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High-resolution Longitudinal Assessment of Flow and Permeability in Mouse Glioma Vasculature: Sequential Small Molecule and SPIO Dynamic Contrast Agent MRI

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Abstract

The poor prognosis associated with malignant glioma is largely attributable to its invasiveness and robust angiogenesis. Angiogenesis involves host-tumor interaction and requires *in vivo* evaluation. Despite their versatility, few studies have employed mouse glioma models with perfusion MRI approaches, and generally lack longitudinal study design. Employing a micro-MRI system (8.5T), a novel dual bolus-tracking perfusion MRI strategy was implemented. Using the small molecule contrast agent Magnevist, dynamic contrast enhanced MRI was implemented in the intracranial 4C8 mouse glioma model to determine K^{trans} and v_e , indices of tumor vascular permeability and cellularity, respectively. Dynamic susceptibility contrast MRI was subsequently implemented to assess both cerebral blood flow and volume, utilizing the macromolecular superparamagnetic iron oxide, Feridex, which circumvented tumor bolus susceptibility curve distortions from first-pass extravasation. The high resolution parametric maps obtained over 4 weeks, indicated a progression of tumor vascularization, permeability, and decreased cellularity with tumor growth. In conclusion, a comprehensive array of key parameters were reliably quantified in a longitudinal mouse glioma study. The syngeneic 4C8 intracerebral mouse tumor model has excellent characteristics for studies of glioma angiogenesis. This approach provides a useful platform for noninvasive and highly diagnostic longitudinal investigations of anti-angiogenesis strategies in a relevant orthotopic animal model.

Keywords

glioma; mouse; dynamic contrast MRI; perfusion

INTRODUCTION

The most lethal primary brain tumors are malignant gliomas, the most common of which is glioblastoma multiforme (GBM). Prognosis remains extremely poor for GBM, largely due to the robust angiogenic and invasive characteristics of this tumor. (1) Angiogenesis is an essential component of tumor progression (2) and as malignant gliomas are among the best-vascularized tumors, (3) anti-angiogenic compounds may offer particular promise, and have the added advantage that blood brain barrier passage is not required to reach the target cell. (2) The development of sensitive methods for noninvasively monitoring anti-angiogenic effects is critical, both for clinical and preclinical evaluation. As the angiogenic process involves tumor-host interaction, preclinical evaluation must employ *in vivo* models. Among the most versatile models for such studies are the mouse intracranial models of malignant glioma. Mice are well defined genetically, and are extensively used for brain tumor and other neurologic and neurovascular research. Transgenic variants enable examination of the genetic role in numerous CNS diseases, including transgenic mice that spontaneously develop malignant brain tumors. Immunocompromised mice can be implanted with human glioma cell lines. Alternatively, syngeneic (mouse) cell lines provide the advantage of a compatible host-tumor interaction, which promotes an aggressive angiogenic response similar to that observed clinically. In comparison to mice, rats are less well defined genetically, provide fewer options in regards to glioma models, do not lend themselves as easily to developing transgenic variants, and are more expensive and difficult to maintain. However, due to size advantages, preclinical MRI approaches generally employ rat models. The application of perfusion MRI methodologies to the noninvasive monitoring of the angiogenic process in models of mouse glioma is relatively new, uncharacterized and presents unique challenges. (4–6) These challenges include acquiring bolus tracking data with sufficient time and spatial resolution for appropriate vascular input function (VIF) determination and the generation of parametric maps with sufficient resolution for diagnostic utility. In addition to challenges specific to mouse models, the vascular permeability that characterizes gliomas also complicates blood flow measurements with dynamic susceptibility contrast (DSC)-MRI, due to first pass contrast agent extravasation, which correction algorithms or preloading strategies may not sufficiently address. The use of macromolecular blood pool contrast agents, such as the superparamagnetic iron oxides (SPIO, molecular diameter 150 nm) could be used to circumvent first pass extravasation, but they have only recently been employed in rodent glioma and require validation and comparison to monomeric Gd(III) chelates in this context. (7) Furthermore, despite the importance of noninvasively monitoring the progression of tumor growth and angiogenesis, few longitudinal perfusion MRI studies in rodent glioma models have been reported. Employing an 8.5T micro-MRI system in order to obtain the necessary spatial and temporal resolution, we devised appropriate strategies for determination of the vascular input functions for DSC-MRI as well as dynamic contrast enhanced (DCE) MRI approaches in a mouse glioma model. DCE-MRI was implemented using Gd-DTPA, in order to assess tumor vascular permeability and cellularity. Using a novel dual bolus-tracking approach, DSC-MRI was subsequently implemented in the same imaging session, using an SPIO contrast agent, in order to assess cerebral blood flow and volume. (6) This use of this agent, Feridex, was characterized and shown to have important advantages over, the small molecule agent Gd-DTPA. Using this approach, a longitudinal study was implemented which obtained a highly diagnostic array of high resolution parametric maps from which quantitative summary data were derived. These data comprehensively and noninvasively defined the progression of tumor growth and provided key indices of tumor blood flow/ volume, vascular permeability, as well as tumor cellularity/necrosis. The syngeneic intracranial model of mouse 4C8 glioma was demonstrated to have robust invasive and angiogenic properties congruent with that of human malignant glioma. The approach

presented here, provides a useful platform for noninvasive and highly diagnostic longitudinal investigations of anti-angiogenic/anti-tumor strategies.

