD11b/C antibody coupled to FITC was used at a dilution of 1:100 and followed by cy3-coupled anti-rabbit secondary antibody (1:200, Jackson Immunoresearch).
total number of nuclei per field. GLUT-1 staining was quantified in at least two separate fields in every section. There were two sections per animal and a total of four animals per group. The quantitation was done using the Metamorph software (Molecular Devices Corp.).

Statistical methods. Changes in tumor volume, mean FDG uptake, and ADC values of the treatment groups were compared at days 3 and 7 using an ANOVA with a Tukey's multiple comparison statistical test. Differences in cell count, MAC-1, and GLUT-1 between groups were assessed using an unpaired Student's t test. All statistical tests were done using a statistical software package (SPSS, Inc.) with significance assessed at $P < 0.05$.

Results

FDG-PET treatment response. FDG-PET images were spatially registered to the corresponding MRI data sets. FDG color overlaid images on coronal T2-weighted MR images are shown in Fig. 1, which allowed for a more accurate definition of tumor boundaries for prescribing regions of interest within individual image slices. FDG images of control and Dex-treated (data not shown) animals revealed no major change in FDG uptake over days 3 and 7. Inspection of FDG images of BCNU-treated tumors revealed a noticeable decline in tumor FDG uptake at day 3 posttreatment with a large increase in FDG uptake at day 7 posttreatment (Fig. 1). However, treatment of animals with BCNU+Dex showed a drop in FDG uptake at day 3, which persisted at day 7 post-BCNU treatment (Fig. 1). Quantification of the average results for all animals was done to provide for statistical comparisons between treatment and time (Fig. 1). At 3 days posttreatment initiation, the decrease in mean %SUV observed for both BCNU-treated groups (BCNU, $−30.9 ± 1.2%$; BCNU+Dex: $−31.4 ± 9.4%$) were significantly lower than controls ($4.1 ± 3.0%$; $P < 0.0001$ and $P < 0.0001$, respectively) and Dex treatment ($−3.0 ± 2.1%$; $P = 002$ and $P = 0.003$, respectively). In contrast, no difference was observed between BCNU-treated groups. By day 7, BCNU+Dex ($−24.7 ± 6.5%$) continued to have a significantly lower mean %SUV than controls.
(5.4 ± 3.7%; \(P = 0.033\)). In contrast, animals treated with BCNU alone showed an increase of 20.1 ± 7.1% from baseline that resulted in a significantly higher mean % SUV than was observed for BCNU+Dex-treated animals (\(P = 0.002\)).

**Diffusion MRI treatment response.** Color maps of apparent diffusion values (ADC) for 9L treatment groups (control, BCNU, BCNU+Dex) were overlaid on coronal T2-weighted MR images as shown in Fig. 2. Control tumor ADC maps revealed little overall change in the ADC distributions as the tumor increased in size. Images of Dex-treated animals revealed very little change in tumor diffusion values over time (data not shown). Treatment with BCNU resulted in a time-dependent increase in tumor diffusion values as shown in Fig. 2 at days 3 and 7 posttreatment. The increase in tumor diffusion values seemed to be fairly spatially uniform throughout the tumor mass over time. To understand the effect of macrophage involvement on influencing ADC changes, results were then compared with those of BCNU+Dex-treated tumors (Fig. 2). Treatment with the combination of BCNU+Dex also resulted in an increase in tumor ADC values on days 3 and 7. Mean ADC values for each tumor were quantified at days 0, 3, and 7 and the percentage change of ADC from baseline for each group was plotted for days 3 and 7 posttreatment (Fig. 2). BCNU-treated tumors revealed a time-dependent increase in tumor diffusion values from 10.5 ± 3.2% to 24.4 ± 7.2% at day 3 and day 7 posttreatment, respectively, which were significantly higher than controls (−6.7 ± 1.1%; \(P = 0.008\) and \(P = 0.011\), respectively). When compared with controls, the BCNU+Dex-treated group also exhibited a statistically larger percentage increase in tumor diffusion values on day 3 (11.0 ± 1.1%; \(P = 0.012\)) as well as on day 7 (21.2 ± 3.1%; \(P = 0.029\)). Only the BCNU-treated group at day 7 was found to be significantly different from Dex-treated animals (−1.2 ± 5.7%; \(P = 0.032\)).

