Magnetic resonance imaging of in vitro glioma cell invasion

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Object. An understanding of single glioma cell invasion has been limited by the static picture provided by histological studies. The ability to nondestructively assess cell invasion dynamically in a full 3D volume would improve the quality and quantity of information available from both in vivo and in vitro experiments. The purpose of this study was to observe glioma cell invasion in a 3D in vitro model using a microimaging protocol at 1.5 tesla and to assess the uptake of micron-sized particles of iron oxide (MPIO) and the consequent effects on cell function.

Methods. Rat C6 glioma cells were labeled with MPIO to a sufficient extent to allow single cell detection in vitro without significant effects on cell proliferation or plating efficiency. When placed on agar-coated plates, the cells formed stable multicellular tumor spheroids (MCTSs), which were embedded in collagen type I gel and serially visualized using magnetic resonance (MR) imaging and phase-contrast microscopy over 8 days. The MCTSs initially appeared as large susceptibility artifacts on MR images, but within 2 days, as cells moved away from the main MCTS, small discrete areas of signal loss, possibly due to single cells, could be observed and tracked.

Conclusions. Glioma cell invasion can be nondestructively observed using MR imaging. The sensitivity of MR imaging, along with its ability to represent full 3D volumes noninvasively over time, makes it ideal for longitudinal in vivo cell tracking studies.

Key Words • glioma • cell invasion • magnetic resonance imaging • cellular imaging

GLIOMAS, which derive from glial cells or their progenitors, are the most common primary tumors of the central nervous system. Often they present an extremely poor prognosis, with mean survival times of less than 1 year in patients with the most aggressive forms. This poor prognosis has been attributed to the extensive invasion of glioma cells into surrounding brain tissue, which makes complete resection nearly impossible and limits the success of radiotherapy and chemotherapy.

Glioma cell invasion is a complex process well adapted to the microenvironment of the central nervous system, involving a large array of molecules and interactions. Cells detach from the primary tumor mass, adhere to molecules in the ECM, and degrade ECM components, thus creating space into which to migrate. This invasion process is unique in that single cells invade diffusely and actively and do not enter the bloodstream, contrary to the behavior of systemic malignancies.

The invasion of gliomas and the mechanisms underlying this process are being studied using a wide variety of methods. Interactions of glioma cells with ECM components, with host cells, and with each other are important, but the specific mechanisms involved remain mostly unknown. The roles of various ECM components in cell migration can be studied most simply by using 2D in vitro assays such as Boyden chambers, colloidal-gold cell tracking, or radial dish assays, combined with time-lapse videomicroscopy, electron microscopy, and phase-contrast microscopy. Three-dimensional models that more closely mimic the in vivo situation have also been developed. For instance, MCTSs cocultured with normal brain cell aggregates or brain slice cultures represent relevant models that have been studied using confocal laser scanning microscopy, but this technique is limited to the top few hundred microns of the system. The ability to dynamically assess the invasion process in an entire 3D volume nondestructively would greatly improve the quality and quantity of information obtainable from both in vivo and in vitro studies.

Magnetic resonance imaging has the potential to provide a full 3D dynamic assessment of cell invasion. This imaging modality is sensitive, noninvasive, and nondestructive; generates high-resolution images with excellent soft-tissue contrast; is not limited by tissue opacity like optical techniques; and does not rely on radioactive isotopes, which may be an important advantage for longitudinal studies. Cellular MR imaging is a newly emerging field of research that combines the ability to generate high-resolution MR data with contrast agents for labeling cells. The utility of...
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Iron oxide–based contrast agents for cellular MR imaging has been demonstrated in many disease applications. These agents include a variety of dextran-coated SPIO and MPIO. The presence of intracellular iron oxide is indicated by areas of signal loss on T2* or T2*-weighted images. The extensive magnetic susceptibility of these agents affects an area much larger than the actual size of the particles, an effect known as a “blooming artifact,” and leads to an exaggeration of the region occupied by the labeled cells.

