NMR IMAGING OF TUMOR ANGIOGENESIS

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NMR Imaging of Tumor Angiogenesis

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ABSTRACT

Cancer remains a major medical problem accounting for over 500,000 deaths in the US annually. A common feature of most human tumors is their ability to induce the proliferation of new blood vessels, i.e. angiogenesis. Considerable evidence now exists which demonstrates that these tumor vessels are associated with a distinct range of morphological and physiological properties which are not present in normal tissue vasculature. Several studies now document that in a wide variety of tumor models, average tumor vessels have diameters two times those of normal tissue vessels. NMR techniques based on magnetic susceptibility mechanisms are sensitive to varying sizes of blood vessels. By using gradient echo (GE) and spin echo (SE) pulse sequences and different concentrations of an exogenous contrast agent, it is possible to determine the signal contribution from small versus large vessels by examining the change in T2 and T2* rates (ΔR2 and ΔR2*), i.e. the ratio of ΔR2* to ΔR2 increases with vessel size. This ratio provides an index for the average size of vessels within a voxel. The central goal of this research was to utilize such a tool in order to obtain a regional picture of the tumor vascular bed.

Rats, inoculated with C6 glial cells, underwent an MR imaging series nineteen days after implantation, which comprised conventional SE and GE images prior to and following serial injections of an equilibrium iron oxide contrast agent (MION). Regions within the tumor and in the contralateral normal gray matter were identified. The change in the T2 rate and T2* rate (ΔR2 and ΔR2*) were calculated for each region. Since susceptibility contrast mechanisms designed to study the distribution of vessel sizes rely entirely on the compartmentalization of the contrast agent within the vasculature, the first set of experiments was designed to demonstrate the stability of MION to remain within the vasculature, despite the disruption in the blood brain barrier. The second experiments measured ΔR2 and ΔR2* as a function of contrast agent concentration and TE. The MR measurements were compared with predicted values of ΔR2 and ΔR2* made from histological assessment of vessel sizes and theoretical Monte Carlo simulation results.

The steady state measurements of ΔR2 and ΔR2* in the first experiments demonstrated that once the maximum contrast agent concentration had been reached, the values of ΔR2 and ΔR2* remained stable over 90 minutes, suggesting that MION remains within the vasculature. In the second experiments, significant differences were observed between the tumor and contralateral deep gray matter. Specifically, the ratio of R2*/R2 was greater in the tumor than the normal brain, by a factor of 1.9 ± 0.2. From histologic
sections and numerical simulations, the corresponding ratio was predicted to be $1.9 \pm 0.1$. These ratios are suggestive of a greater relative density of large vs small vessels. Maps of the ratio $\Delta R2^*/\Delta R2$ were also produced on a pixel by pixel basis. Regions of high intensity on these maps (indicating a higher ratio of $\Delta R2^*/\Delta R2$) corresponded well with the location of the tumor as determined using conventional images.

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To Bruce

for your support and friendship
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Chapter I
INTRODUCTION

Cancer. It is the second leading cause of death in the United States, exceeded only by heart disease. Although some cancers such as cervical, colon, rectal and testicular have seen a decline in mortality over the past twenty years, there has been an overall 7% increase in death rate per 100,000 population. This year alone, over 560,000 people will die of cancer, and over 1,350,000 new invasive cancers will be diagnosed - not including carcinoma in situ or basal and squamous cell carcinoma. In 1990, there were 20,500 new cases of primary brain tumors and over 20,700 new cases of metastatic brain tumors. Seventy-five percent of these patients will die within a year of diagnosis. Primary malignant brain tumors are the second most common cause of cancer death in people under 34 years of age. Despite concentrated efforts to improve both radiation therapy and chemotherapy, the five year survival rate for patients suffering from brain tumors has only improved by 11% in the past thirty years (18% in 1960 to 29% in 1990) (1). This type of cancer remains one of the deadliest. New diagnostic and therapeutic strategies are required to increase survival in these patients.

The cause of primary brain tumor is unknown, as is the case for most cancers, despite advances in molecular biology. Environmental agents, familial tendencies and viral causes are all under investigation. Brain tumors originate from various cells: neuromas are tumors of the nerves themselves; astrocytomomas arise from astrocyte cells; lymphomas are cancers of the immune system; ependymomas arise from ependymal cells which form the lining of the ventricles and central canal of the spinal cord; hemangioblastomas originate from blood vessels; meningiomas arise from the meninges; pituitary adenomas are slow growing tumors of the pituitary gland. Some of these tumors are usually benign (neuromas, hemangioblastomas, meningiomas and pituitary adenomas) while most gliomas (tumors arising from the glial or supportive tissue of the brain) can present at various stages
of growth and aggressiveness, from low grade to high grade. Often, tumors contain several grades of cells. The highest or most malignant grade of cell found determines the grade of the tumors, even if most of the tumor is a lower grade. Tumor diagnosis by name and by grade is very important for both treatment and prognosis (1).

Brain cancer can be very difficult to diagnose because of the protective nature of the skull. A physician is unable to see or feel a brain tumor during a routine examination. Non-invasive imaging studies such as those performed using CT and MRI can obtain a picture of the brain to provide clues which may suggest a particular type of tumor. Only a sample of tumor obtained via biopsy and examined under a microscope can provide an exact diagnosis. However, because of the inherent heterogeneity of tumors, biopsies are subject to potential sampling errors because routinely only one or two small portions of the tumor are assayed and repeat biopsy is not feasible. It is possible that in the future, tumor markers may help identify the presence of a tumor. Relatively non-invasive techniques such as blood tests could screen for the presence of various tumor markers to determine the presence or regrowth of certain tumors. Unfortunately, these markers do not indicate the location or extent of a tumor, only its presence. Many tests would have to be carried out to determine the exact diagnosis of the tumor, since these markers are cell specific.

Imaging techniques, including Computed Tomography (CT), Magnetic Resonance Imaging (MRI) and Positron Emission Tomography (PET) have become very useful in the diagnosis and follow-up of cancers. Brain tumors often cause much of their damage due to their size and hindrance of vital sections of the brain. This mass effect can usually be seen on anatomical scans as deviations from normal anatomy. However, a simple anatomical image does not provide information about grade or aggressiveness of a tumor. Radiation necrosis, which is caused by the radiation treatments given patients, may also have similar mass effects and thus look identical to recurring tumor. Tumors are known however to differ in function from normal tissue and from necrosis. For example, high grade lesions because of their aggressive nature and uncontrolled growth require a large
amount of nutrients. In the brain, that nutrient is primarily glucose. By injecting glucose which has been labeled with radioactive fluorine (FDG), the increased intake of glucose by the tumor can be monitored via PET as shown in figure 1-1. Low grade tumors have a slower metabolism, requiring less energy. A low grade tumor would therefore not appear as an FDG hot spot. Although this is a significant improvement to simple anatomical images, PET suffers from low resolution. It may not be able to resolve very small focal increases in glucose uptake, which would be indicative of a local region of high grade tumor.

![Post Gd FDG](image)

**Figure 1-1:** High grade tumor. The image on the left is a post-Gd image enhancing the location of the lesion due to the disruption of the Blood Brain Barrier (BBB). The PET FDG image on the right shows the lesion with high glucose uptake, characteristic of a high grade tumor.

Another common feature of solid tumors is their increased vasculature. Tumors are in fact capable of inducing the proliferation of new blood vessels - a process known as angiogenesis. The microvascular system in the adult remains quiescent without capillary growth for prolonged periods (2). However, within a short time, it appears to be capable of responding to both physiologic and pathophysiologic demands. The term angiogenesis was coined in 1935 by Hertig (3) to describe the neovascularization in the developing placenta. It is also found in the development of the corpus luteum following ovulation and
in the granulation tissue of wound healing. Angiogenesis also plays a major role in many
diseases (4) such as diabetic retinopathy (5), atherosclerosis (6), rheumatoid arthritis (7)
and most importantly cancer (8). For over 100 years, solid tumors have been known to be
associated with an increased vascular supply, but detailed studies of tumor angiogenesis
only began about 50 years ago. In 1939, Ide et. al. (9) surmised that "it is probable that
tumors may be elaborating a vessel growth stimulating substance". Algire et. al. (10) then
showed that tumor implants began to grow in size only after the initial vascularization
occurred, implying that the capacity of tumors to stimulate perpetual growth of new
capillaries from the host may be the fundamental difference between the malignant cell and
the normal cell from which it arose. In 1966, Folkman et. al. (11) reported that when
tumors were grown in isolated perfused organs where blood vessels do not proliferate,
they reach a maximum size of only 1-2 mm\(^3\). However, on transplantation to mice these
tumors induce vascularization and expand rapidly to 1-2 cm\(^3\). Based on the data cited
above, Folkman (12) proposed the hypothesis that "tumor growth is angiogenesis-
dependent". This hypothesis can be stated as follows: "In its simplest terms, once tumor
take has occurred, every increase in tumor cell population must be preceded by an increase
in new capillaries converging on the tumor" (4).

