Manganese-Enhanced Magnetic Resonance Imaging for Early Detection and Characterization of Breast Cancers

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Abstract

Very early cancer detection is the key to improving cure. Our objective was to investigate manganese (Mn)-enhanced magnetic resonance imaging (MRI) for very early detection and characterization of breast cancers. Eighteen NOD scid gamma mice were inoculated with MCF7, MDA, and LM2 breast cancer cells and imaged periodically on a 3 T scanner beginning on day 6. $T_1$-weighted imaging and $T_1$ measurements were performed before and 24 hours after administering MnCl$_2$. At the last imaging session, Gd-DTPA was administered and tumors were excised for histology (hematoxylin-eosin and CD34 staining). All mice, except for two inoculated with MCF7 cells, developed tumors. Tumors enhanced uniformly on Mn and showed clear borders. Early small tumors ($\leq 5$ mm$^3$) demonstrated the greatest enhancement with a relative $R_1(1/T_1)$ change of 1.57 ± 0.13. $R_1$ increases correlated with tumor size ($r = -0.34, p = .04$). Differences in $R_1$ increases among the three tumor subtypes were most evident in early tumors. Histology confirmed uniform cancer cell distribution within tumor masses and vasculature in the periphery, which was consistent with rim-like enhancement on Gd-DTPA. Mn-enhanced MRI is a promising approach for detecting very small breast cancers in vivo and may be valuable for very early cancer detection.

EARLY CANCER DETECTION remains a critical goal toward reducing mortality and improving the chances for successful treatment. In the management of breast cancer, mammography is a proven and effective method for screening and works by detecting masses and microcalcifications that are an early sign of breast disease. However, these findings may not be caused by cancer, and not all cancers are found. Only on suspicious findings is further diagnosis performed: ultrasound imaging to differentiate solid tumors from cysts; magnetic resonance imaging (MRI) to delineate tumor extent with fine detail, stage, and provide more diagnostic information such as tumor vascularity; and image-guided breast biopsy for definitive diagnosis and treatment planning. MRI is also used in place of mammography to screen young high-risk women with dense breasts, to assess treatment response, and to follow-up posttherapy.$^{1,2}$ For these high-risk patients, MRI is considered the screening modality of choice for early breast cancer detection.$^3$ When MRI is performed, contrast is typically administered via intravenous injection of a gadolinium (Gd) chelate such as gadolinium–diethylenetriamine pentaacetic acid (Gd-DTPA) to increase the sensitivity of tumor detection and delineation. However, this method requires the presence of increased tumor vascularity to mediate contrast uptake. A dilemma arises with this screening approach because it relies on the tumor vasculature, yet it is known that tumor angiogenesis is not the first presenting sign of cancer. In fact, most early developing primary solid tumors that are 1 to 2 mm diameter are in a state of avascular growth.$^4$ Evidently, with no information from imaging on cancer at the cellular level, we cannot detect sparse cancer cells in the absence of gross changes. This is a severe limitation considering that biopsy can only sample regions indicated as suspicious on imaging. More importantly, breast cancer, like most cancers, has a long period of growth in years before reaching a size detectable on mammography.
During this sojourn period, some aggressive breast cancers may spread to other parts of the body even before the patient becomes symptomatic. Detecting early small cancers must, therefore, resort to a method that images the cancer cells directly, not indirectly through the tumor blood supply.

Manganese (Mn)-enhanced MRI is a cellular imaging method that has the potential for detecting early small tumors. It generates positive signal contrast as a result of cell uptake of the Mn ion, a trace mineral found naturally in our bodies. The majority of Mn-enhanced MRI applications to date have been on studies of brain function and myocardial viability, but there have been some, albeit far fewer, applications to cancer. In cancer imaging, there is evidence that the administration of Mn as a contrast agent is useful for tumor detection and distinction from normal tissue. However, virtually all in vivo studies on Mn-enhanced cancer imaging in the literature have been performed when tumors were large and in the late stages of growth. Early detection of small tumors using Mn-enhanced MRI has not, to our knowledge, been explored.

The aim of this study was to investigate the potential of Mn-enhanced MRI to detect early small breast tumors in vivo. A secondary aim was to determine if differential Mn enhancement among different breast cancers recently observed in vitro translates to the in vivo setting and, if so, how the enhancement patterns evolve as tumors grow in size.