Several groups have demonstrated that single cells labeled with SPIO can be detected using MR imaging in vitro, but most studies have been conducted with field strengths exceeding 4.7 tesla. We previously demonstrated that single SPIO-labeled cells can be detected in vitro at the clinically relevant field strength of 1.5 tesla using our optimized microimaging protocol, which includes a custom-built gradient insert coil and the fully balanced steady-state free precession pulse sequence FIESTA (GE Healthcare). More recently, we have demonstrated that this same system allows single cell detection in vivo in the mouse brain.

The primary purpose of this work was to apply our microimaging protocol to track invading glioma cells in a 3D in vitro model of cell invasion. The label uptake by cells and the effects of the incorporated label on cell behavior were also assessed to ensure the validity of future studies. This work represents the first step toward in vivo imaging and tracking of single glioma cells that leave a primary tumor and invade surrounding brain tissue.

Materials and Methods

Cell Culture and Labeling

The rat C6/lacZ glioma cell line (CRL-2303, American Type Culture Collection) was chosen because it is well characterized and forms tumors that exhibit important features of human gliomas, including single cell invasion. Cells were maintained at 37°C in 5% CO2 in high glucose DMEM (Gibco) supplemented with 10% fetal bovine serum (Gibco) and were passaged 2 to 3 times per week. For magnetic labeling, cells were placed in six-well plates at 5 × 10^5 cells per well in 1.5 ml DMEM and incubated for 24 hours. At that time, the medium was aspirated to remove dead cells and replaced with 1 ml fresh DMEM. The MPIO (0, 2, 4, 8, 16, 32, 64, 128 μg/cm², Bangs Laboratories, Inc.) was added. The particles used had a mean diameter of 0.9 μm and were embedded with a fluorescent tag (either Dragon green or Flash red). These MPIO were chosen because they are known to be taken up by many different cell types and are not degraded by cells (unlike the dextran-coated particles), which may allow them to be used for longer studies. After 24 hours of incubation, the cells were washed in the plates three times with PBS (Gibco), treated with trypsin containing 0.25% ethylenediaminetetraacetic acid (Gibco) for 5 minutes, quenched with DMEM/10% fetal bovine serum, and washed three or four more times by resuspension in PBS and centrifugation (100 G at room temperature for 5 minutes). Before the final spin, cells were counted using a hemocytometer and subsequently resuspended at the desired concentration in PBS or DMEM, according to the needs of the experiment.

Bead Uptake and Effect on Cell Function and Behavior

Cellular iron content was measured using a susceptometry technique, the Reilly-McConnell-Meisenheimer method, modified to use multiecho imaging, as described previously by Bowen et al. Cells were uniformly suspended at a known concentration of 2 to 3 × 10^6 cells/ml in 4% gelatin in PBS and loaded into nuclear MR tubes. The local magnetic dose, which is defined as the average increase in magnetization due to the presence of superparamagnetic particles, was calculated from magnetic susceptibility measurements and converted to an iron concentration using calibration data. The average iron content per cell could then be obtained.

Cell viability was measured using the MTT assay (Sigma-Aldrich Canada, Ltd.). Washed cells were resuspended in DMEM without phenol red and plated at 1 × 10^5 cells in 100 μl per well in 96-well plates. On the day of the assay, the MTT reagent was added to the cells and metabolized by the mitochondria of viable cells to form insoluble purple formazan crystals, which were then solubilized by the addition of a detergent. The amount of color produced, as indicated on a plate reader at 590 nm (background measured at 650 nm and subtracted), was proportional to the number of viable cells. Growth curves were generated for all iron loadings by measuring cell viability each day for 6 days.

To assess the effect of the label on the ability of the cells to form colonies from single cells, the cells were labeled with MPIO as described above, plated on 10-cm-diameter Petri dishes (100, 200, and 500 cells/plate), and incubated for 10 days. The medium was then removed, plates were rinsed with PBS, and cells were fixed with 10% formalin for 10 minutes. The fixative was removed and cells were stained with 1% crystal violet for 10 minutes, followed by washing with distilled water until no color remained. The cell colonies were then counted to determine the plating efficiency.