Over the last 20 years, many lines of evidence have accumulated supporting
Folkman's hypothesis (8, 13) including the discovery of various angiogenic factors which
are released by tumor cells themselves (14-17). While much of this work has been
performed on tumor and cell lines from outside the brain, it has also been demonstrated that
glioma cells have high levels of expression of multiple immunoreactive forms of angiogenic
growth factors (18, 19). In fact, of the following list of histologic characteristics of
glioblastomas: high cell density, cellular pleomorphism, mitoses, necroses with palisading
cells and prominent vascularization with endothelial cell proliferation (20), Burger et. al.
(21) noted that in a study of 1440 malignant astrocytic gliomas, only vascular proliferation
differentiated anaplastic astrocytomas into short and long-term survival categories. This
increased vascularization therefore offers another opportunity by which to characterize lesions in the central nervous system (CNS) by using imaging techniques which highlight the vasculature.

PET can measure Cerebral Blood Volume (CBV) by using carbon monoxide labeled $^{15}$O which remains in the vasculature by binding with the hemoglobin present in red blood cells (22). The increased vasculature present in tumors is located within the microcirculation, i.e., within vessels on the micron scale. In normal brain, the microvascular system makes up only 4% of tissue. In a low resolution scan such as PET, where a single voxel is on the order of a cm$^2$, signal from the microcirculation is lost. Only voxels which consist of pure blood vessels will register as high intensity. Therefore, PET CBV images are mostly representative of large arteries and veins.

Magnetic resonance imaging techniques have been developed which can map both normal and abnormal brain microvascular hemodynamics, including CBV. These techniques, based on magnetic susceptibility contrast mechanisms are more sensitive to the microvasculature (see chapter two) than are the PET techniques and can therefore provide better information about the increased microcirculation of brain neoplasms. Briefly, a paramagnetic contrast agent is injected into the patient's vein. As the agent circulates through the brain, it will induce inhomogeneities in the local magnetic field in the tissues surrounding the vasculature. With high speed imaging techniques such as echo planar imaging (EPI), the dynamic first pass effects of the contrast agent can be recorded before the agent is washed out of the vasculature. Areas of high microvascular blood volume will cause increased changes in the surrounding tissue, thus appearing as bright regions in CBV maps, as shown in figure 1-2. This figure is representative of a high grade glioma associated with an increase blood volume. Evaluation of patients with primary brain tumors using high speed susceptibility contrast techniques can have significant impact on the diagnosis, prognosis and/or management of patients. Studies have been conducted which show a positive correlation between relative CBV and tumor grade (23, 24). These
studies are used to aid in initial diagnosis, provide important information on patient prognosis, guide surgical interventions and biopsies and provide early surveillance for evidence of malignant dedifferentiation and tumor recurrence post treatment.

![T2 CBV map](image)

**Figure 1-2:** High grade glioma. Image on the image is a T2 weighted image. Image on the right is the rCBV map showing a high intensity region corresponding to the increased vasculature present in high grade tumors.

Along with increased vasculature, it is well known that tumor vessels are associated with a distinct range of both morphological and physiological properties which are not present in normal tissue vasculature. Qualitatively, tumor vessels are disorderly and dominated by large, dilated and tortuous vascular structures with increased vessel spacing. Deane and Lantos (25) studied the various phases of vascularization of rat glioma and discovered that in the late phase of growth, microvessels with diameters on the order of 40 μm could be found in the intermediate zone of the tumor, i.e. the zone between the necrotic core and the proliferating edge. These vessels also varied in shape and size. Using scanning electron microscopy on tumor vascular casts, Zama et al. (26) were able to assess the three dimensional growth patterns of rat gliomas. They found large budding vessels which attained diameters of 250 μm. These buds, although filled with blood, did not have normal blood flow characteristics. Dewhirst et al. compared the newly forming blood vessels in tumors with those of granulation tissue found in wound healing (27).
They discovered that on average, tumor vessels had diameters two to three times greater than those of normal proliferating vessels.

The mechanism of image contrast associated with magnetic susceptibility contrast techniques is dominated by the compartmentalization and delivery of the contrast agent. Both theoretical and empirical data (28-33) have suggested that microscopic tissue properties such as blood vessel size and density affect susceptibility-sensitive imaging techniques. These effects will be described in greater detail in chapter two. Briefly, the compartmentalization of paramagnetic contrast agents within the vasculature induces long-range magnetic field perturbations which shorten the T2 and T2* of the tissue. The range at which these effects are measurable depends on the size of the blood vessel containing the contrast agent. The enhancement of the transverse magnetization relaxation rate, known as relaxivity, can be expressed as follows:

\[
\Delta R_2 = \frac{1}{T2_{\text{post}}} - \frac{1}{T2_{\text{pre}}} \approx -\frac{1}{TE} \ln \left( \frac{S_{\text{post}}}{S_0} \right)
\]

\[
\Delta R_{2*} = \frac{1}{T2^*_{\text{post}}} - \frac{1}{T2^*_{\text{pre}}} \approx -\frac{1}{TE} \ln \left( \frac{S_{\text{post}}}{S_0} \right)
\]

where T2_pre and T2_post are the tissue T2 in the absence and presence, respectively, of field perturbations (similarly for T2*), S0 and S_post are the signal intensities in the absence and presence of field perturbations, respectively.

Both \( \Delta R_2 \) and \( \Delta R_{2*} \) have been shown to vary with vessel size (31, 32), as shown in figure 1-3. Shown here for a concentration of contrast agent comparable to physiologic injections of Gd-DTPA, \( \Delta R_2 \) and \( \Delta R_{2*} \) first increase as a function of vessel size. At larger vessel radii (> 8 \( \mu m \)), \( \Delta R_2 \) decreases to zero whereas \( \Delta R_{2*} \) maintains a constant value, independent of vessel size. \( \Delta R_2 \) peaks for vessels with radii on the order of 3-4 \( \mu m \), corresponding to normal microvasculature. It is important to note the ratio of \( \Delta R_{2*} \) to \( \Delta R_2 \) increases as the size of the vessels increases. This ratio could thus be used as an index of the average vessel size present within a voxel.
Based on this theory and the fact that tumor vessels can have diameters two to three times greater than normal tissue capillaries (27), MRI could therefore provide another marker for tumor growth, by obtaining a regional picture of tumor vascular morphology.

Specific Aims

To examine whether tumor vessels have different morphologic characteristics than normal vessels, this research will study an established rat glioma model - the C6 model. \( \Delta R_2 \) and \( \Delta R_2^* \) measurements will be made as a function of contrast agent concentration and as a function of echo time. An equilibrium iron oxide agent known as MION (Monocrystalline Iron Oxide Nanoparticle) will be used as the contrast agent.

Aside from these morphologic properties unique to tumor vessels, tumor vasculature is also characterized by a range of physiologic properties not shared by normal tissue capillaries. Specifically, tumor capillaries are known to be hyperpermeable (14). In the brain, a barrier known as the blood-brain-barrier (BBB) controls the transport of various chemicals, hormones and nutrients from the vasculature into the brain parenchyma.
Blood vessels in brain tumors are known to have a disruption of the BBB, making them more permeable to agents injected within the vasculature. Additionally, factors released by the tumor cells themselves such as vascular endothelial growth factor, also known as vascular permeability factor (VEGF/VPF) increase the permeability of capillaries to solutes. These combining factors make brain tumor capillaries hyperpermeable.

Susceptibility contrast mechanisms rely entirely on the compartmentalization of the contrast agent within the vasculature. If the agent were to leak out of the vessels faster than its first passage through the microcirculation, no signal changes would be recorded. It is therefore important to determine whether the contrast agent used to measure the relaxivity ($\Delta R2$ and $\Delta R2^*$) remains intravascularly. Although MION has a diameter of approximately 40 nm (34) which is too large to cross the blood brain barrier, it may be possible that with a disruption of the BBB, this agent will leak out of the vessels. The first set of experiments shown will examine the measurements of $\Delta R2$ and $\Delta R2^*$ as a function of time, establishing the stability of MION to remain within the vasculature.