MATERIALS AND METHODS

Brain tumor induction

Mouse studies were conducted with the approval of the University of Alabama at Birmingham Institutional Animal Care and Use Committee. Female C57BL/6 × DBA/2 F₁ hybrid mice (B6D2F₁) were purchased from the NCI Frederick Animal Production Program, Frederick, MD. Brain tumors were induced by intracerebral injection of 4C8 cells, harvested from log-phase growth in tissue culture. Cells were suspended in serum-free DMEM/F12 with 5% methyl cellulose and injected intracerebrally (right caudate nucleus) in a volume of 5–10 µl (0.5–1 × 10⁶ cells) using a Stoelting stereotaxic frame as previously described.⁽⁸⁾

MRI procedures

Starting at 4 weeks after tumor cell inoculation, mice (n=5) were imaged weekly using contrast-enhanced (200 µl 10X diluted Magnevist i.p.) T₁ weighted MR imaging to determine tumor growth status. An initial latency period of variable duration characterized by negligible tumor growth was generally observed. Rapid tumor growth generally commenced when tumor cross sectional area reached approximately 5 mm², between 9 and 12 weeks after tumor inoculation. Perfusion MRI studies were initiated at this stage for a minimum of 4 consecutive weeks. Mice were initially anesthetized with a ketamine/xylazine mixture (1.5 mg xylazine/10 mg ketamine/100g). Cannulation of one of the lateral tail veins was performed using a 30 gauge needle attached to sterile PE 10 tubing, filled with sterile saline containing 15U heparin/ml. MR imaging employed a Bruker-Biospin 8.5T vertical wide-bore DRX-360 with an AVANCE console, a Paravision 3.0.2 software platform, a Mini0.5 imaging system with a 56 mm inner diameter gradient set (30G/cm), and a 20 mm birdcage mouse head RF coil insert. The mice were positioned (anterior up) with their heads immobilized with a specially designed head holder with adjustable ear pieces. Body temperature was maintained using a water-jacketed heating blanket. Respiration was continuously monitored using a PhysioGard system. Isoflurane (0.5–2%) in 100% oxygen was administered upon placement in the MR probe, and continuously adjusted and maintained while monitoring respiration.

A coronal 16 slice T₁ weighted image set was obtained using a spoiled gradient echo sequence (Paravision FLASH sequence, 256×256 matrix, 98 µm in-plane resolution, 0.5 mm slice thickness, TR 500 msec, TE 3.5 msec, FA 60°, 1 signal average, 2.1 min acquisition time). This was used to locate the tumor and position the perfusion MRI slice, which was matched to that used in previous imaging sessions (if any) using anatomical features and skull contours. Using slice geometry identical to that of the perfusion slice, MR angio scans precisely defined the major blood vessel locations, (Paravision gradient echo FC2D sequence, 128×128 matrix, FOV 2.5 cm, TR 30 msec, TE 6 msec, FA 90°), using either anterior or posterior inflow saturation to distinguish arterial and venous circulation. A single “fully relaxed” (M₀) spoiled gradient echo precontrast image was obtained at the position of the subsequent DCE dynamic series, (Paravision FLASH sequence, TR 6000 ms, TE 2 ms, FA 90°, 128×128 matrix, 1 slice, 1 mm slice thickness, 195 µm in-plane resolution). An inflow saturation slab (5 mm thick, 0.5 mm gap, adiabatic 90° pulse) was positioned anterior to the slice to eliminate spin refreshment at the jugular vein, to enable use for the vascular input function. The subsequent DCE dynamic T₁ weighted 200 image series employed the same parameters except with TR 15.6 and FA 20°, (2.0s/image). Prior to the DCE image series, a calibrated amount of Gd-DTPA (Magnevist, Berlex Inc, *i.v.*, 10X diluted, 3.0 µl/g,

0.15 mmol/kg) was loaded into the infusion line with a Hamilton syringe and injected 30s (~1 ml/min) after initiation of the perfusion image series, using a 150 μ l saline/heparin chase. Subsequently, a post-contrast T_1 weighted multislice image set was acquired (4 averages, acquisition time 8.5 min, other parameters above), followed by a T_2 weighted multislice image set (Paravision spin echo RARE sequence, 16 slices, 0.5 mm slice width, matrix 256 $\times$ 256, 98 μ m in-plane resolution, TR 4500 msec, $TE_{\text{effective}}$ 60 msec, RARE factor 8, 4 averages, acquisition time 9 minutes). DSC-MRI was then implemented with 150 rapid T_2^* weighted images, (Paravision FLASH sequence, TR 12 ms, TE 6 ms, FA 5°, 128 $\times$ 128 matrix, 1 slice, 1 mm slice thickness, 195 μ m in-plane resolution, 1.5s/image). Feridex, an SPIO agent (Berlex Inc, 4X diluted, 2.4 μ l/g, 26.9ug iron/g) was injected 30s (~1 ml/min) after initiation of the DSC-MRI perfusion image series using a 150 μ l saline/heparin chase. In a few cases, for comparative purposes, a second DSC-MRI protocol was implemented 24 hours later using Gd-DTPA (Magnevist, 2.9 μ l/g 3X diluted, 0.49 mmoles/kg, *i.v.*), ten minutes after implementing a 25% Gd-DTPA preload (Magnevist, 0.97 μ l/g, 3X diluted, 0.12 mmoles/kg, *i.v.*).