To determine whether the label is lost during cell division, cells were labeled and replated in T75 flasks at 2 × 10^5 cells/flask and allowed to grow for 1 to 3 days. On each day, cells were harvested, counted, and prepared for susceptibility measurements of iron content. Based on both the initial cellular iron content and the cell counts, the expected iron content, assuming none was degraded or expelled by the cells, was calculated and compared with susceptometry measurements.

Single Cell and MCTS Phantoms

To validate single cell detection using MR imaging, 30 to 50 labeled cells were sparsely distributed in 350-μl plastic microwells in a single plane sandwiched between a lower layer of 8% gelatin in PBS and an upper layer of 1% gelatin in PBS. Magnetic resonance images of the phantoms were overlaid with corresponding phase-contrast microscopy images to correlate areas of signal void with the true location of cells.

The MCTSs were grown using the simplified method described by Yuhas et al. Cells were plated at 2 × 10^5 cells/ml on 1.5% agar base–coated six-well plates for approximately 96 hours, during which time they formed MCTSs with diameters of 200 to 500 μm. Single MCTSs were picked up using capillary pipettes, embedded in 350-μl plastic microwells containing collagen type I gel (Vitrogen-100, Cohesion Technologies), and covered with DMEM. These plastic microwells are optically transparent, allowing for the acquisition of both MR and optical images for careful validation of cell detection. The phantoms were visualized serially with MR imaging on the day of implantation (Day 0), Days 1 to 4, 6, and 8. Corresponding optical images were obtained on Days 0, 2, 3, and 6.

To make a semi quantitative assessment of whether the label affects the cells’ ability to invade, we obtained daily (except on Day 4) phase-contrast microscopy images of collagen-embedded unlabeled and labeled MCTSs for 7 days. The radius of the main invasive edge and the distance traveled by the furthest cell were measured relative to the original radius of the MCTS.

Magnetic Resonance and Optical Imaging

Magnetic resonance imaging was conducted on a 1.5-tesla GE CV/i clinical unit with a custom-built gradient insert coil (inner diameter 17.5 cm, maximum gradient strength 600 mtesla/m, and slew rate > 2000 tesla/m/sec) using a customized solenoid radiofrequency coil (1-cm diameter and 1-cm length). Samples were visualized using the 3D FIESTA pulse sequence (TR 7.1 msec, TE 3.6 msec, flip angle 30°, and bandwidth ± 20.83 kHz) at 100-μm isotropic resolution with four averages (scan time ~12 minutes).

Phase-contrast microscopy was performed on an Olympus IX50 inverted microscope equipped with a Sony 3CCD color digital camera. Bright field images of the single-labeled cells and the implanted MCTSs and invading cells were captured at high power.
Results

Bead Uptake and Effect on Cell Function and Behavior

Rat C6 glioma cells incorporated MPIO in an iron incubation concentration-dependent manner that can be described by an exponential function of the form $Fe_{\text{max}} = Fe_{\text{init}}(1 - \exp[-(Fe/k)])$, where $Fe_{\text{init}}$ is the average iron content per cell (in picograms), $Fe_{\text{max}}$ is the maximum iron content per cell, $[Fe]$ is the iron incubation concentration (in micrograms per squared centimeter), and $k$ is a rate constant (Fig. 1). The mass/area was found to be the more relevant measure of iron incubation concentration than mass/volume, probably because the beads settle quickly onto the adherent monolayer of cells.

Figure 2 shows the growth curves generated from cell viability data measured using the MTT assay for cells labeled with different quantities of iron. The data were analyzed using a one-way ANOVA, which indicated a significant effect at the earliest time point only. Results of a subsequent Tukey post hoc test revealed significant differences between some of the iron loadings (6 pg/cell differed from control [0 pg/cell], 14.5 pg/cell, and 19 pg/cell, and each of these loadings differed significantly from 10.5 pg/cell [p < 0.05]). Note, however, that there was no trend as to higher or lower proliferation with increasing iron loading and that the differences did not continue beyond the first time point.