The second set of experiments will examine the ratio of $\Delta R2^*$ to $\Delta R2$ as a function of contrast agent concentration and echo time. Based on the theory, this ratio should be larger in the region of the tumor where larger microvessels should dominate the microcirculation system. It is also possible to create maps of $\Delta R2^*/\Delta R2$ on a pixel by pixel basis in order to examine the regional picture of the microvascular bed.

Finally, in order to demonstrate the validity of this model, histologic assessment of vessel size will be performed in both the tumor and the contralateral grey matter of the brain. A direct comparison of the histologic data and the MR data will be described and results shown.

**Outline of thesis**

Chapter two will provide the theoretical background upon which this theory is based. The basic principles of NMR will be briefly outlined, followed by a presentation of studies which have described the effects of vessel size on relaxivity. Chapter three will
present experimental methods and materials as well as the analysis methods used in these experiments. The results from the experiments and the data analysis will be presented in chapter four. Finally, a discussion of the material presented will be offered in chapter five.
Chapter II
THEORY OF MAGNETIC SUSCEPTIBILITY CONTRAST

The discovery of NMR dates back some fifty years to the work of Bloch (35) and Purcell (36) in 1946. The impact of their work was immediate with applications in physics, chemistry, biology and eventually in medicine. The advent of Nuclear Magnetic Resonance Imaging (MRI) took somewhat longer to develop. In 1973, Lauterbur (37) was the first to publish an image of two tubes of water, which was obtained via a series of back-projections using field gradients. The first human image of a live finger was reported in 1976 (38). Since then, NMR images have improved in quality, resolution, contrast to noise and tissue discrimination.

MRI presents several distinct advantages over other imaging modalities. First and foremost, it does not use ionizing radiation like x-rays, CT, PET or SPECT. In contrast with ultrasound, the energy used in MRI is capable of penetrating bony structures without attenuation. The relaxation parameters inherent to MRI are able to provide not only excellent anatomical information, but also physiologic information not previously seen with other modalities (i.e. multiple sclerosis plaques). Finally, unlike CT, MRI can visualize an object in axial, coronal or sagittal slices, or slices of arbitrary orientation (39).

2.1 THE NMR SIGNAL

Nuclear magnetic resonance can be performed on any element which possesses a magnetic moment and spin. The nuclei which possess this property have an odd number of either protons or neutrons. Such nuclei are characterized by an inherent property known as the gyromagnetic ratio. For example, hydrogen, with only one proton, has a gyromagnetic ratio of 42.58 MHz/T. Although any such nuclei could be used for imaging the human body, hydrogen, with a spin of 1/2, is most often used because of its high natural
abundance as well as its high gyromagnetic ratio. As a consequence, standard clinical images are often referred to as proton images.

Because of the magnetic moment inherent to protons, these nuclei behave somewhat like a tiny bar magnet. Much like the compass of a needle which aligns itself with a magnetic field, protons will act in a similar manner when exposed to an external magnetic field. Unlike the needle however, quantum mechanics dictate that the longitudinal component of the nuclear magnetization will take on one of two states: parallel or anti-parallel to the external magnetic field. These two alignments correspond, respectively to lower and higher energy states. Within this applied magnetic field, there is always an small excess of protons, also referred to as spins, which fall into the lower energy state. As such, a net magnetization ($\mathbf{M}$) will exist in the direction of the applied field (figure 2-1). By convention, the external magnetic field is applied along the $z$-direction. The net magnetization is proportional to the strength of the external field ($B_0$). The larger the applied magnetic field, the larger the magnitude of $\mathbf{M}$. This will increase the MRI signal, which explains why the MRI scanners are heading towards higher and higher field strengths.

![Figure 2-1](image)

**Figure 2-1:** Because more spins align with the external magnetic field, a net magnetization will exist in the sample
Although the protons are said to be aligned with or against $B_0$, in reality, they are precessing around the main magnetic field at a frequency known as the Larmor frequency:

$$\omega = \gamma B_0$$

(2.1)

where $\omega$ = Larmor frequency (MHz), $\gamma$ = gyromagnetic ratio (MHz/T) and $B_0$ = applied magnetic field strength (T). The individual magnetizations of the protons therefore have projections along the $z$-axis which form the net magnetization $M$ but the projections on the $x$-$y$ plane sum to zero.

If a second, smaller magnetic field ($B_1$) is applied along either the $x$ or $y$ direction, i.e., perpendicular to $M$, the net magnetization will rotate away from the $z$-axis at the Larmor frequency (figure 2-2). However, as soon as $M$ is tipped slightly from the $z$-axis, $B_0$ will tend to bring it back because of its relatively greater strength. If $B_1$ also precesses at the Larmor frequency, then it will remain perpendicular to $M$. This will cause the net magnetization to continue to move away from the $z$-axis. Its final position with respect to the $z$-axis is dependent upon the duration and strength of $B_1$ and its final angle with respect to the $z$-axis is known as the flip angle. Since this applied field is rotating at a frequency in the MHz range which is in the radiofrequency (RF) range, it is referred to as the RF pulse. For example, if the net magnetization lands in the $x$-$y$ plane, also known as the transverse plane, the RF pulse is a 90° RF pulse. To simplify the motion, it is easiest to view this process in a rotating frame of reference. If the coordinate system rotates at the Larmor frequency, then the 90° RF pulse is seen to produce a simple tilt of the magnetization into the transverse plane (figure 2-3).

Once the net magnetization is in the transverse plane and the RF pulse is removed, $M$ will precess about the $z$-axis. According to Faraday's law, a time varying magnetic field will induce a voltage in an antenna surrounding the sample. This voltage which is proportional to the magnitude of the magnetization is the MRI signal (figure 2-4).
Figure 2-2: Effect of a 90° pulse on the net magnetization $M$ is to tilt $M$ into the transverse plane in a spiraling motion.

Figure 2-3: Effect of a 90° RF pulse as seen in the rotating frame of reference.
2.2 RELAXATION

There are several tissue-related factors which affect the strength of the NMR signal. The most important of these is known as relaxation and refer to the process by which spins return to their equilibrium state following the perturbing effects of the RF pulse. There are two mechanisms by which this may occur: 1) $T_1$ relaxation also known as spin-lattice relaxation and 2) $T_2$ relaxation, also known as spin-spin relaxation. Each of these parameters is a characteristic of each tissue or substance. They can however be altered by changing the temperature of the sample and/or the external magnetic field or by adding contrast agents to the sample.

2.2.1 $T_1$ Relaxation

$T_1$ relaxation is most easily understood in terms of $M$. If $M$ is tipped into the transverse plane by a 90° RF pulse, there will be no component remaining along the z-axis.
The $T_1$ relaxation time is an exponential time constant that relates to the period required for the longitudinal, or $z$, component to recover from zero to its maximal value (figure 2-5). Although the NMR signal originates from the transverse magnetization, there must exist a longitudinal magnetization in the $z$-axis in order to create the transverse magnetization. Therefore, if any experiment is to be repeated (as most NMR experiments are), the repetition time (TR) must allow for sufficient longitudinal magnetization to recover. TR is thus dependent upon the $T_1$ of the sample.

$$M_l(t) = M_0(1-e^{-t/T_1})$$

**Figure 2-5:** After the 90° pulse, the longitudinal magnetization will return to its initial value in an exponential manner with a time constant of $T_1$.

A $T_1$-weighted image takes advantage of the varying $T_1$ values present within a sample. As a simple example, consider a sample contains two substances, one with a short $T_1$ and the other with a long $T_1$. If the repetition time is chosen such that the substance with the short $T_1$ has recovered most of its longitudinal magnetization while the substance with the long $T_1$ has only recovered a small amount (figure 2-6), then the signal following the 90° RF pulse will be weighted by the amount of magnetization which has recovered. This difference provides contrast between the two substances.
Figure 2-6: By choosing the repetition time appropriately, differences in $T_1$ will provide image contrast.

2.2.2 $T_2$ Relaxation

$T_2$ can be most easily understood if the net transverse magnetization is seen as the sum of individual magnetic moments. Once the RF pulse is removed, each spin will precess about the $z$-axis at or near the Larmor frequency. Local magnetic field inhomogeneities cause some nuclei to experience a slightly stronger magnetic field and others to experience a weaker field. Since the precessional frequency is direction proportional to the magnetic field experienced by the protons, those in the stronger field will precess faster while those in the weaker field will precess more slowly. This loss of phase coherence between the protons is known as dephasing. It will result in a loss of net transverse magnetization, as shown in figure 2-7. $T_2$ is the exponential time constant which relates to the loss of this transverse magnetization. Since the transverse magnetization is responsible for the NMR signal, its reduction will cause signal loss (figure 2-7).