The doses/dilutions of the contrast agents were empirically calibrated so as to remain in a range where blood concentration changes during the bolus were linear with changes in relaxation.

Image processing

Perfusion MRI data were processed using the Jim software package (Xinapse Systems LTD, Northants, UK). Calculation of DCE-MRI parameters followed the general approach described in Tofts PS *et al.*(9), using the modified model which includes a blood plasma volume term (v_p).(10) We employed this model because to date it is the most commonly employed quantitative DCE-MRI modeling approach. Comparison of this standard model to recently developed approaches which allow for variations of transcytolemmal water exchange out of the “fast-exchange” regime, were beyond the scope of this study.(11,12) For DCE-MRI, the jugular veins were used to determine VIF, the precise positions of which were determined from the angio images. The jugular vein indicated substantially improved VIF consistency in comparison with major brain arterial vessels. Consistent with other small animal studies, this is likely due to its reduced flow pulsatility and considerably larger diameter, a key consideration in mouse brain (see Figure 1). (13) At the perfusion slice positions employed in the current study, the sagittal sinus was comparatively small and provided VIFs of poor quality and dynamic range. The approximately 4–20 fold greater cross sectional area of the jugular vein (~20 pixels) in comparison to other visible circulation (~1–5 pixels), provided increased signal/noise and reduced partial voluming. The small mouse brain geometry limits VIF dispersion and arterial-venous lag, the latter of which can be readily determined via visual inspection of the serial image data and adjusted for with image processing software settings. Parametric maps were generated for K^{trans} , the volume transfer constant for the contrast agent, a measure of vascular permeability, and v_e , the extra-vascular extra-cellular space volume fraction. The Jim image analysis software incorporates intensities from the precontrast fully relaxed M_0 image into the analysis to obtain fully quantitative parametric maps of K^{trans} and v_e . It employs Eqn 1 below, to calculate R_1 ($R_1=1/T_1$) from the DCE image signal intensities, where M and α are the signal intensity and the flip angle, respectively, of the FLASH dynamic series images.(14) Insertion of the reported T_1 value for mouse brain (1.66 sec, 8.5T) (4) into Eqn 1 indicates that the M_0 images acquired in our study (TR 6 sec) closely approximated the fully relaxed condition, reporting 97% of theoretical intensity. For region of interest (ROIs) analyses, (histograms, means) “non-fitting” pixels were removed from the DCE data, as indicated by out of range v_e values ($v_e=0$ or >1), constituting a mean $15.5 \pm 2.3\%$ (SE) of pixels within

tumor perimeters, and greater in brain parenchyma outside of tumor margins, due to minimal contrast agent uptake.

$$M=(M_0)\frac{(\sin\alpha)(1-\exp(-TR\times R_1))}{(1-\cos(\alpha)\exp(-TR\times R_1))}$$
Eqn 1

Calculation of DSC-MRI perfusion parameters followed the model-independent method described in Ostergaard L. *et al.* (15) For DSC-MRI, the VIF was determined from (12–15) brain pixels identified to have VIF characteristics (large, early, rapid intensity changes) by a two stage automatic scanning and selection routine, from which tumor regions and major brain vessels were excluded. This approach targets brain parenchymal arterial micro-circulation, circumventing dispersion and susceptibility saturation effects at major vessels, and empirically provided the most consistent data. DSC data analysis was restricted to the first 75 images in the series, which bracketed the susceptibility bolus intensity changes. Parametric maps were generated for cerebral blood flow (CBF in ml blood/100g tissue/minute), cerebral blood volume (CBV in blood volume percentage of total tissue volume), and mean transit time (MTT in seconds).

Histology

Briefly, selected mice were sacrificed, their brains were removed and fixed overnight at 4°C in neutral buffered formalin. Fixed tissues were sectioned coronally at 1 mm intervals and embedded in paraffin. Paraffin-embedded fixed sections 5–8 µm thick were mounted on positively charged slides, dried (2 hr, 60 °C), deparaffinized, cleared and rehydrated. Histology included serial sections for hematoxylin and eosin staining adjacent to immunohistochemistry for microvessel-associated vonWillebrand Factor with methyl green counter-staining. Sections were also stained for apoptotic cells by TUNEL immunostaining. For immunostaining, tissues were subjected to antigen retrieval (100 mM citrate buffer, pH 6.1, 95 °C 10 min) Endogenous peroxidases were blocked using 3% H₂O₂ in methanol. Non-specific binding sites were blocked. For microvessel composition, blood vessels were reacted sequentially with a rabbit polyclonal primary antibody to vonWillebrand Factor (Factor VIII-related antigen), a biotinylated goat anti-rabbit Ig, streptavidin-horseradish peroxidase and 3,3'-diaminobenzidine. Tissues were counter-stained with methyl green, rinsed, dehydrated and permanently mounted. Paraffin imbedded sections were stained to detect apoptotic cells using the ApopTag peroxidase In Situ Detection Kit (Chemicon, Temecula, CA), according to manufacturer's instructions.

Statistics

Values are expressed as mean ± SEM. Using repeated measures ANOVA with Fisher's protected LSD method (NCSS statistical analysis software, Kaysville, Utah), parameter values at week 1 were tested for differences from weeks 2, 3, and 4.