Tumor volumes were assessed using MR images at days 0, 3, and 7 to follow treatment response. As shown in Fig. 3, control and Dex animals were found to grow in percent volume from day 0 to day 3 (200 ± 59% and 186 ± 85%, respectively) and day 7 (631 ± 135% and

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**Fig. 2.** Diffusion MRI. Representative coronal images of intracerebral 9L tumors ADC (\(\times 10^{-11}\)m²/s) maps shown as color overlays on registered T2-weighted MR images of a control-, BCNU-, and BCNU+Dex-treated animal over time at pretreatment (day 0) and repeated on days 3 and 7 posttreatment. Change in ADC. Percentage change in tumor ADC for control, Dex-, BCNU-, and BCNU+Dex-treated animals at days 3 and 7 posttreatment.
A reduced percent increase in tumor volume in animals treated with BCNU (70 ± 24% and 198 ± 34% at days 3 and 7, respectively) and BCNU+Dex (38 ± 11% and 86 ± 36% at days 3 and 7, respectively) over control animals was found (Fig. 3). No significant difference in percentage change in tumor volume was observed between all groups at day 3 versus day 0. BCNU+Dex was found to have significantly lower percentage change than controls at day 7 (P = 0.027), whereas the BCNU group was found to have a lower tumor volume than control animals (P = 0.060).

Quantification of histology. Evaluation of the effect of treatment-associated responses on the imaging biomarkers was accomplished using H&E, MAC-1, and GLUT-1 for quantification of cell number, macrophage number, and the glucose transporter-1 protein, respectively. These studies were undertaken to provide supportive evidence related to the underlying metabolic and cellular changes that occurred following treatment. GLUT-1 was included as GLUT-1 expression is a major reason why tumor cells are FDG positive in PET scans and treatment-dependent changes in expression could affect FDG uptake by the tumor.

Histologic analysis of tumor tissue for the nontreated and day 7 post-BCNU treatment tumors revealed that significant loss of tumor cellularity had occurred (Fig. 4A and B). A significant reduction of the number of cell nuclei from 611 ± 40 to 84 ± 10 was observed for the non-treated and BCNU-treated tumors, respectively, 7 days following BCNU treatment (Fig. 4C). The data revealed that BCNU therapy had resulted in a significant therapeutic effect as it resulted in a massive eradication of viable tumor cells within the tumor mass. To verify that Dex treatment inhibited the inflammatory response, tumor tissue harvested on day 7 posttreatment was interrogated for the presence of macrophages using MAC-1 staining (Fig. 5). Immunofluorescence quantification of the tumor samples revealed that control and BCNU+Dex-treated tumors had small (7.8 ± 1.7) and slightly elevated (20.9 ± 2.4, P < 0.001) levels of macrophages present, respectively, per microscopic field within the tumor tissue. In comparison, BCNU-treated tumors were determined to have highly elevated numbers of macrophages (44.7 ± 7.0) per microscopic field, which was significantly elevated over control (P < 0.001) and BCNU+Dex-treated (P < 0.005) tumors (Fig. 5). To determine if BCNU treatment also produced a decrease in the expression levels of

![Fig. 3. Change in tumor volume. The mean percentage change in intracerebral 9L tumor volumes determined from T2-weighted MR images are shown for control, Dex-, BCNU-, and BCNU+Dex-treated animals at days 3 and 7 posttreatment.](image-url)
the glucose transporter system in the glioma cells, an immunofluorescent stain for GLUT-1 was used. As shown in Fig. 6, immunofluorescence micrographs of 9L tumor tissue revealed that no significant changes ($P > 0.05$) in the GLUT-1 expression levels could be found between control (0.44 ± 0.04 AFU), BCNU-treated (0.47 ± 0.07 AFU) and BCNU+Dex-treated (0.47 ± 0.06 AFU) animals (values are normalized as a percent area stained per number of nuclei).