The local magnetic field inhomogeneities noted previously are produced by two factors: 1) microscopic effects due to magnetic interactions with neighboring molecules or 2) macroscopic effects due to inhomogeneities of the external magnetic field $B_0$. 

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Dephasing which is due to molecular interactions alone is called $T_2$ while dephasing due to both processes is called $T_2^*$. With a perfect magnet, $T_2^*$ would be identical to $T_2$. In reality, $T_2^*$ is always shorter than $T_2$.

Imaging sequences can take advantage of these inherent parameters to produce $T_2$-weighted images. The NMR signal is usually read at a time interval known as the echo time (TE) after the RF pulse has been applied. As TE increases, more dephasing can occur due to $T_2$ effects, leading to greater signal loss. For a given TE, the signal intensity of a substance with a long $T_2$ will be greater than that of a substance with a short $T_2$. These differences are translated into image contrast.

![Diagram showing loss of transverse magnetization due to differences in local magnetic fields experienced by the protons.](image)

**Figure 2-7:** Loss of transverse magnetization due to differences in local magnetic fields experienced by the protons.
2.3 PULSE SEQUENCES

Different combinations of RF pulses can be used to produce different types of MR signals and different types of images. The two most commonly used sequences are the spin echo and the gradient echo.

2.3.1. Spin Echo

The spin echo pulse sequence consists of two RF pulses, 90° and 180°, separated in time by an interval equal to TE/2 (figure 2-8). The signal is read at a time of TE. The 90° RF pulse will tip the net magnetization to the transverse plane. Because of T2 effects the spins will lose phase coherence during the time TE/2. The 180° RF pulse is applied along either the x or y direction. Its effect is to cause the spins to rotate 180°, but without loss of precessional frequency. In the equivalent time TE/2, the protons will realign, causing an "echo" in the signal measurement (figure 2-8). The 180° RF pulse has in effect, rephased or refocused static inhomogeneities in the sample (such as the imperfections of the external magnetic field). It is unable however to refocus dynamic or nonstatic inhomogeneities inherent within the sample which are responsible for T2. Therefore, spin echo sequences are T2-weighted rather than T2*-weighted.

![Figure 2-8: A typical spin echo sequence. The 90° RF pulse is separated from the 180° RF pulse by a time TE/2. An echo occur at a time TE.](image)

2.3.2. Gradient Echo


The gradient echo pulse sequence consists of a 90° RF pulse and externally applied linear magnetic field gradients. These gradients are applied between the RF pulse and the signal read out which occurs at a time TE. The first gradient will dephase the spins and is thus called a dephasing gradient. The second gradient is usually equal in magnitude but reversed in sign. It rephases the spins and is therefore called a rephasing gradient. This gradient reversal will cause a gradient echo to occur at the time TE. The absence of the 180° refocus pulse generates images which are weighted by $T_2^*$ rather than $T_2$. Gradient echo images are therefore sensitive to the inhomogeneities in the externally applied magnetic field, but also to static inhomogeneities present within the sample, such as large iron deposits (such as sites of hemorrhage). These effects, known as magnetic susceptibility effects will be discussed in the following sections.

2.4 MAGNETIC SUSCEPTIBILITY

Magnetic susceptibility is a substance's ability to attract or repel the lines of force of an externally applied magnetic field. These effects are dominated by the electrons of a sample, especially unpaired electrons which have both spin and orbital angular momentum.

2.4.1. Diamagnetism

Most organic and many inorganic compounds of low molecular weight which have paired electrons are diamagnetic. Such materials weakly repel magnetic lines of force and therefore reduce the local magnetic field. Although most tissues are diamagnetic, the signal changes which occur due to this property are more typically overwhelmed by larger effects of signal loss, such as relaxation. NMR imaging artifacts at air-tissue interfaces are nonetheless due to this effect.

2.4.2. Paramagnetism

Many transition metal ions and lanthanide metals have unpaired orbital electrons, with a spin of 1/2, which behave much like the hydrogen nucleus when exposed to an external magnetic field. That is to say that they tend to align themselves parallel or anti-
parallel to the applied field. Since more align with the field than against it, the local magnetic field surround this atom or molecule is enhanced. Such materials are known as paramagnetic materials. The amount by which the local field is enhanced is proportional to the strength of the applied magnetic field $B_o$:

$$M = \chi B_o$$

(2.2)

where the constant of proportionality, $\chi$, is called the magnetic susceptibility of the material. Elements such as gadolinium and dysprosium are used as contrast agents in NMR. A thorough review of paramagnetic metal complexes as contrast agents for MRI is presented by Lauffer et al. (40). The effect of a contrast agent on the relaxation rates $1/T_1$, $1/T_2$ and $1/T_2^*$ can be divided into two groups. Changes in $T_1$ occur because of a magnetic field interaction between the electron magnetic dipole and the proton nuclear magnetic dipole. The molecules in the contrast agent are also able to enhance the local magnetic fields which increases the inhomogeneities seen by the protons in the environment. This effect, known as susceptibility induced relaxation will shorten the $T_2$ and $T_2^*$. In either situation, the changes may be expressed as follows:

$$\Delta R_1 = \frac{1}{T_{1\text{post}}} - \frac{1}{T_{1\text{pre}}}$$

$$\Delta R_2 = \frac{1}{T_{2\text{post}}} - \frac{1}{T_{2\text{pre}}}$$

$$\Delta R_{2^*} = \frac{1}{T_{2^*\text{post}}} - \frac{1}{T_{2^*\text{pre}}}$$

(2.3)

One of the most common contrast agents used in clinical MRI is a gadolinium atom chelated with diethylenetriaminepentaacetic acid (Gd-DTPA). Following intravenous administration, this agent travels through the vasculature and diffuses into the extracellular compartment, except in the brain because of the presence of an intact blood brain barrier. The localization of the contrast agent within the vasculature of the brain induces a magnetic susceptibility difference between the intravascular and extravascular spaces of $2.8 \times 10^{-8}$ /mM Gd (41). This will shorten the tissue $T_2$ such that a decrease in signal intensity in $T_2$-
weighted images can be seen during the first pass of the contrast agent through the vasculature (figure 2-9).

2.4.3. Superparamagnetism

Materials such as iron oxide are considered to be superparamagnetic because they enhance the local magnetic field to an even greater extent than paramagnetic agents (42). The field induced by these agents are also proportional to the applied external magnetic field at low field strengths, but they saturate at high fields. A superparamagnetic agent known as MION (Monocrystalline Iron Oxide Nanoparticle) was used in this study (34, 43). It is composed of a core of iron oxide measuring approximately 4.5 nm in diameter, surrounded by dextran molecules which gives the particle a total diameter of approximately 40 nm.

2.5 MR SUSCEPTIBILITY AND PULSE SEQUENCES

The compartmentalization of paramagnetic contrast agents within the vasculature or cells induces long-range magnetic field perturbations that extend over many microns which shorten the T2 and T2* of tissue. Assuming monoexponential decay, the enhancement of the transverse magnetization relaxation rate caused by these agents and can be expressed as follows:

\[
\Delta R_2 = \frac{1}{T2_{post}} - \frac{1}{T2_{pre}} \approx -\frac{1}{TE} \ln \left( \frac{S_{post}}{S_o} \right)
\]

\[
\Delta R_{2*} = \frac{1}{T2*_{post}} - \frac{1}{T2*_{pre}} \approx -\frac{1}{TE} \ln \left( \frac{S_{post}}{S_o} \right)
\]

(2.4)

where \(T2_{pre}\) and \(T2_{post}\) are the tissue T2 in the absence and presence, respectively, of field perturbations (similarly for \(T2*\)), \(S_o\) and \(S_{post}\) are the signal intensities in the absence and presence of field perturbations, respectively.
a) Dynamic passage of paramagnetic contrast material

Figure 2-9: Dynamic cerebral passage of Gd-DTPA (0.2 mmol/kg). (a) Six SE echo planar images (TR = 1 sec, TE = 100 msec) from the series reflect the parenchymal signal attenuation during transit of agent. (b) Time course from a cortical region of interest.
The magnitude of $\Delta R2$ and $\Delta R2^*$ is dependent upon the diffusion of tissue protons in the vicinity of susceptibility-induced field perturbations. Because spin echo sequences use an $180^\circ$ RF pulse, they are capable of refocusing static inhomogeneities. However, susceptibility differences combined with proton diffusion introduce nonstatic inhomogeneities which are not completely refocused by spin echo sequences. As the echo time is increased with respect to the diffusional correlation time, SE acquisitions become more sensitive to magnetic susceptibility inhomogeneities. Gradient echo sequences on the other hand are sensitive to static magnetic field inhomogeneities (like $B_0$ imperfections) as well as the effects of proton diffusion. Even in the absence of diffusion, considerable signal attenuation will occur in the presence of susceptibility differences due to the range of intravoxel Larmor frequencies induced.