The effect of MPIO uptake on the cells’ plating efficiency is illustrated in Fig. 3. After 10 days, individual cell colonies were easily identifiable with the naked eye and were not overlapping. As indicated by the results of a one-way ANOVA, labeled cells retained the ability to form colonies to the same extent as unlabeled cells (p > 0.1).

In Fig. 4 the measured average iron content per cell over time as cells divide (black diamonds) is compared with the calculated iron content expected (for the initial iron loading and measured cell counts) if the only mechanism of decrease in label intensity was through dilution due to cell division (that is, assuming no label is degraded or expelled by the cells). Data are normalized to the initial iron content. The measured iron content agrees extremely well with the calculated predictions (p > 0.1, paired samples t-tests). These data support the belief that the MPIO particles are not degraded by cells and indicate that the label is not lost by other means as cells divide.

Validation of Single Cell Detection

Glioma cells could be labeled with MPIO to a sufficient extent to allow single cell detection. Magnetic resonance and phase-contrast microscopy images of a small number of cells sandwiched between layers of gelatin in microwells are shown in Fig. 5. Discrete areas of signal void on the MR images correlated excellently with single cells (or occasionally two cells as shown by the arrows in Fig. 5) under the microscope. Phantoms containing unlabeled cells showed no areas of signal void on MR images (not shown).

Multicellular Tumor Spheroid Formation and Cell Invasion

When labeled rat C6 glioma cells (mean iron content ~30 pg/cell) were plated at $2 \times 10^6$ cells/well in 1.5% agar base–coated six-well plates, they formed well-defined and stable MCTSs of 100- to 300-μm diameter in approximately 96 hours. Figure 6 shows two examples of a single MCTS embedded in collagen type I gel and the subsequent invasion into the gel over 8 days. The two examples represent what was typically observed and thus will be described together. The MCTS adhered well to the gel, and cells began to invade within the first few hours (Day 0 in Fig. 6). Invading cells appear elongated, indicating that they are in active motion. By Day 2, cells were visible on phase-contrast images up to approximately 450 to 600 μm from the original implantation site (indicated by the arrows in Fig. 6). The corresponding MR images showed mild invasion on Day 2, as the large blooming artifact produced by the MCTS prevented the detection of invading cells close to the implantation site. By Day 6, however, extensive invasion could be observed on both the MR and optical images.

Figure 7 shows a comparison of the distances of invasion as measured on optical images of unlabeled and labeled MCTSs. Figure 7A is a typical example of how the measurements were taken; the circle delineates the main invasion.
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Fig. 3. Bar graph showing the plating efficiency for cells loaded with different quantities of MPIO. Intracellular incorporation of MPIO had no significant effect on the ability of C6 cells to form colonies from single cells, as determined using a one-way ANOVA (p > 0.1). Error bars represent standard error of the mean.

Fig. 4. Graph displaying the average iron content per cell as labeled cells are allowed to divide, normalized to the initial cell loading. Black diamonds indicate the measured average iron content per cell at different times points; white diamonds, the calculated iron content expected (for the measured initial iron loading and cell counts) if one assumes that the only mechanism of label intensity decrease is due to cell division (that is, no label is expelled or degraded by the cells). No significant difference between the measured data and the calculations was observed (p > 0.1, paired samples t-tests). Error bars represent the standard deviation.

Discussion

Multicellular tumor spheroids approximate gliomas in that they exhibit single cell invasion, and if allowed to grow to a large enough size, they will develop a necrotic core. In the present study, the MCTS model was chosen as a simple in vitro model of a glioma that would have MR signal characteristics similar to in vivo tumors fully labeled with SPIO, without the confounding effects presented by vasculature and interactions with surrounding brain tissue. The model was used to assess the capability of our microimaging protocol in detecting changes in the MCTS and cell invasion as an important step toward single glioma cell tracking in vivo.