RESULTS

Figure 1 is a compilation of typical brain images obtained with the dual bolus-tracking DCE/DSC-MRI strategy.(6) The angio image in Figure 1A depicts venous circulation, precisely revealing the jugular vein locations, used for the VIF determination, and notably are much larger than the two internal carotid arteries at the base of the brain, visible in Figure 1B. Figures 1B and 1C depict two images from the subsequent DCE dynamic series, indicating images prior to and 22 seconds after the arrival of contrast agent to the major brain blood vessels, respectively. Figure 1C indicates substantial image intensity enhancement occurring

on a very rapid timescale, resulting from contrast agent permeation into the extra-vascular/extra-cellular space of the tumor. Figure 1D demonstrates a high resolution post-contrast T_1 image at the position of the perfusion slice, obtained after the DCE series, indicating persistent contrast enhancement at the tumor. At the same slice position Figures 1E and F reveal images from the subsequent DSC-MRI dynamic series, both prior to, and during the Feridex bolus, respectively (non-brain areas masked). The reduction of intensity is clearly visible in Figure 1F within the tumor region. The precontrast signal/noise of the DCE and DSC dynamic images (contralateral brain/background) was very consistent, 5.6 ± 0.3 and 6.4 ± 0.2 , respectively, for the ($n=5$) mice. Figures 2 and 3 indicate images and parametric maps obtained at the same geometric position from a single mouse implanted with 4C8 mouse glioma, in which the dual bolus-tracking DCE/DSC methodology was implemented over four consecutive weeks during a period of significant tumor growth. The series of T_1 weighted post-contrast images in Figure 2A clearly indicate the presence of a contrast-enhanced tumor which undergoes substantial growth while maintaining an approximately spherical shape. Figures 2B and C represent the v_e and K^{trans} parametric maps, respectively, and demonstrate impressive spatial resolution. The v_e parameter quantifies the interstitial space that is accessible to the contrast agent (units: fraction of tissue volume). The v_e maps provide an excellent visual representation of where contrast agent uptake occurs, and were used to accurately define the tumor perimeter for ROI analyses. The K^{trans} parameter (units: min^{-1}) is an index of vascular permeability. The intense but heterogeneously distributed K^{trans} values of this tumor increase over time. Figure 3 contains the CBF and CBV parametric maps, respectively, obtained from the Feridex DSC-MRI experiments obtained subsequently to the Gd-DTPA DCE-MRI experiments (non-brain areas masked), and also indicate impressive spatial detail/resolution. Figure 3A shows an elevated but heterogeneous CBF within the tumor, with flow “hot spots” indicating values exceeding five times that of contralateral brain regions. Comparison of the CBF maps to the K^{trans} maps in Figure 2 indicates differing patterns, indicative that they assess different vascular properties and provide complementary information. Consistent with this is the relatively low pixel-to-pixel correlation between K^{trans} and CBF ($R^2=0.28$) within the tumor perimeter. Comparison of Figures 3A and B indicates that some tumor regions report more robust CBV levels than CBF, particularly in weeks 3 and 4, (color scales were adjusted to reflect relative to contralateral values). The areas of difference between the CBV and CBF, or CBV-CBF mismatch, are best revealed by viewing the MTT parametric maps in Figure 3C. MTT is essentially the ratio CBV/CBF , and provides an index of vascular efficiency. Particularly in week 4, the MTT is elevated at the tumor perimeter, consistent with the presence of inefficient tumor neovasculature. Co-registered histological sections obtained at the end of the longitudinal MRI study for the mouse brain shown in Figures 2–3 are shown in Figure 4. Coregistration was facilitated by the parallel coronal orientation of the two modalities, and employed anatomical brain features (ventricles, etc) in tandem with precise knowledge of the geometrical distances separating the slices/sections. Figure 4A indicates a hematoxylin-eosin section (H&E) which indicates significant patches of necrosis (areas of weak staining). The sections indicated in Figure 4B also indicate a number of regions exhibiting apoptotic cells, identified by TUNEL immunostaining which overlap strongly with those indicating necrosis. The histological detection of necrotic/apoptotic tumor regions strongly corroborates the increased local tumor v_e intensities observed in Figure 2B. Figures 4C and 4D indicate enlarged areas from a section utilizing microvessel-associated vonWillebrand Factor with methyl green counter stain, and clearly delineate microvessels (brown) and the tumor region (green). In contrast to normal brain, the tumor perimeter was characterized by a large number of micro-vessels, with a large proportion of them being of extremely large diameter and in many cases oddly shaped. Mean vessel density, (MVD, mean $\#/\text{25mm}^2$ grid/ $200\times$ field $\pm$ std. dev.) a standard histological measure of vascularization, indicated that for the region depicted in Figure 4D, MVD was 27.6 ± 4.9 versus 9.0 ± 2.4 for a contralateral (non-tumor) region. MVD does not consider vessel diameter or function. Notably, the

abnormal vasculature apparent from the histological sections is very consistent with the pattern observed in the MTT parametric map at week 4 in Figure 3C which indicates inefficient vascular flow at the tumor perimeter.