### Materials and Methods

#### Tumor Induction in Mice

All procedures were approved by our institutional animal care committee and conducted in accordance with the national standards on animal care. Human breast adeno-carcinoma cell lines MCF-7, MDA-MB-231, and 231/LM2-4, hereafter referred to as MCF7, MDA, and LM2, respectively, were used. Both MCF7 and MDA were obtained from American Tissue Culture Collection (ATCC, Manassas, VA). The MDA cell line is a highly metastatic triple negative breast cancer, which is clinically important as it is the most aggressive and has the poorest prognosis, with no targeted therapy available and a high risk of recurrence. The LM2 cell line, a highly metastatic variant of MDA-MB-231, was obtained after two rounds of metastasis selection in mice. The least aggressive is MCF7, which is amenable to hormone therapy. Cancer cells were maintained in 1640-RPMI medium (Sigma-Aldrich Canada, Oakville, ON). The medium was supplemented with 10% fetal bovine serum and 0.5% penicillin-streptomycin at 37°C and 5% CO₂. Cells were harvested by washing 80% confluent flasks with phosphate-buffered saline (PBS) and adding 0.05% trypsin-ethylenediaminetetraacetic acid (EDTA) (Gibco, Carlsbad, CA) to detach cells. Cells were then harvested, counted, and resuspended in complete PBS.

Eighteen 6-week-old female NOD scid gamma (NSG) mice (Jackson Laboratory, Bar Harbor, MN) weighing approximately 25 g were used in this study. Mice were anesthetized with 2.5% isoflurane, and approximately 10⁶ cells were injected in the right flank, six mice per cell line. Estrogen pellets for 60-day release (Innovation Research of America, Sarasota, FL) were inserted subcutaneously into the back of the neck for the estrogen-dependent cell line MCF7.

#### In Vivo MRI

Beginning at 6 days after tumor induction, mice were imaged periodically (weekly for the first 4 weeks and then biweekly for the slower-growing cell lines) until tumors reached a size of 1 cm in diameter or animals showed signs of distress, such as severe dehydration or weight loss. Imaging was performed on a 3 T clinical magnetic resonance scanner, using an eight-channel wrist coil for signal detection. Mice were induced on 2.5% isoflurane in pure oxygen (2 L/min flow rate) and maintained on 2% isoflurane during imaging. Mice were placed prone within the coil, resting on top of a water blanket maintained at 36°C (HTP-1500, Adroit Medical Systems, Loudon, TN). A dose of 0.3 mmol/kg of manganese chloride (MnCl₂) was injected subcutaneously at the back of the neck. Prior to and 24 hours after MnCl₂ administration, a high-resolution T₁-weighted spin-echo scan and T₁ mapping were acquired. The T₁-weighted spin-echo scan used a two-dimensional acquisition with the following parameters: repetition time (TR) = 507 ms, echo time (TE) = 14 ms, number of signal averages (NSA) = 4, 10 cm field of view (FOV), 14 1 mm thick slices, and 0.5 × 0.5 mm in-plane resolution. T₁ mapping consisted of a three-dimensional spoiled fast field echo (T₁-FFE) sequence repeated at flip angles of 2°, 10°, and 20°; other parameters were TR = 6.9 ms, TE = 3.7 ms, NSA = 16, 10 cm FOV, 14 1 mm thick slices, and 0.5 × 0.5 mm in-plane resolution.

At the terminal imaging session for each mouse, Gd-DTPA (Magnevist, Bayer, Toronto, ON) was injected to provide a comparison between Mn enhancement and Gd enhancement patterns. Multiple Gd-DTPA injections were not possible during the course of the study as the smallest
catheters commercially available were comparable to the size of the tail vein. Catheter placement required surgical guidance and was an end-point procedure. An animal surgeon operated on the tail to insert a 26-gauge angiocath into the lateral tail vein and attached it to a three-way stopcock. At the MRI scanner, the stopcock was connected to a 1 mL line tubing through which Gd-DTPA was delivered as a bolus at a dose of 0.05 mmol/kg. Immediately after imaging, animals were sacrificed and the tumors were excised.