The effects of proton diffusion in magnetic fields have been studied extensively since Hahn's original work (44). In uniform gradients, Carr and Purcell derived an expression for spin echo signal attenuation due to proton diffusion (45):

$$S = e^{-\sigma^2/2} = e^{-\gamma^2 G^2 T_E D/12}$$

where $G$ is the gradient amplitude, $g$ is the gyromagnetic ratio and $D$ is the diffusion coefficient. The effects of nonlinear gradients have also been studied by various investigators (46-48). Glasel et. al. (49) investigated systems of heterogeneous water and glass beads and concluded that the field gradients at the surface of small spheres can be very large even if the susceptibility difference between the spheres and the surrounding water is small. These results were later applied to physiological origins of susceptibility variations, for example in transport and metabolism of cells (50), erythrocyte suspensions (51, 52), and the oxygenation state of hemoglobin (53, 54).

The complete analysis of susceptibility-induced relaxivity must take into account the diffusional rate of protons with respect to the size of the magnetic field perturbations. Three regimes have been described which are determined by the magnetic, geometric and dynamic properties of the system. These regimes, defined as the "motionally narrowed",

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"intermediate" and "linear gradient" regimes are best defined in terms of the diffusive correlation time of the protons with respect to the magnetic field inhomogeneities, $\tau_D$, and the characteristic variation in Larmor frequency due to field perturbations, $\delta\omega$ (55). For localized magnetic field perturbers, $\tau_D$ is the time required for diffusion past the perturber, and $\delta\omega$ is the change in frequency on the surface of the perturber, expressed as (55):

$$\tau_D = \frac{r^2}{D}, \quad \delta\omega = \gamma B_{eq}(r).$$

(2.6)

where $r$ is the radius of the perturber and $B_{eq}(r)$ is the magnetic field on the surface of the perturber.

In the motionally narrowed regime, diffusion is fast compared to spatial variations of field perturbations, such that $\tau_D \delta\omega \ll 1$. This condition holds in regions where the size of high-susceptibility is small, where water diffusion rates are high or where the frequency shifts are small. In such a regime, Gillis and Koenig (56) found that the relaxation rate due to a spherical perturber can be expressed as:

$$\Delta R^2 = 16 \tau_D f(\delta\omega)^2 / 135$$

(2.7)

which varies quadratically with magnetic field and the radius of the perturber ($r$). In the case of intravascular paramagnetic contrast agents, this would imply that for small vessels, $\Delta R^2$ would vary as $r^2$.

At the opposite end of the spectrum, the linear gradient regime, $\tau_D \delta\omega \gg 1$, holds for cases of slow water diffusion or large field perturbations. In such a situation, the variation of fields can be locally approximated by a linear gradient. Under these conditions, Majumdar and Gore (29) showed that the transverse relaxation enhancement in a spin echo experiment is well describe by:

$$\Delta R^2 = (\gamma \sigma_G^2 \text{TE})^2 D / 12$$

(2.8)

where $\sigma_G^2$ is the variance of the internal gradient distribution induced by the perturbation. Weisskoff et. al. (31) used the modified Bloch equations with diffusion terms to produce scaling rules which can be used for more general cases. Weisskoff noted that the diffusion
coefficient \( D \) should enter any relaxivity expression only the form \( D/r^2 \). As such, the expression for \( \Delta R_2 \) in the linear gradient regime as derived by Majumdar and Gore (29) is inversely proportional to the square of the radius of the perturber. Therefore, in the case of intravascular paramagnetic contrast agents, the SE relaxivity will approach zero as the size of the vessels increases.

Gradient echo relaxivity on the other hand has been described by Brown (57, 58) who derived an expression for \( \Delta R_2^* \) induce by magnetite particles in the linear gradient regime:

\[
\Delta R_2^* = \left( \frac{2t}{3V} \right) f \delta \omega
\]

(2.9)

He concluded that in this regime, \( \Delta R_2^* \) is independent of diffusion and by the same argument as above, independent of perturber radius.

When \( \tau_D \delta \omega \gg 1 \), the analytical approximations and solutions described above cannot be applied to the intermediate regime. However, numerical techniques have been applied to predict the response of both \( \Delta R_2 \) and \( \Delta R_2^* \) (28, 31, 59-61). For example, Monte Carlo simulations performed by Weisskoff et. al. (31) have estimated the enhanced relaxivity of both SE and GE acquisitions in the presence of intravascular paramagnetic contrast agents as a function of vessel size. Their results, shown in figure 2-10, not only support the theoretical data described above, but also show the values of \( \Delta R_2 \) and \( \Delta R_2^* \) in the intermediate regime. Shown here for a concentration comparable to physiologic Gd-DTPA injections, \( \Delta R_2 \) and \( \Delta R_2^* \) first increase as a function of vessel size, corresponding to the motionally narrowed regime. At larger vessel radii (> 8 \( \mu m \)), \( \Delta R_2 \) decreases to zero whereas \( \Delta R_2^* \) maintains a constant value, independent of vessel size. This corresponds to the linear gradient regime. \( \Delta R_2 \) is seen to peak for vessels on the order of 3-4 \( \mu m \), corresponding to normal microvasculature, making SE pulse sequences more sensitive to capillary blood volume.

Theoretical data courteously provided by Dr. Jerry Boxerman generated the plot shown in figure 2-11 (see (32)). For a susceptibility difference of \( \Delta \chi = 10^{-6} \) and a TE =
20 msec, the ratio of $\Delta R_2^*$ to $\Delta R_2$ was computed as a function of radius. These results show an almost linear relationship between the ratio $\Delta R_2^*/\Delta R_2$ and radius. This plot supports the hypothesis that measurements of $\Delta R_2$ and $\Delta R_2^*$ and most importantly their ratio could be used to obtain an index of the average vessel size within a voxel.

Based on this theory and the fact that tumor vessels can have diameters two to three times greater than normal tissue capillaries (27), this research set out to obtain a regional picture of tumor vascular morphology by measuring $\Delta R_2$ and $\Delta R_2^*$ as a function of contrast agent concentration in a rat glioma model.

![Figure 2-10: Effect of vessel radius on $\Delta R_2$ and $\Delta R_2^*$ at $\Delta \chi = 10^{-6}$ and TE = 20 msec.](image)
Figure 2-11: $\Delta R2^*/\Delta R2$ as a function of vessel radius for $\Delta \chi = 10^{-6}$ and TE = 20 msec. This ratio could be used to obtain an index of the average vessel size within a voxel.
The basic aim of these experiments was to measure the changes in the relaxation rates (both $1/T_2$ and $1/T_2^*$) as a function of contrast agent concentration and TE. Two sets of experiments were performed. First, since susceptibility contrast mechanisms designed to study the distribution of vessel sizes rely entirely on the compartmentalization of the contrast agent within the vasculature, it is important to demonstrate that the disruption in the blood brain barrier within the tumor does not cause the contrast agent to leak out of the vasculature for the entire duration of the experiment. These experiments were performed on two animals. Second, experiments were performed to measure $\Delta R_2$ and $\Delta R_2^*$ as a function of contrast agent concentration and TE in four animals.

3.1 ANIMAL PREPARATION

Adult female Fisher 344 CD rats weighing 150-200g were used for this study. Standard pelleted food and water ad libitum were given to the animals and a 12h light-dark cycle was continuously maintained.

3.1.1 Day 1: Brain tumor implantation

Rats were anesthetized using 1% halothane. After immobilizing the heads in a small rodent standard stereotactic apparatus (D. Kopf Instruments), the top of the heads were treated with alcohol and a linear skin incision was placed over the midline. A 1 mm burr hole was drilled in the skull approximately 3.5 mm lateral to the midline and 0.5 mm behind the bregma on the left side. A Hamilton 10 µL gas-tight syringe (Hamilton-Comp., Reno, Nevada) was used for injection of 10 µL C6 glioma cell suspension (10^6 cells cultured in DMEM) into the left frontal lobe at a depth of 3.5 mm relative to the dural surface. The injection time was one minute, after which the needle was slowly retracted for two minutes. Bone wax (Ethicon) was used to occlude the burr hole and to prevent leakage.
of CSF. The skin was closed with stitches and the animals were allowed to recover from the anesthesia under permanent observation. The entire procedure required approximately fifteen minutes.