Figure 5 indicates tumor summary variables obtained from MRI experiments with 5 mice, over 4 weeks. The mean tumor volume growth curve in Figure 5A, determined from post-contrast 16 slice image sets, indicates the occurrence of substantial tumor growth. Figure 5B indicates the mean rCBF, rCBV and rMTT plotted over time. Relative values were used to focus on the differences between normal and tumor flow, to reduce measurement error, and minimize effects from alterations in intracranial pressure, blood pressure, and depth of anesthesia. The contralateral ROIs were positioned symmetrically in the contralateral hemisphere, and were of similar shape and area to the tumor ROI's. The mean tumor rCBF and rCBV increased throughout the 4 week period. The increases in rCBV were substantial ($p < 0.05$ week 3,4 vs week 1), and outpaced those of rCBF ($p < 0.05$ week 4 vs week 1), indicating CBV-CBF mismatch, particularly in weeks 3 and 4. This is also reflected in the increase in rMTT over time ($p < 0.05$ weeks 3,4 vs week 1). These patterns indicate there to be substantial increases in tumor vascular volume in tandem with decreased efficiency of tumor vasculature in the latter stages of tumor growth. Figure 5C indicates that K^{trans} increased steadily in parallel with tumor growth, reaching elevated levels at week 4, indicating the development of high levels of vascular permeability over time in these tumors ($p < 0.05$ week 3,4 vs week 1). The mean v_e parameter, a measure of contrast agent accessible space, also indicated a substantial increase (Figure 5D, $p < 0.05$ week 4 vs week 1), particularly in weeks 3 and 4, consistent with decreasing cellularity and increasing tumor necrosis. Also indicated is v_p , known as plasma volume, which was quantified from the DCE-MRI data. The mean v_p indicated substantial increases over the 4 week period ($p < 0.05$ week 4 vs week 1), from 0.035 ± 0.005 to 0.067 ± 0.017 . This increase is consistent with that observed with rCBV, given the margins of error.

Figures 6A and 6B indicate tumor histograms of rCBF and K^{trans} for week four of the mouse depicted in Figures 2 and 3, respectively. The rCBF histogram indicates that a large proportion of the tumor has flow that is 2–3 fold above contralateral, and significant regions reporting 5–7 fold higher. The analogous contralateral ROI histogram (see inset) indicates a narrower profile of values. The K^{trans} histogram indicates there to be a wide distribution of values across the tumor. Figure 6C indicates mean tumor fractions that are greater than certain threshold values, for the five mice over the 4 week period. The thresholds, while arbitrary, were selected to demarcate abnormal parameter levels. Different from examining tumor mean values, this approach provides a summary distribution parameter. The data indicate that the tumor fraction in the high K^{trans} range ($> 0.1 \text{ min}^{-1}$) and the high v_e range (> 0.2) increases 4-fold to 59% and 3-fold to 76%, respectively ($p < 0.05$, weeks 3,4 vs week 1). Analogously, tumor fractions in the high rCBF and rCBV ranges, indicated 3- and 9-fold increases, respectively, over the four weeks ($p < 0.05$ for rCBV).

Figure 7 compares DSC bolus susceptibility curves obtained with the macromolecular contrast agent Feridex and the small molecule contrast agent Gd-DTPA. During the fourth study week, a DSC protocol using Gd-DTPA was performed at the same slice position 24 hours after the dual bolus protocol. It employed a Gd-DTPA preload to enable more appropriate comparison with the preceding experiment, which is also a standard approach employed to circumvent T_1 related bolus curve distortions resulting from first pass extravasation. It would not however, be expected to saturate potential T_2/T_2^* related bolus curve extravasation distortions, occurring at higher tissue Gd-DTPA concentrations. Given sufficient vascular permeability, these could be substantial given the T_2/T_2^* sensitivity of the DSC protocol. For appropriate comparison the Feridex bolus curves are scaled to match the analogous Gd-DTPA curves. The Gd-DTPA and Feridex bolus curve shapes from the

contralateral (non-tumor) area of the brain, are remarkably similar during the early (first pass) portion of the curves. They gradually diverge because Feridex, a blood pool agent, maintains a stable blood concentration, while progressive Gd-DTPA distribution throughout the mouse tissues induces a return to baseline. In contrast, the Feridex and Gd-DTPA tumor bolus curves differ substantially. While the Feridex tumor DSC curve shape is similar to that of the contralateral bolus curves, the Gd-DTPA tumor bolus curve exhibits a prolonged recovery from the initial deflection. It does not exhibit the often observed (positive) baseline “overshoot”, likely because of the minimal T_1 weighting in combination with the Gd-DTPA preload. Also shown, is the intensity bolus curve at the same ROI obtained from the DCE protocol implemented prior to the Feridex DSC protocol. This indicates that the DCE intensity increases occur very rapidly, indicating elevated vascular permeability to Gd-DTPA, consistent with the tumor K^{trans} values shown in Figures 5 and 6. The timing of the bolus curves in Figure 7 can be directly compared because their individual timescales were shifted to synchronize their VIF. As expected, the DCE intensity increase is slightly delayed in comparison to the initial DSC downward deflection, but critically, it coincides perfectly with the period of delayed recovery exhibited by the DSC Gd-DTPA bolus curve. The direction of the distortion (intensity decrease) of the DSC Gd-DTPA bolus curve indicates T_2/T_2^* causality. Taken together, the data strongly indicate that the slowed recovery of the Gd-DTPA DSC tumor bolus curve is caused by T_2/T_2^* effects resulting from the rapid tissue Gd-DTPA uptake. For the same ROI, the Feridex and Gd-DTPA experiments indicated a rCBF of 1.69 and 1.54, a rCBV of 1.91 and 2.68, and a rMTT of 1.05 and 1.52, respectively, indicating a greatly increased rCBV and rMTT for Gd-DTPA in comparison to Feridex. This is consistent with the prolonged recovery of the Gd-DTPA bolus intensity curve, which elevates the area under the corresponding contrast agent concentration-time curve.