**In Vitro MRI**

Cell pellets were also prepared in triplicates and imaged to assess uptake and retention of MnCl\(_2\). Medium containing 1.0 mM of MnCl\(_2\) was added to cells growing in the exponential growth phase for 1 hour, after which cells were rinsed with fresh medium and trypsinized. This concentration maximized differences in enhancement among cell lines without affecting cell viability. Cells were centrifuged at 440 g for 10 minutes to create cell pellets with a depth of approximately 1 cm in borosilicate glass tubes (Life Science Products Inc., Frederick, CO). Imaging and T\(_1\) mapping were performed as described previously.\(^{16}\)

**Data Analysis**

MRI data were transferred to an independent workstation for quantitative data analysis using in-house software developed in Matlab version 8.1 (MathWorks, Natick, MA).

In vivo data were first analyzed for T\(_1\) relaxation times before and after contrast administration. T\(_1\) maps were generated by calculating T\(_1\) relaxation times on a pixel-wise basis on every slice in three-dimensional imaging volume using a previously described method.\(^{19}\) Regions of interest (ROI) were drawn on T\(_1\) maps to encompass the entire tumor; the mean T\(_1\) value was then determined in the ROI. The measured T\(_1\) was then converted to the relaxation rate R\(_1\) (\(= 1/T_1\)). The change in R\(_1\) postcontrast injection relative to baseline (ie, precontrast injection) was determined for each tumor following both MnCl\(_2\) and Gd-DTPA administration. Tumor volumes were also calculated by summing up all pixels across all slices that showed enhancement after MnCl\(_2\) administration.

In vitro data were also analyzed on a pixel-wise basis for T\(_1\) relaxation times in cell pellets immediately and 24 and 72 hours after incubation with MnCl\(_2\). The average T\(_1\) and R\(_1\) were calculated in each cell pellet.

**Statistical Analysis**

Tumor volumes were first classified as small (\(\leq 5 \text{ mm}^3\)), medium (\(> 5 \text{ and } \leq 20 \text{ mm}^3\)), or large (\(> 20 \text{ mm}^3\)). Changes in R\(_1\) in tumors following contrast administration were sorted according to tumor size and cell line. Differences in contrast-induced R\(_1\) change were compared using one-way analysis of variance (ANOVA), with the main effect being the tumor size or the cell line. Post hoc Tukey-Kramer testing for multiple comparisons was then performed at the 95% confidence level. Significance is reported at a p value of 5% unless otherwise stated.

**Histology and Immunohistochemistry**

Excised tumors were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned into 5 \(\mu\)m thick slices. Sections were stained with hematoxylin and eosin and CD34. Light microscope (Olympus BX60) images were taken to confirm that growths were cancer cells and to assess tumor composition and structure.

**Results**

Tumor induction was successful in 16 of the 18 mice. The two animals that did not develop tumors were both inoculated with MCF7 cancer cells. The in vivo growth characteristics of all three breast tumor xenografts, MCF7, MDA, and LM2, are shown in Figure 1. The LM2 cell line consistently demonstrated the most rapid growth in vivo. At the other extreme is MCF7, which showed a wide range of growth rates and time of first detection on imaging.

**Figure 1.** In vivo growth characteristics of breast tumor xenografts in mice. Tumor volume as measured on imaging versus days after cancer cell inoculation in mice for three breast cancer cell lines: MCF7, MDA, and LM2. Shown are mean values ± SD.
Figure 2 illustrates in vivo contrast-enhanced T₁-weighted spin-echo images acquired post-MnCl₂ injection during both very early and late tumor development for all cell lines. Two observations should be noted. One was that during early tumor growth, Mn-enhanced MRI consistently improved the detection and delineation of small tumors. The other observation was that when tumors were much larger, Mn-enhanced MRI continued to provide a clear depiction of tumor borders and even interior structures.