3.1.2 Day 2-19: Post-operative care

During the first three postoperative days, the rats were monitored twice daily for incomplete wound closure, pain or distress. In case of pain, ibuprofen was given into the drinking water at a dose of 400 mg/bottle.

Nineteen days after implantation of the tumor cells, the animals underwent the imaging protocol described below. Rats were anesthetized with 1.5% halothane and the femoral vein was catheterized for contrast agent administration. The animals were placed supine on a water-heated pad and placed on the imaging table. Imaging took place on a 2T 11cm bore SISCO NMR image Spectrometer (Varian Associates, Fremont, CA) using a custom built 40mm cosine volume coil.

3.2 IMAGING PROTOCOL

Conventional SE and GE images were acquired prior to and following each injection of the equilibrium iron oxide contrast agent MION. For the spin echo sequence, TR = 3000 msec was used and a series of echo times were obtained: TE = 20, 40, 60, 80 msec. Gradient echo sequences used a similar TR = 3000 msec but TE = 15, 20, 30, 40 msec. Both imaging sequences had the following parameters: FOV=3.5 x 3.5 cm, 64 x 64 resolution, slice thickness of 2 mm, and the number of slices ranged from three to five to cover the entire tumor. Each image acquisition required 13 minutes. A post Gd-DTPA SE image (TR=500 ms, TE=25 ms, 16 averages) was acquired to delineate the location of the tumor. The animals were then sacrificed via carbon dioxide inhalation, the brain was removed and fixed in OMNIFIX 2000 (Aaron Medical Industries Inc.) for at least 24 hours.

3.2.1. Equilibrium measurements
For this set of experiments, three injections of MION were given at a concentration of 8 mg/mL. The imaging protocol described above was performed at the beginning of the experiments and following each injection. After the last injection, the imaging protocol was repeated three times, for a total of approximately 90 minutes.

3.2.2. AR2 and AR2* experiments

In these experiments, five serial injections of 5 mg/mL of MION were given, preceded and followed by the imaging protocol described above.

3.3 IMMUNOHISTOCHEMISTRY

Immunohistochemical staining was performed in three tumors. Sections 2 mm thick covering the location of the tumor were cut from the fixed brain and embedded in paraffin (1 hr in 75% ethanol, 1 hr in 95% ethanol, 4 x 1 hr in 100% ethanol, 2 x 30 min in xylene, 1 hr in melted paraffin at 57°C). Five micron slices were obtained through the tumor and placed on standard histology slides. After one hour in a 60°C oven, the slides were allowed to cool overnight.

To stain the slides, a standard immunohistochemical technique for laminin was used to stain the vascular endothelial cells (62). The slides were deparaffinized by immersing them for 2 x 10 min in xylene, followed by 3 min in 100% ethanol and 1 min each in 95%, 75% and 50% ethanol. The slides were then soaked in deionized water for 5 minutes followed by 5 minutes in phosphate buffered saline (PBS). Endogenous peroxidase activity was blocked with methanol containing 0.3% H2O2 for 30 minutes. The slides were treated with trypsin-EDTA (Gibco) for 1 min at 37°C and then immersed in cold PBS for 5 min. Following a 20 minute incubation with 10% normal goat serum (Zymed Laboratories Inc., CA) tissue sections were incubated with the polyclonal anti-laminin antibody (Sigma, ) at 1:100 for 30 minutes. The slides were rinsed with PBS 2 x 5 min and incubated with biotinylated secondary anti-rabbit IgG antibody (Zymed Laboratories Inc., CA) for 10 minutes. Following a PBS rinse (2 x 5 min), the slides were incubated with streptavidin
peroxidase conjugate solution for 5 minutes (Zymed Laboratories Inc., CA). Following a
PBS rinse, diaminobenzidine (DAB) was placed on the slides long enough for sufficient
staining to occur, as monitored under a microscope. Finally, the slides were counterstained
with hematoxalin (15 seconds), rinsed in tap water for 2 minutes and dehydrated using the
same alcohol baths as above, but in reverse order. Coverslips were placed on the slides
and allowed to dry overnight.

Morphometric analysis and quantification of microvascularization were performed
using BioQuand System IV morphometry software package (R&M Biometrics, Nashville,
TN) interface with an IBM microcomputer. Over 300 tumor vessels and over 200 grey
matter vessels were measured.

3.4 DATA ANALYSIS

From the MR images, a 4x4 pixel region of interest (ROI) was selected in the tumor
core and in the contralateral deep grey matter. The signal intensity averaged over these
ROIs was obtained at each point in the experiment (i.e. at baseline and following each
injection of contrast agent). The standard error of the ROI was used as an estimate of the
error. This may overestimate the error because the measurement includes the biological
variability inherent to the region of interest . ΔR2 and ΔR2* measurements were made for
each MION dose and each echo time according to equation (2.4). Standard error
propagation analysis was used to calculate the errors on ΔR2 and ΔR2*. Plots of ΔR2 and
ΔR2* as a function of dose and TE were made for each animal as well as a summary plot
of ΔR2 vs ΔR2* for all five animals.

Maps of ΔR2*/ΔR2 were produced on a pixel by pixel basis for one animals. At
each dose, both the SE and GE data as a function of TE were fit to a monoexponential
curve and interpolated to a value of TE = 20 ms. ΔR2, ΔR2* and their ratio (ΔR2*/ΔR2)
at TE = 20 ms were calculated at each dose and averaged to obtain a ratio map. In addition,
a total blood volume (CBV) map was obtained from the ΔR2* data (63, 64). Using the
same ROIs as described above, tumor blood volume was compared to grey matter blood volume.

To validate the vessel size dependence of our $\Delta R2^*/\Delta R2$ metric, we combined our empirically measured distributions of vessel size in both normal grey matter and tumor tissue with previous Monte Carlo simulation of the functional relation between $\Delta R2$ and $\Delta R2^*$ and vessel size (32) to estimate a histologically realistic weighted average of $\Delta R2^*/\Delta R2$ ratio. The values of $\Delta R2^*$ and $\Delta R2$ from theoretical simulations performed at $TE = 20\, ms$ and $\Delta\chi = 10^{-6}$ were weighted by the actual vessel sizes obtained from our histologic data, as follows:

$$
\frac{\Delta R2^*}{\Delta R2} \approx \frac{\sum r^2 f(r) A_{GE}(r)}{\sum r^2 f(r) A_{SE}(r)}
$$

where $r$ is the radius of the vessel, $f(r)$ is the number of vessels with radius $r$ and $A_{GE}(r)$ and $A_{SE}(r)$ are the amplitudes of the Monte Carlo determined values of $\Delta R2^*$ and $\Delta R2$ at radius $r$, respectively. To obtain an error estimate on these calculations, random distributions were generated from the measured vessel distributions. With each newly generated distribution, the ratio $\Delta R2^*/\Delta R2$ was computed according to Eq. [5] in both tumor and grey matter. The quotient of these ratios (tumor/grey) was obtained and compared with the measured MR ratio.
Chapter IV
RESULTS

Data from experiments described in chapter III will be presented in this chapter.

4.1 RAW DATA

Figure 4-1 shows a representative series of spin echo (TE = 60 msec) and gradient echo (TE = 15 msec) images from one animal. Because gradient echoes decay with the time constant $T_2^*$ which is shorter than $T_2$, TE values for gradient echo images are shorter than for spin echo images. These images show the decrease in signal intensity associated with serial injections of the contrast agent MION. Because MION is an equilibrium agent, it accumulates within the vasculature throughout the experiment, causing a cumulative decrease in signal intensity with consecutive injections. In figure 4-1, the spin echo signal intensity decreases by $= 13\%$ following each injection whereas the gradient echo signal intensity decreases by $= 17\%$.