**Discussion**

Initial preclinical investigations (48) reporting a decrease in FDG uptake hypothesized that a decrease in uptake values is indicative of positive treatment response, thus leading to its possible application as a prognostic imaging biomarker of treatment outcome. An early preliminary glioma clinical investigation reported an increase in FDG uptake at day 1 following radiotherapy, which was followed by a decrease in FDG uptake by day 7 (50). The reports of the ability to predict response to therapy based on FDG changes are conflicting with some, suggesting that they may correlate with outcome (10, 49) whereas others suggesting they do not (56). Furthermore, [18F]-FDG has been shown to have diagnostic limitations due to the high rate of glucose metabolism in normal brain tissue especially for the detection of low-grade tumors and recurrent high-grade tumors (57, 58). However, coregistration of PET images with MRI as done in this study has been reported to improve the performance of [18F]-PET image interpretation (59). The results presented in this study provide some context to these conflicting reports if consideration is given to the fact that the amount of tumor FDG uptake will not only depend on the net metabolic rate of the tumor cells themselves, but also on the extent of infiltrating immune cells. Moreover, the temporal interplay of differing populations of cell types and their net effect on the overall %SUV of FDG becomes significantly complex to interpret following successful treatment, as the decline and increase of tumor and inflammatory cell populations may occur at different and unknown rates. In addition, the glucose uptake of the different cell types involved in the dynamic processes associated with a pathophysiologic response to therapy may also have varied rates, which would led to an even more complicated scenario for deconvolving the relative contributions of the cell types to their overall contribution to the measured %SUV. The current study was initiated in...
an effort to better understand the dynamic changes in % SUV for FDG in a tumor treated with chemotherapy as well as the effect and interplay of the effects of the ensuing inflammatory response that occurs in concert with tumor cell death.

Modulation of the immunologic response following tumor treatment was accomplished using a corticosteroid, which is used in the management of brain tumor patients for reducing the debilitating effects of brain edema (60). Dex is effective in suppressing macrophage infiltration; therefore, this compound was used to evaluate the effect of modulating the immune response on tumor FDG uptake values and diffusion MRI imaging readouts following tumor treatment. As Dex may affect FDG uptake (61–63) and alter tumor ADC values (64), a cohort of Dex-only treated animals were also evaluated and served as an additional "control" arm for interpreting the effects of BCNU +Dex treatment on tumor FDG uptake. Immunohistochemical analysis of tumor specimens revealed that by 7 days post-BCNU treatment, a host therapeutic-associated immune response was induced, resulting in a large infiltration of macrophages into the tumor. However, cotreatment with Dex (BCNU+Dex) was found to greatly reduce the influx of immune cells to the tumor at this time point (Fig. 5). It was found that baseline levels of tumor FDG uptake (%SUV) were very stable over the 7-day time period for control and Dex-treated animals, revealing that Dex produced minimal effect on glucose uptake over this 1 week time period (Fig. 1). BCNU treatment resulted in a significant and early decrease in tumor glucose uptake at day 3 posttreatment (−30.9%) compared with controls, indicating a significant number of tumor cells were eradicated. This is consistent with a previous report which showed by quantification of histologic sections of treated tumors that a significant reduction of 9L tumor cells occurred between 3 and 7 days following BCNU treatment (21), which corresponds with our observed loss in glucose uptake at day 3.

Furthermore, in the current study, we observed a dramatic decrease in the percent increase in tumor volume (Fig. 3) as well as the approximate 6-fold loss of tumor cells within the tumor mass (Fig. 4) compared with control tumors. These findings reveal that the decrease in tumor FDG uptake at 3 days posttreatment can be attributed to a treatment-induced cell loss that does not recover by day 7. At day 7, a significant reduction in tumor cell density was shown (Fig. 4) along with a large net influx of activated macrophages (Fig. 5), which corresponded with the observed increase in tumor FDG uptake over baseline levels (Fig. 1). These early changes in imaging readouts also correlate with response as BCNU treatment of the 9L tumor resulted in an attenuation of tumor volumes from the time of treatment to days 3 to 7 (Fig. 3). Overall, this BCNU dose results in the killing of ~90% of the cells estimated from the significant delay in tumor growth, indicating that early changes in tumor imaging biomarkers can be used to predict outcome (20). Moreover, the increased FDG uptake observed on day 7 posttreatment cannot be attributed to tumor regrowth as the tumor cells had not repopulated the mass at this time interval based on the H&E data (Fig. 4) and the increased ADC values (Fig. 2). The confirmed presence of a significant inflammatory response within the tumor at day 7 posttreatment corresponds with the increase in FDG uptake (Fig. 5), thus it is the most likely macrophage infiltration that accounts for increased FDG activity.