Quantitative measurements of relative changes in R₁ relaxation rates following MnCl₂ administration are shown as a function of tumor volume for all cell lines in Figure 3. There was a clear trend toward larger Mn-induced R₁ increases in smaller tumors, and the correlation between relative R₁ increase and tumor volume was significant (r = −.34, p = .04). This implies that even very small tumors can be detected, with high sensitivity. The increase in relative R₁ change from medium-sized to small tumors or from large to medium-sized tumors was significant (p < .05) (Figure 4). Figure 5 classifies R₁ changes according to cell line. Differences in Mn-induced R₁ change among cell lines were not significant at any stage of tumor growth. However, early small tumors appeared to suggest cell line–dependent differences (p < .25), with MDA cells enhancing the most and LM2 cells the least. This in vivo observation is compared against in vitro measurements of Mn-induced R₁ change, where both MDA and LM2 cells demonstrated the greatest R₁ change and a significant reduction 24 hours after exposure to MnCl₂ (p < .05) (Figure 6).

The pattern of Mn enhancement was very different from that of Gd enhancement. Figure 7 shows contrast-enhanced images after MnCl₂ administration and after Gd-DTPA injection for the three cell lines. In all cases, Mn-enhanced images demonstrated uniformly enhancing masses that were easily distinguished from surrounding normal tissue. On the other hand, Gd-enhanced images showed typical rim enhancement patterns, but the tumors themselves were difficult to distinguish in some cases. Histology using hematoxylin-eosin and CD34 staining confirmed that all lesions identified on imaging were solid tumors with a dense distribution of cancer cells throughout. Vasculature was present but confined mainly to the tumor periphery, with the MCF7 subtype demonstrating higher vascularity than the other two subtypes.

Discussion

Early cancer detection is recognized to be a key factor to improving patient survival and outcome. However, current contrast-enhanced MRI methods based on administering...
Gd-DTPA for highlighting tumor vasculature may not be the best option for early detection because most small developing solid tumors are in a state of avascular growth. Furthermore, different tumor types have varying degrees of vascularity. For these reasons, an intuitively more sensitive method to detect early tumors is to image the cancer cells directly. In this study, we employed MnCl$_2$, an intracellular magnetic resonance contrast agent, to image cancer cells and investigated its potential to detect early small breast tumors in vivo. We also determined whether or not the ability of Mn-enhanced MRI to differentiate breast tumor subtypes in vitro could be achieved in the in vivo setting. It was shown in breast tumor–bearing mice at 3 T that Mn-induced enhancement was greatest in the smallest early tumors.

Figure 3. Mn induced relative $R_1$ increase in vivo versus tumor volume for all tumors. $R_1$ increase relative to precontrast injection levels is shown in relation to tumor volume for all tumor subtypes. There is a significant negative correlation between relative $R_1$ increase and tumor volume ($r = -0.34, p = 0.04$).

Figure 4. Relationship between Mn-induced relative $R_1$ increase in vivo and tumor volume. Average relative $R_1$ increases for small ($\leq 5$ mm$^3$), medium ($> 5$ and $\leq 20$ mm$^3$), and large ($> 20$ mm$^3$) tumors are significantly different: *$p < .05$. Shown are mean values ± SD.

Figure 5. Relationship between Mn-induced relative $R_1$ increase in vivo and tumor subtype. Average relative $R_1$ increases for MCF7, MDA, and LM2 tumors are compared across different tumor volumes: small ($\leq 5$ mm$^3$), medium ($> 5$ and $\leq 20$ mm$^3$), and large ($> 20$ mm$^3$). Differences were not significant: *$p < .25$. Shown are mean values ± SD.

Figure 6. Cellular retention of MnCl$_2$ in vitro. Measurements of $R_1$ in cancer cell pellets at various times postincubation with MnCl$_2$. Shown are mean values ± SD. A decrease in $R_1$ is significant at 24 hours postincubation for MDA and LM2 cells: *$p < .05$. 
tumors. At later stages of development, Mn-enhanced MRI continued to provide consistently uniform contrast throughout these solid tumors and clearly delineated the borders, which is very different from the rim-like enhancement observed on Gd-enhanced MRI that was difficult to detect in some cases. The ability to characterize tumor subtype based on Mn enhancement may be feasible in vivo only during the early stage of tumor growth.