4.2 EQUILIBRIUM EXPERIMENTS

To verify the stability of the contrast agent in the vasculature, steady state measurements of $\Delta R2$ and $\Delta R2^*$ were performed as described. Figures 4-2 and 4-3 plot $\Delta R2$ and $\Delta R2^*$ as a function of time for both the tumor and grey matter ROIs for two animals at TE = 20 msec. The closed squares represent $\Delta R2$ in the tumor core while the open squares are measurements of $\Delta R2$ in the contralateral deep grey matter. The closed circles are measurements of $\Delta R2^*$ in the tumor core and the open circles are measurements of $\Delta R2^*$ in the grey matter. Error bars are present on all data points, although some are too small to appear on the graph. The first three points represent the measurements following three consecutive injections of MION. Following the third injection, the imaging protocol was repeated three times, for a duration of 90 minutes. For both tumor and grey matter,
the values of \(\Delta R_2\) and \(\Delta R_2^*\) remain constant, within errors, over 90 minutes. The values of \(\Delta R_2\) and \(\Delta R_2^*\) within the tumor of the two animals did not change by more than 12\% over the course of the steady state measurements, indicating that the contrast agent is remaining within the vasculature.

Figure 4-1: Spin and gradient echo images of a single slice following consecutive injections of MION.
Figure 4-2: ΔR2 and ΔR2* as a function of time for one animal (10-10-96) at TE = 20 msec. Measurements of ΔR2* and ΔR2 remain constant, within errors, for 90 minutes.
Figure 4-3: $\Delta R_2$ and $\Delta R_2^*$ as a function of time for one animal (04-30-97) at $TE = 20$ msec. Measurements of $\Delta R_2^*$ and $\Delta R_2$ remain constant, within errors, for 90 minutes.
4.3 ΔR2 AND ΔR2* EXPERIMENTS

There is also a difference in the ratio of ΔR2* to ΔR2 between the grey matter and the tumor. For each dose and each stability point of figures 4-2 and 4-3, this ratio is consistently larger in the tumor than in the contralateral deep grey. This trend was reproduced in four animals who received five consecutive injections of MION. Figures 4-4 through 4-7 show plots of ΔR2 and ΔR2* as a function of contrast agent dose for each animal at TE = 20 msec. As seen in all animals, the ΔR2* measurements were consistently larger than the corresponding ΔR2 values, and both increase approximately linearly with dose. As hypothesized, the ratio of ΔR2*/ΔR2 is greater for tumor than normal brain.

All measurements in these experiments were performed at various echo times. The results from each animal at dose 3 are shown in figure 4-8 through 4-11. These are plots of ΔR2 and ΔR2* as a function of TE. The ΔR2 measurements in each animal remain quite independent of TE. The ΔR2* measurements in both tumor and grey matter show different trends in each animal. Some decrease with increasing TE (figure 4-8) while others increase with increasing TE (figure 4-9). The average of all four animals is shown in figure 4-12. Within the standard deviation of the measurements, ΔR2 and ΔR2* for both the tumor and the grey matter were independent of TE.
Figure 4-4: $\Delta R_2$ and $\Delta R_2^*$ measurements for one animal (07-03-96) at $TE = 20$ msec as a function of dose. The closed squares represent $\Delta R_2$ in the tumor, the open squares are $\Delta R_2$ measurements in grey matter. The closed circles represent $\Delta R_2^*$ in the tumor and the open circles are $\Delta R_2^*$ measurements in grey matter.
Figure 4-5: ΔR2 and ΔR2* measurements for one animal (07-17-96) at TE = 20 msec as a function of dose. The closed squares represent ΔR2 in the tumor, the open squares are ΔR2 measurements in grey matter. The closed circles represent ΔR2* in the tumor and the open circles are ΔR2* measurements in grey matter.
Figure 4-6: ΔR2 and ΔR2* measurements for one animal (07-31-96) at TE = 20 msec as a function of dose. The closed squares represent ΔR2 in the tumor, the open squares are ΔR2 measurements in grey matter. The closed circles represent ΔR2* in the tumor and the open circles are ΔR2* measurements in grey matter.
Figure 4-7: \( \Delta R_2 \) and \( \Delta R_2^* \) measurements for one animal (08-07-96) at TE = 20 msec as a function of dose. The closed squares represent \( \Delta R_2 \) in the tumor, the open squares are \( \Delta R_2 \) measurements in grey matter. The closed circles represent \( \Delta R_2^* \) in the tumor and the open circles are \( \Delta R_2^* \) measurements in grey matter.
Figure 4-8: $\Delta R_2$ and $\Delta R_2^*$ measurements for one animal (07-03-96) at dose 3 as a function of echo time. The closed squares represent $\Delta R_2$ in the tumor, the open squares are $\Delta R_2$ measurements in grey matter. The closed circles represent $\Delta R_2^*$ in the tumor and the open circles are $\Delta R_2^*$ measurements in grey matter.
Figure 4-9: ΔR2 and ΔR2* measurements for one animal (07-17-96) at dose 3 as a function of echo time. The closed squares represent ΔR2 in the tumor, the open squares are ΔR2 measurements in grey matter. The closed circles represent ΔR2* in the tumor and the open circles are ΔR2* measurements in grey matter.
Figure 4-10: $\Delta R_2$ and $\Delta R_2^*$ measurements for one animal (07-31-96) at dose 3 as a function of echo time. The closed squares represent $\Delta R_2$ in the tumor, the open squares are $\Delta R_2$ measurements in grey matter. The closed circles represent $\Delta R_2^*$ in the tumor and the open circles are $\Delta R_2^*$ measurements in grey matter.
Figure 4-11: $\Delta R_2$ and $\Delta R_2^*$ measurements for one animal (08-07-96) at dose 3 as a function of echo time. The closed squares represent $\Delta R_2$ in the tumor, the open squares are $\Delta R_2$ measurements in grey matter. The closed circles represent $\Delta R_2^*$ in the tumor and the open circles are $\Delta R_2^*$ measurements in grey matter.
Figure 4-12: $\Delta R2$ and $\Delta R2^*$ measurements averaged over four animals at dose 3 as a function of echo time. The closed squares represent $\Delta R2$ in the tumor, the open squares are $\Delta R2$ measurements in grey matter. The closed circles represent $\Delta R2^*$ in the tumor and the open circles are $\Delta R2^*$ measurements in grey matter.
A summary of all four animals is shown in figure 4-13 which plots $\Delta R2^*$ versus $\Delta R2$ for all doses at TE = 20 msec. The closed squares represent the data for the tumor core while the open squares represent data from the contralateral deep grey. As seen on this graph, the values of $\Delta R2^*$ are greater than the values of $\Delta R2^*$. This difference can be expressed quantitatively by taking the ratio of $\Delta R2^*$ to $\Delta R2$. By fitting the data to straight lines through the origin, the following ratios were obtained:

$$
\left( \frac{\Delta R2^*}{\Delta R2} \right)_{tumor} = 9.2 \pm 1.0
$$

$$
\left( \frac{\Delta R2^*}{\Delta R2} \right)_{grey} = 4.8 \pm 0.3
$$

The ratio of these values was:

$$
\frac{\left( \frac{\Delta R2^*}{\Delta R2} \right)_{tumor}}{\left( \frac{\Delta R2^*}{\Delta R2} \right)_{grey}} = 1.9 \pm 0.2
$$

From the histology sections, over 100 tumor vessel diameters in each of the three tumors and over 200 grey matter vessel diameters were measured. The distribution of vessels in the tumors and the grey matter are shown in figure 4-14. The average tumor vessel diameter was $12.5 \pm 6.8 \mu m$ and the average grey matter vessel diameter was $6.6 \pm 2.1 \mu m$. The predicted ratio of $\Delta R2^*/\Delta R2$ computed using Eq. [5] and the MRI measured ratio of $\Delta R2^*/\Delta R2$ for tumor to grey matter are as follows

$$
\left( \frac{\Delta R2^*}{\Delta R2} \right)_{tumor} = 6.6 \pm 0.6
$$

$$
\left( \frac{\Delta R2^*}{\Delta R2} \right)_{grey} = 3.6 \pm 0.1
$$

The relative ratio of these values was:

$$
\frac{\left( \frac{\Delta R2^*}{\Delta R2} \right)_{tumor}}{\left( \frac{\Delta R2^*}{\Delta R2} \right)_{grey}} = 1.9 \pm 0.1
$$
Averaging over all four tumors, total tumor blood volume was 15% greater than the blood volume in the contralateral grey matter. A GE CBV map for one animal is shown in figure 7, along with a post-Gd T2 weighted SE image and a map of ΔR2*/ΔR2. In the ratio map, an area of high intensity in the upper right region of the brain corresponds to the location of the tumor as shown in the post-Gd image. Recalling that this ratio increases as the average vessel increases (figure 2-11), this is suggestive of relatively larger microvessels in the tumor than in the contralateral grey matter.
Figure 4-13: $\Delta R^2_\text{tumor}$ versus $\Delta R^2$ for all four animals. The closed squares represent the tumor and the open squares represent the contralateral grey matter.
Figure 4-14: Distribution of vessels in tumor (a) and contralateral grey matter (b). The average tumor vessel diameter was 12.5 ± 6.8 μm and the average grey matter vessel diameter was 6.6 ± 2.1 μm.
Figure 4-15: Map of $\Delta R^*/\Delta R^2$ for one animal (08-07-96). The post Gd T$_1$ SE image on the left highlights the location of the tumor in the right hemisphere. The region of high intensity in the corresponding location in the $\Delta R^*/\Delta R^2$ map indicates that the tumor has a greater density of larger microvessels than the contralateral hemisphere.
Chapter V
DISCUSSION