DISCUSSION

The goal of the current study was to develop comprehensive perfusion micro-MRI procedures for reliably quantifying key indicators of angiogenesis, growth and necrosis in mouse glioma. To achieve this, we employed a novel dual bolus approach, in conjunction with a macromolecular agent to circumvent extravasation issues. Mouse brain parametric maps of unprecedented resolution were obtained in a longitudinal study to quantify the progression of tumor vascular parameters. The study documented the robust neovascularization in 4C8 mouse glioma model and its suitability for studies of angiogenesis in malignant glioma.

Employing high-field MRI equipment with RF coils specifically designed for mice, the current study obtained sub-200 μm resolution, greatly exceeding that obtained in previous rodent perfusion MRI studies. (4,5,7,16,17) VIF were determined directly from the dynamic images, circumventing the inaccuracies resulting from obtaining them *ex vivo* or from separate animals, as is sometimes employed in DCE-MRI studies. (16,17) The VIF determination enabled calculation of rCBF and rMTT, (in addition to rCBV), which to our knowledge has not been previously demonstrated in an intracranial mouse glioma model. We demonstrated that mean tumor rCBF, as well as the high rCBF tumor fraction, steadily increased with tumor growth. This progressive vascularization was confirmed histologically, which included MVD quantification. The increase in tumor rCBV over time substantially outpaced that of rCBF, as rMTT also increased. The findings are consistent with the concept of tumor angiogenesis promoting rapid development of haphazard and inefficient vascular networks. (18) Comparison of the MTT maps with histological sections confirmed that areas of low vascular efficiency on the tumor rim correlated well with the presence of numerous vessels of abnormally large diameter. The capability to monitor vascular efficiency is important for assessing the status of tumor neovascularization and anti-angiogenic treatment.

Anti-angiogenic treatment has been hypothesized to provide a vascular “normalization” window, the timing of which could be determined from the pattern of changes in MTT, CBV and CBF.(7,18) The term normalization, refers to anti-angiogenic pruning of inefficient vasculature, coupled with decreases in vascular permeability and interstitial fluid pressure, which potentially provides a period during which chemotherapeutic agents could be more effectively delivered to the tumor.(18) As our experimental approach also provides K^{trans} , an index of vascular permeability, it is uniquely suited to evaluate a normalization process in tumor vasculature. Figures 5 and 6 indicated steady increases in mean tumor K^{trans} and the high K^{trans} tumor fraction. Each increased by ~4-fold over the four week period, strongly suggesting an increasing vascular permeability within the tumor. This increasing permeability is consistent with a robust neovascularization process and a significant presence of immature and/or defective vasculature. The progression of tumor K^{trans} values, to some unknown extent, may also reflect the increasing blood flow. K^{trans} is determined by the permeability of the microvessels, their surface area, and depending on conditions, by blood flow, with dependence on blood flow increasing as extravasation approaches a flow-limited condition. (19)

The novel dual bolus tracking approach employed for this study, differs from a previous study, in which sequential administration of extra- and intra-vascular agents was implemented in a murine melanoma tumor model.(20) The absence of VIF determination permitted only approximations of K^{trans} and v_e , rCBF was not measured, and rCBV was obtained from pre- and post-contrast agent images, rather than with dynamic MRI. Towards the same goal, dual-echo approaches have been devised using the small molecular contrast agent Gd-DTPA, in order to acquire T_1 and T_2^* weighted images simultaneously.(21,22) These also show promise, as extravasation effects on T_1 weighted images can be used to extract permeability information and correct the T_2^* weighted images for extravasation related T_1 contamination, but these approaches may insufficiently correct for extravasation related T_2/T_2^* effects. A few studies have reported measurements of tumor blood flow and volume in addition to measures of permeability, exclusively employing T_1 weighted DCE-MRI approaches.(23,24) Accurate blood flow modeling from these data can be limited by the presence of elevated vascular permeability, and its effect on the critical first-pass portion of the bolus curve. To address this, some studies have employed intermediate-sized macromolecular contrast agents with such approaches in experimental tumor models.(25,26) While potentially promising, these strategies involve complex multiple parameter fits and require further validation. The high K^{trans} values and rapid Magnevist uptake (see Figure 7) observed in our study, in combination with the slightly lower data collection rate compared to the DSC-MRI protocol, would limit the accuracy of flow parameter extraction from the DCE-MRI protocols. The DCE-MRI quantitative modeling did incorporate a v_p term, which indicated changes in mean tumor values consistent with those of rCBV, indicating a 90% increase over the 4 weeks which was within experimental error of the 70% increase reported for rCBV. Despite the consistency of mean tumor increases, the v_p -rCBV tumor pixel-to-pixel correlation was relatively low ($R^2 = 0.28$, $n=5$). The v_p individual pixel variation was relatively large; the v_p coefficient of variation for the 5 mice (range = 0.7 – 1.6) was invariably larger than for rCBV (range = 0.5 – 0.8). Using data simulations, Buckley *et al* (27) noted that quantitative modeling approaches such as the one used in the current study can underestimate v_p by 2%–96%. Consistent with this, Weidensteiner *et al* (13) used the same quantitative DCE modeling approach as in the current study, and reported the difficulty of quantifying v_p in the presence of high tumor vascular permeability/ K^{trans} , and documented the advantage of employing a medium molecular weight contrast agent for this purpose, versus a small molecule contrast agent. Harrer *et al* (28), however, noted the utility of quantitative models which include a v_p term, in terms of reducing K^{trans} overestimation in prominently vascularized regions.