The observation that very small tumors can be sensitively detected on Mn-enhanced MRI has two important implications. One is that primary tumors may be found much earlier than is currently possible, it is hoped at a stage before significant vasculature has developed and metastasis has already begun. The second implication is that metastasis, once it has occurred, may also be identified much earlier if the presence of small metastatic lesions that are otherwise occult can now be sensitively detected. What enables these small tumors to be detected is cell uptake of MnCl$_2$, as previously demonstrated in vitro. However, why the relative enhancement increases significantly with smaller tumor volumes is unclear. Perhaps cell density is greatest during the initial stages of tumor growth and then gradually declines as the tumor mass expands and develops necrotic patches. Another possibility is that cancer cell activity changes during tumor progression, thus altering uptake of MnCl$_2$. An in-depth investigation to elucidate the underlying mechanisms would need to incorporate measurements of cell activity (eg, proliferation markers) and absolute Mn content on a per cell basis at different stages of tumor growth.

Several important similarities and distinctions exist between the in vivo and the in vitro setting for Mn-enhanced MRI of breast cancer. For the MDA and MCF7 cell lines, the more aggressive subtype, MDA, demonstrated higher Mn-induced enhancement than MCF7 both in vivo and in vitro. The LM2 cell line, however, which is a more aggressive variant of MDA, demonstrated a much lower enhancement in vivo, even lower than MCF7. A very likely explanation is simply a difference between the in vivo and in vitro setting: in vitro, cells are exposed to MnCl$_2$ for an hour and then removed, whereas in vivo cells are exposed to MnCl$_2$ over many hours, which gives rise to a situation where some tumor cells may attain equilibrium and start releasing contrast agent, whereas other tumor cells may continue to accumulate contrast agent so long as MnCl$_2$ is present in the body. In the case of LM2 cells, which release Mn in vitro much more rapidly compared to MCF7 cells, they may already have begun to release Mn ions in vivo, whereas MCF7 cells may not have reached the equilibrium point yet. This last point emphasizes the importance of finding an optimal imaging time interval that balances cell uptake and release of contrast agent.

Perhaps a more interesting observation is that differences in Mn-induced enhancement among the breast cancer subtypes are greatest in early small tumors but
disappear as tumors grow. We can only speculate at this point as to why this is the case. It may be possible that during early tumor development, the mass consists mainly of cancer cells, whereas at later stages, necrosis, debris, and vasculature also occupy a portion of the tumor mass and exert their own influences on signal contrast. Therefore, it is at the early stage of in vivo tumor growth, which most closely mimics the setting of in vitro cell study, where differential Mn enhancement among cancer subtypes would be most readily observed.

There are a number of considerations for future investigation to optimize the technique and to understand our observations. One area to investigate is the choice of imaging time point on the positive contrast that is achieved. Our previous experience had shown repeatedly that enhancement at 24 hours was much greater than at 2 to 4 hours postinjection. However, the interval between 4 and 24 hours has not been investigated. We may find, for example, the LM2 cell line exhibiting much higher signal contrast, closer to that observed in vitro, if the time interval were less than 24 hours post-MnCl₂ administration. Furthermore, we may find greater differences among the cell lines at the optimal time point. The dose of MnCl₂ administered will also need to be optimized, but the subcutaneous route of administration is deemed best for slow distribution of contrast and gradual uptake by the tumor (our previous experience with intravenous injections failed to produce marked signal contrast). To understand the mechanisms underlying our observations, a comprehensive experiment that incorporates measurement of absolute Mn content, cellular mechanisms (eg, proliferation, cell cycle), and histology needs to be conducted at different stages of tumor growth. Also, to test the postulation that early-stage cancer in a state of avascular growth can be reliably detected on Mn-enhanced MRI, we will need to compare against Gd-DTPA administration at very early time points, which this study has identified to be within the first 2 weeks postinoculation. Finally, to investigate the universality of this method, we need to apply it not only to small primary tumors, as we have done in this work, but also to detecting small metastatic cancers.

Conclusion

In vivo Mn-enhanced MRI is shown to detect early small breast tumors, with the highest sensitivity. As tumors grow, MnCl₂ continues to provide enhancement of the entire tumor mass, depicting morphology and tumor borders more clearly than Gd-DTPA. Cancer subtype–dependent differential Mn enhancement is most evident at the early stage of tumor development. The results of this study suggest that Mn-enhanced MRI may play an important role in very early detection of primary tumors and possibly of small metastatic lesions in early metastasis.

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