We showed that rat C6 glioma cells can be easily and reproducibly labeled with MPIO to a sufficient extent to allow single cell detection (Figs. 1 and 5) and that the spatial and temporal progression of glioma cell invasion from an MCTS through a collagen gel matrix can be monitored with our microimaging protocol at 1.5 tesla (Figs. 6 and 8). In most cases, cellular imaging has been conducted at high magnetic field strengths (≥ 4.7 tesla). Our ability to detect single cells at 1.5 tesla relies on the high signal-to-noise ratio efficiency and high iron sensitivity of the 3D FIESTA pulse sequence, as well as on our custom-built gradient insert coil and customized radiofrequency coils.

Whereas most cellular imaging studies aim to follow large numbers of cells, studying glioma cell invasion, during which cells invade as single cells, requires technology with the sensitivity to detect individual cells. As yet, we are unaware of any other noninvasive imaging modality with this level of sensitivity. Positron emission tomography has been applied in cell tracking, but the best sensitivity achieved has been on the order of hundreds of cells, and the resolution is intrinsically limited to the millimeter range. Similarly, bioluminescence techniques are limited by tissue opacity and are sensitive only to hundreds or thousands of cells.

At the early stages of cell invasion, a large blooming artifact was produced around the labeled MCTSs on the MR images, and it was impossible to identify individual cells. As seen in Fig. 8, the volume of signal void initially decreased, most likely because as the MCTSs become less tight, the iron density decreases, lessening the size of the blooming effect. The volume of signal loss then increases steadily as the cells invade the collagen gel. The
Fig. 5. Magnetic resonance image (A) and overlay of MR and phase-contrast microscopy images (B) of single cells containing approximately 17 pg Fe. Discrete areas of signal void on the MR image correspond very well to single cells (or occasionally two cells, as indicated by the arrows) under the microscope. White dots indicate the position of cells. These results validated our thesis that single glioma cells could be detected with our microimaging protocol.

Fig. 6. Minimum intensity projections through MR image slices and corresponding phase-contrast microscopy images of two MCTSs in collagen type I gel over time. The upper two rows are images of one MCTS on Days 0, 2, 3, and 6, and the lower two rows are representative of a second MCTS at the same time points. At Day 0, the MCTSs are a tight group of cells that have adhered to the gel and just begun to invade, as can be observed on the phase-contrast images. In the corresponding MR images, the MCTSs appear as large blooming artifacts due to the high density of Fe in the MCTSs. By Day 2, single cells are observable under the microscope up to 450 to 600 μm from the initial implantation site, as indicated by the arrows on the Day 2 phase-contrast images. Some invasion is also observable in the corresponding MR images at that time point. As the cells continue to invade the gel, distinct areas of signal void can be observed on the MR images, and extensive invasion can be seen under the microscope. The MR images were acquired at 100-μm isotropic resolution with the 3D FIESTA pulse sequence. Phase-contrast microscopy images were acquired at 100×. Day 6 phase-contrast images are collages of several fields of view to see the entire invasion area at the same magnification as the other images.
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Fig. 7. Phase-contrast microscopy image (A) of invading MCTS on Day 3 showing main invasive edge and furthest cell measurement, and bar graph (B) indicating invasion distances for unlabeled and labeled MCTSs over time. A: The circle outline delineates the main invasive edge and the position of the furthest invading cell is indicated by the radial line. The initial radius of the MCTS (measured on Day 0) was subtracted from the subsequently measured radii to give the distance invaded, relative to the size of the MCTS. B: No significant difference is observed at any time point between invasion distances of unlabeled compared with labeled MCTSs (p > 0.1, one-tailed Student t-test).