In a tumor environment with permeable vasculature, the v_e parameter is an index of tumor necrosis, and an inverse index of tumor cellularity.(19,29) The marked increase in v_e in the final two weeks of the study is consistent with our histological detection of substantial areas of necrosis and apoptosis the fourth week of tumor growth. It is also very consistent with literature documentation of the appearance of necrosis in latter growth stages of malignant glioma.(30) The v_e parameter is a potentially a very useful, and to date perhaps an underutilized, indicator of tumor treatment effect. Recent studies have shown that diffusion-weighted MRI images (MRI), specifically, the apparent diffusion constant (ADC) maps derived from them, can provide sensitive markers of tumor necrosis and early predictors of treatment efficacy, by assessing the pixel-by-pixel ADC temporal correlation over the therapy period. It has been hypothesized that this is due to sensitivity to alterations in water compartmentalization, in effect, tumor cellularity.(31) While evidence suggests that the ADC parameter has empirical utility for tracking tumor response, the relation between ADC and tissue cellularity is complex, indirect, and controversial.(32)

Coupled with novel acquisition protocols, our study developed quantification procedures which enabled consistent inter-animal and longitudinal data comparison. Such procedures are required to successfully monitor the changes in the tumor environment that occur with tumor growth and tumor treatment. The v_e maps provided clearly discernable tumor perimeters, which were employed for the mean and histogram ROI analyses. The current study represents the first report of a longitudinal perfusion MRI study in a mouse glioma model. Notably, it also employed non-invasive MRI to determine the timing of rapid growth initiation, and the accompanying perfusion MRI examination schedule. This approach can provide an important advantage by determining a more valid reference timepoint than that of tumor cell implantation, given the variable latency period which can exist prior to the rapid growth phase in various tumor models. Our findings with the 4C8 mouse glioma model indicated that the tumor growth (see Figure 5) and the progression of tumor vascular parameters were highly consistent when referenced to the point of growth initiation, which could be readily assessed with anatomical MRI scans.

The dual bolus approach used in the current study, demonstrated excellent DCE-DSC compatibility. The Gd-DTPA DCE experimental dose is lower than the amount required for a DSC experiment (3-fold lower), and in the range commonly used for DSC-MRI contrast agent pre dosing strategies employed to minimize contrast agent extravasation effects.(33,34) The DSC-MRI bolus tracking experiment depends on intensity changes, and with sufficient time provided for T_2^* image intensity changes (if any) to stabilize from the DCE experiment, the subsequent experiment is unaffected. Feridex DSC experiments with and without a prior Gd-DTPA DCE protocol, done on consecutive days, indicated CBV/CBF maps which were in excellent agreement (not shown).

The current study validated the strategy of using an SPIO contrast agent to circumvent first-pass extravasation, comparing Feridex and Gd-DTPA DSC experiments within the same tumor. Using a novel approach, these were also compared to Gd-DTPA DCE experiments, enabling direct comparison of the Gd-DTPA extravasation time-course to the DSC bolus time-courses. These unique experiments provided direct evidence that Feridex circumvents the significant T_2/T_2^* related distortions of the tumor bolus susceptibility curve observed with Gd-DTPA. Furthermore, while the Gd-DTPA preload strategy can effectively reduce T_1 related alterations of the DSC tumor bolus curve, our data demonstrates it does not address the T_2/T_2^* distortions that can occur with first-pass Gd-DTPA extravasation which greatly elevates the measured rCBV values. Hence, Feridex greatly improves the accuracy of the DSC measurement, eliminating systematic measurement errors which are not easily corrected. Various strategies have been employed to minimize the effects of extravasation in the DSC experiment, such as using a contrast preload, dual echo-acquisition or post-

processing correction algorithms.(22,33,34) Importantly, however, these primarily counteract the T_1 effects of extravasation on the susceptibility bolus curve, and ineffectively eliminate T_2/T_2^* effects, which can be quite significant.

In our experiments, no evidence of Feridex accumulation within tumors was observed with repetitive Feridex DSC experiments separated by periods ranging from 24 hours to weeks, and those done on consecutive days produced CBF/CBV maps that were in excellent agreement (not shown). Human and intracranial rat tumor studies employing MRI as well as histology (H&E and Prussian Blue), have clearly indicated that Feridex does not cross the vasculature and accumulate in brain tumors.(35,36) This was attributed to the particular configuration of the dextran coating of Feridex, which greatly impacts endocytotic processes. Feridex is rapidly taken up into the reticuloendothelial system of the liver. Although it is a “blood pool” agent because its large size prevents permeation across the vasculature, its half-life in human blood plasma has been reported to be less than 30 minutes, in contrast to 25–30 hours for Ferumoxtran-10, (35) another SPIO agent.