Although tumor angiogenesis is essential to the growth of all solid tumors, there exists no direct or non-invasive method of assessing its role. Many studies have found via invasive histological or three-dimensional casting techniques that tumor blood vessels are associated with a wide range of both morphologic and physiologic characteristics not found in normal capillaries (see e.g. (25-27, 65, 66)). Of particular interest to this study is the fact that tumor capillary diameters can be two times greater than normal microvessels (65). Magnetic susceptibility contrast mechanisms predict that larger microvessels will have a greater ratio of $\Delta R2^*$ to $\Delta R2$ than normal smaller microvessels. For all animals in the study, this ratio was indeed greater in the tumor than in the contralateral deep grey matter, suggesting a higher relative density of larger vessels. On average, the measured tumor ratio of $\Delta R2^*/\Delta R2$ was a factor of 1.9 greater than the grey matter ratio, a value which was in good agreement with that predicted by our own direct histologic assessment of vessel size and previous susceptibility contrast modeling. From figure 1b, the average vessel size within the tumor and grey matter ROIs can be determined from the MRI and histologically predicted values of $\Delta R2^*/\Delta R2$. Although the absolute MRI values of $\Delta R2^*/\Delta R2$ were significantly larger than the predicted values resulting in larger estimates of vessel sizes, the relative relationship between tumor and grey matter measurements are similar. This difference in the absolute $\Delta R2^*/\Delta R2$ values may be accounted for by a systematic bias in our assessment of the true vascular distribution (potentially secondary to the paraffin blocking process), or by differences between cylindrical and true vascular geometry in our Monte Carlo simulations.

From the direct histology measurements, the relative size of the average tumor vessel compared to the average grey matter vessel diameter was also $1.9 \pm 0.6$. The good agreement between the histologically determined ratio of vessel size between tumor and
grey matter and the ΔR2*/ΔR2 ratio between tumor and grey matter indicates that this technique can be used to obtain a relative index of vessel size in areas of pathophysiologically altered vascular proliferation.

Although the ΔR2 and ΔR2* measurements in this study were made at various echo times, our results showed little TE dependence at the doses used. Boxerman et. al. (32) showed that ΔR2 at Δχ = 3 x 10^-8 decreased as a function of TE and the peak susceptibility change also shifted towards smaller radii. This trend was not reproduced in this study, perhaps due to the higher concentrations (hence Δχ's) studied. It is possible that trends as a function of TE may have been masked by the interanimal variation, especially in the exact values of Δχ with each injection. This variability could be more properly addressed by obtaining quantitative Δχ measures on blood samples following each injection. Should such differences as a function of TE ultimately be observed, these may allow not only the mean vessel diameter within a voxel to be determined, but may also provide enough information to calculate the distribution of vessels.

While previous MR studies have shown a correlation between increased blood volume in tumors and tumor grade (23, 24, 67), none have addressed the question of vessel size. A recent study by van Dijke et. al. (67) which measured the plasma volume in a mammary carcinoma model using contrast-enhanced MR imaging techniques, showed a positive correlation between histologically determined capillary number density and MR measurements of plasma volume. Their relationship was not linear however. With increasing tumor aggressiveness, an exponential rise in plasma volume was detected. They suggested therefore that a simultaneous increase in size of vessels must accompany the increase in vessel number. This is in agreement with our direct measurements of increased vessel size at a late phase of tumor growth. It is of interest to note that the relative CBV of the C6 gliomas calculated here was elevated by only about 15% compared to contralateral grey matter compared, to a 90% increase in average vessel size. This suggests that an
increase in relative vessel size may provide unique, independent information on tumor vessel physiology which complements relative CBV maps.

Tumor angiogenesis is a complex process which can be triggered by various factors including stress and hypoxia (19, 68, 69). As such, angiogenic activity can be heterogeneous throughout the tumor (15, 70), making it important to sample as much of the tumor as possible. Although histologic data obtained from biopsy is the gold standard for examining vascular morphology, it is subject to sampling errors because only one or two portions of a tumor are probed during routine biopsy. In our C6 rat glioma model, it was possible to examine much of the tumor on a pixel by pixel basis as shown in figure 5 (map of ΔR2*/ΔR2). With multi-slice techniques, the entire volume of the tumor can be assayed as opposed to small regions obtained with histologic techniques. Equally important, this MR technique is non-invasive and can therefore be repeated over time.

Our results in these 19 day old tumors show a relatively homogeneous ratio of ΔR2*/ΔR2 throughout. Further longitudinal studies should reveal a more complex pattern, allowing us to examine the dynamics of vessel size evolution during tumor growth. Deane and Lantos (25) found that vessels in a rat glioma model varied as the tumor progressed: during the early phase, small vessels were found throughout the tumor; at late phases, small vessels were only found at the proliferating edge of the tumor and larger microvessels were found between the edge and the necrotic core. The tumors in this study were examined only at one specific time point. Since the size of vessels within a tumor are known to vary with the aggressiveness of the tumor (66), it may further be possible to find a correlation between tumor grade and vessel size for various human tumors. Future studies using methods similar to the one described here could be used to longitudinally assess the fate of tumor blood vessels as a function of time and tumor size. This technique may therefore not only help diagnose the stage and grade of tumors, but it may ultimately be useful in assessing the response of tumors to therapy directed at the angiogenic process (18, 71).
The conclusions drawn from these data may be limited in various ways. Several studies have shown that the perfusion efficiency of brain tumors is significantly lower than normal cortex (72-74). For example, Yuan et. al. (73) found that red blood cell velocity in pial vessels may be an order of magnitude faster than that in tumor vessels of the same diameter. Low flow could affect the MR measurements of $\Delta R2^*$ and $\Delta R2$ which rely on the homogeneous distribution of the contrast agent. However, by using a long lived intravascular agent and a prolonged imaging protocol (= 13 minutes), all perfused vessels within the tumor should be adequately filled with contrast agent during the experiment since even with flow rates an order of magnitude lower than those found in normal tissue, vascular transit times are still less than one minute. Furthermore, modeling studies show only a weak dependence of susceptibility contrast itself as a function of perfusion (32). We therefore think it unlikely that low tumor perfusion significantly perturbs our measurements.

Results from the C6 rat glioma model may also not be directly applicable to human brain tumors or other tumors in general. Low grade tumors which have been known not to be associated with as dramatic an increase in blood volume as high grade (23) may not exhibit the corresponding increase in vessel diameter. Correlations between MR imaging and histologic characteristics will differ with tumor type and level of angiogenic activity. Furthermore, equilibrium blood pool contrast agents have yet to be approved for human use. If this technique were to be applicable to humans today, similar data could be obtained from first pass dynamic contrast agents such as Gd-DTPA. Unfortunately, these agents are small enough to leak out of some disrupted tumor vasculature in high grade human brain tumors, and normal vasculature outside the brain. In a tumor whose endothelial permeability is greatly increased, Gd-DTPA may thus not be able to provide adequate susceptibility-induced signal loss for measurement of $\Delta R2$ or $\Delta R2^*$. Techniques relying on first pass dynamic measurements of $\Delta R2^*$ and $\Delta R2$ would also be subjected to regional variations in tumor perfusion. The low perfusion efficiency reported in tumors (72-74)
may therefore prevent the assessment of average vessel size via methods described in this study due to the lack of signal attenuation. However, the assessment of tumor perfusion efficiency itself may provide interesting information concerning the regional hemodynamics present in tumors.

Despite these limitations, the technique described in this study shows that tumor vessel sizes can be compared between normal and diseased tissue by injecting an equilibrium blood pool contrast agent. With the advent of human approved intravascular agents, magnetic susceptibility contrast mechanisms, which have already been useful in determining relative cerebral blood volumes, may serve to provide further information about human tumor vasculature and their response to therapy. This technique may be of particular interest in studying, non-invasively, the effects of novel anti-angiogenic drugs on the existing tumor vasculature.
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