Fig. 8. Three-dimensional rendering of the volume of signal loss on serial images of the same two invading MCTSs as in Fig. 6. The appearance of the MCTS on each day is shown on the left in the figure and can be matched with its appearance in the larger images on the right. The MCTS initially appears as a strong blooming artifact, similar to a large air bubble, because of the high density of iron in the tight MCTS. The volume of the signal void decreases slightly on Day 1 compared with Day 0 as the iron density decreases while the MCTS becomes less tight, thus decreasing the blooming effect. The volume of the signal void then increases steadily, and as the distance between invading cells and the main MCTS increases, small discrete areas of signal void become visible (Days 2 and onward). The larger images on the right are overlays of all seven days, giving a more correlative view of the progression over time. Arrowheads point to some cases in which small areas of signal void appear to change position over consecutive days, demonstrating the potential for tracking cells over time.
bellowing artifact is typical of superparamagnetic agents. Other contrast agents that do not lead to such an artifact, including Gd-based T₁ agents and fluorine-based agents, are also being evaluated. Although these agents may lead to better specificity, their sensitivity is not as high as that of iron oxide agents and to date is limited to large cell numbers.

Whether invading glioma cells do in fact divide is the subject of ongoing debate. Nevertheless, it is important to understand what effect the dilution of MPIO due to cell division has on the MR signal. In this study, with an iron loading of approximately 17 pg/cell, a resolution of 100 μm isotropic, and a signal-to-noise ratio of 90, the mean contrast-to-noise ratio was approximately 50. Data from a previous in vitro study by us have indicated that the minimum amount of iron that could be detected under these conditions is approximately 1 pg (corresponding to a contrast-to-noise ratio of 5). We have observed that MPIO particles are conserved during cell division, meaning that they are not expelled or degraded by the cells (Fig. 4). Assuming that the particles are evenly distributed during cell division, as suggested by previously published data, and based on what we know about the detection limits for MPIO with FIESTA, we should be able to tolerate three or four cell divisions before the cells become undetectable. However, we have noticed that the requirements for single cell detection are more difficult to achieve in vivo than in gelatin. Strategies to increase label uptake by the glioma cells and improve the image quality are being evaluated and will hopefully lead to definite single cell detection in vivo over several cell divisions. The problem of label dilution is also encountered with some histological stains, although with the advent of green fluorescent protein, such dilution can often be avoided. Persistent contrast agents based on reporter gene technology are also being developed for MR imaging, but as yet lack the sensitivity required for single cell detection.

Meaningful information has been, and continues to be, obtained from histological studies and in vitro models of glioma cell invasion. For the most part, in vitro models can be successfully studied with simple light microscopy and confocal laser scanning microscopy, but the destructive nature of histological methods limits their ability to provide dynamic 3D information in vivo. The noninvasiveness, high sensitivity, and 3D digital nature of MR imaging data sets make MR imaging ideal for in vivo longitudinal studies. Until recently the imaging of single cells in vivo had not been possible due to resolution limits, but authors of a few recent studies have reported single cell detection in vivo, and we have successfully achieved in vivo single cell detection using MR imaging with validation by confocal scanning laser microscopy. Most recently, we have been able to monitor the fate of metastatic breast cancer cells in the mouse brain from the single cell stage through the development of tumors. The detection of single, actively invading glioma cells in vivo will present challenges beyond resolution limits and iron loading. For example, the contrast may be affected by the constantly changing environment of the cells due to remodeling of the ECM, the growth of new vasculature, and the influx of inflammatory cells reacting to the tumor.

Cellular imaging presents a new approach to the study of glioma cell invasion, and its success will represent important advances for both the glioma and microimaging communities. Data in this study support future in vivo tracking of single glioma cells.

Conclusions

Rat C6 glioma cells can be efficiently and reproducibly labeled with MPIO particles that allow them to be detected in vitro at the single cell level. The incorporation of MPIO does not have a significant effect on the plating efficiency or proliferation of the cells up to a cellular iron content of 20 pg and does not appear to affect the ability of the cells to invade. The progression of glioma cell invasion through a collagen gel matrix from an MCTS can be observed using MR imaging. Cellular imaging is a new approach in the study of glioma cell invasion, and the application of this technique in vivo will have a significant effect on the study of cell migration and invasion as well as on the field of cellular imaging. This study is an important first step toward in vivo imaging of glioma cell invasion at the single cell level.

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