In studies of tumor angiogenesis, the choice of tumor model is critical. While most preclinical MRI studies have utilized the larger rat model, it is important to develop viable MRI approaches which can utilize the many mouse glioma models which are available, which include syngeneic models, human tumor xenograft models, as well as transgenic. (37,38) While human xenograft models are derived from human tumor lines, they are employed with immune-incompetent animals and have a compromised tumor-host interaction. Critically, this can result in tumors which lack the robust invasive and angiogenic properties observed clinically with malignant glioma, hence negating much of the advantage of studying a human tumor cell line.(4,38) In order to foster a seamless tumor-host interaction, our study utilized the syngeneic 4C8 model in normal wild type mice. The 4C8 glioma line is a clone of the MOCH-1 tumor that arose spontaneously in the brain of a B6D2F1 mouse transgenic for Myelin Basic Protein-promoter-driven c-neu.(39) It grows slowly in culture and as implanted tumors in the brains of syngeneic mice, providing ample time for the development of an attendant neovasculature and glioma-like invasive characteristics. The current study is the first to employ noninvasive MRI approaches to characterize this model and in particular, to investigate its vascular properties. This study documented that the intracranial mouse 4C8 glioma model mimics the clinical observations of malignant glioma, with aggressive growth, development of necrosis, and a robust neovascularization characterized by inefficient and leaky vasculature.

In summary, the current study employed high-field MRI and a novel bolus-tracking approach to obtain a comprehensive set of high resolution parametric maps from a mouse glioma model. The progression of tumor growth, tumor rCBF, rCBV, and indices of vascular permeability, efficiency, and tumor cellularity/necrosis, were quantitatively defined in a longitudinal manner.. The experiments validated the utility of an SPIO contrast agent for DSC-MRI approaches. The 4C8 mouse glioma model was found to be characterized by a robust neovascularization congruent with human malignant glioma. The methodological and quantification approaches presented in this study lay the groundwork for noninvasive and highly diagnostic longitudinal investigations of anti-angiogenic and anti-tumor strategies in a relevant intracranial mouse glioma model.

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Figure 1.

Typical images demonstrating the dual DSC/DCE approach in 4C8 intracerebral mouse tumor model. **A:** Angio image indicating venous circulation; **B–C:** Two images from the DCE dynamic series obtained prior to and after the arrival of contrast agent to the major brain blood vessels, respectively; **D:** High-resolution post-contrast T_1 image; **E–F:** Two images from the DSC-MRI dynamic series, both prior to, and during the Feridex bolus, respectively (non-brain areas masked). See text for details.

Figure 2.

Typical images and parametric maps from a single mouse implanted with 4C8 mouse glioma, in which the dual bolus-tracking DCE/DSC approach was implemented over four consecutive weeks at the same geometric position. **A:** High resolution post-Gd-DTPA T_1 weighted images; **B–C** v_e (units: fraction of tissue volume) and K^{trans} (units: min^{-1}) maps produced from the DCE-MRI experiment, respectively. See text for details.

Figure 3. DSC-MRI parametric maps obtained from the same mouse depicted in Figure 2, obtained from implementing the dual bolus-tracking DCE/DSC approach. **A–B:** CBF and CBV parametric maps, respectively, (non-brain areas masked). **C:** MTT parametric maps. Color scales are relative, with contralateral hues set (approximately) to unity, See text for details.

Figure 4. indicates co-registered histological sections obtained at the end of the longitudinal MRI study for the mouse brain depicted in Figures 2–5. **A:** Hematoxylin-eosin (H&E) section indicating necrosis (areas of weak staining). **B:** TUNEL immunostaining section, indicating regions exhibiting apoptotic cells. **C–D:** Enlarged areas from a section utilizing microvessel-associated vonWillebrand Factor with methyl green counter stain. The abnormal tumor rim vasculature is consistent with the pattern observed in the week four MTT parametric map in Figure 3. See text for details.

Figure 5. indicates longitudinal perfusion MRI data averaged from 5 mice, over 4 continuous weeks. **A:** Mean tumor volume growth curve. **B:** Mean tumor rCBF, rCBV, rMTT progression throughout the 4 week period. The rCBV increases outpaced those of rCBF, consistent with the increase in MTT. **C:** Mean tumor K^{trans} indicating increases which parallel tumor growth. **D:** Mean tumor v_e , indicating an accelerating progression, consistent with decreasing cellularity and increasing necrosis. Mean tumor v_p indicates progressive increases consistent with those of rCBV. *: $p < 0.05$ vs week 1. See text for details.

Figure 6.

A–B: Tumor histograms of rCBF and K^{trans} for week four, respectively, for the mouse depicted in Figures 2 and 3. Pixels within each value category are divided by the total number of tumor pixels to represent tumor fraction. Inset in A) indicates the analogous histogram for the contralateral ROI. **C)** Mean tumor (n=5) fractions above the designated threshold values for the parameters shown, over 4 continuous weeks. *: $p < 0.05$ vs week 1. See text for details.

Figure 7.

compares DSC bolus susceptibility curves obtained with the macromolecular contrast agent Feridex and the small molecule contrast agent Gd-DTPA, obtained from a mouse in the final longitudinal study week. Feridex contralateral and tumor curves (red) are compared with analogous Gd-DTPA curves (black) obtained on the following day at the same perfusion slice position. The magnitude of the Feridex curves are normalized to the analogous DSC Gd-DTPA curves to enable curve shape comparison. The intensity bolus curve from the DCE Gd-DTPA experiment (blue, arbitrary units) implemented in tandem with the Feridex DSC experiments protocol, indicates that the timing of tissue Gd-DTPA uptake/extravasation coincides with the prolongation of the DSC Gd-DTPA bolus susceptibility curve. Individual timescales of the bolus curves were shifted to synchronize their VIF. See text for details.