Mounting an immune response occurs over time following therapy, which assists in the clearance of macromolecular debris arising from tumor cell death. In this study, we found that following BCNU treatment, the death of 9L glioma cells was accompanied by a large influx of macrophages into the tumor site at day 7 posttreatment. The increase in the number of inflammatory cells within the tumor mass correlated with the observed increase in tumor FDG uptake at day 7, which exceeded that observed before therapy by ~20% and was thus significantly higher.
than FDG uptake levels measured at day 7 post-BCNU therapy in BCNU+Dex-treated animals. These results show that the dynamic changes associated with tumor treatment immune response can complicate the interpretation of FDG-PET data.

During the course of effective tumor therapy, a reduction in tumor cell density will occur, which will reduce the extent of the barriers impeding water diffusion within those regions. The utility of DW-MRI for detecting changes within a tumor following treatment was initially shown using a BCNU treatment of the rat 9L glioma model (19) and was successfully extended in a variety of preclinical studies assessing the response to anticancer interventions (3–6, 20, 21, 23–29, 31, 33, 35, 42, 47, 52, 65). Treatment-induced changes in tumor ADC values have been shown to precede changes in tumor regression, which has provided the rationale for developing this imaging biomarker as an early predictive marker of treatment response. However, the effect of posttreatment immune response on tumor diffusion changes has not been previously evaluated. In this study, we found that Dex administration to 9L animals resulted in negligible alterations in tumor ADC levels versus control animals at up to 7 days (Fig. 2). Animals treated with BCNU were found to have a significant increase in tumor diffusion values at day 3, which increased further at day 7 posttreatment as the tumor cells continued to die over time, as shown in previous studies (21). Diffusion values for BCNU+Dex-treated animals were not significantly different from BCNU-treated animals at days 3 or 7 posttreatment compared with the BCNU only treatment group. These data indicate that a chemotherapy-associated immunologic response does not produce a profound effect on the overall detected tumor diffusion response using DW-MRI.

In this study, we used the rat 9L glioma model as a system to investigate the effect of the immune response on imaging biomarker readouts of response. Attenuation of the immune response posttherapy provided for a more complete understanding of the effect and temporal nature of macrophage infiltration on DW-MRI and FDG-PET imaging biomarker readouts of treatment response. Both FDG-PET and diffusion MRI detected early changes in the tumor cellular/microenvironment following chemotherapy. Comparison of the effect of the immune response on the modulation of the two imaging biomarker readouts revealed that by day 7, the effect of BCNU+Dex on ADC measurements were minimal for the BCNU-treated animals compared with the observed large increase in tumor FDG uptake. It seems that changes in FDG-PET were dramatically affected by the treatment-associated immune response over time to the extent that FDG uptake was higher than pretreatment levels at day 7 post-BCNU treatment and day 7 values were significantly higher than day 3 values. However, the temporal response profile of DW-MRI was found to be more consistent longitudinally in that although immune modulation was found to be present, the net effect on tumor ADC values was minimal.

In summary, the treatment-associated immune reaction was observed to not significantly affect MR diffusion changes, whereas for FDG-PET scans, it was shown to significantly reverse the initial decline, thus complicating the interpretation of FDG-PET for treatment response assessment as an increase in signal may be interpreted as tumor recurrence. Overall, data presented revealed that MRI diffusion measurements were less sensitive to dynamic changes in tumor macrophage content relative to FDG-PET measurements.

Disclosure of Potential Conflicts of Interest

T.L. Chenevert, B.D. Ross, and A. Rehemtulla have a financial interest in the underlying diffusion technology presented in this manuscript.

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References


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Evaluation of Treatment-Associated Inflammatory Response on Diffusion-Weighted Magnetic Resonance Imaging and 2-[18F]-Fluoro-2-Deoxy-d-Glucose-Positron Emission Tomography Imaging Biomarkers

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