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MRI detection of early bone metastases in B16 mouse melanoma models

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Abstract

Bone metastasis causes significant morbidity in cancer patients, including bone pain, pathologic fractures, nerve compression syndrome, and hypercalcemia. Animal models are utilized to study the pathogenesis of skeletal metastases and to evaluate potential therapeutic agents. Previously published methods for imaging bone metastasis in rodent models have focused on identifying advanced stage metastasis using simple X-rays. Here we report MRI as a method for detecting early bone metastases in mouse models *in vivo*. B16 mouse melanoma cells were injected into the left cardiac ventricle of C57BL/6 mice and magnetic resonance (MR) images were obtained of the left leg following the development of metastatic disease, when tumor associated bone destruction was histologically present but not visible by X-ray. T1 and T2 relaxation times of bone marrow were measured in healthy control mice and B16 melanoma tumor-bearing mice. Mean T2 values for normal marrow were 28 ms (SD 5) and for diseased bone marrow were 41 ms (SD 3). T2 relaxation time of diseased bone marrow is significantly longer than that of normal bone marrow ($P < 0.0001$) and can be used as a marker of early bone metastases. These studies demonstrate that MR imaging can detect bone marrow metastases in small animals prior to development of cortical bone loss identified by X-ray.

Keywords

animal models; bone metastases; magnetic resonance imaging; melanoma; rodents

Introduction

Bone is the most common site for metastasis in a variety of cancers, including breast, prostate, lung, melanoma, and bladder cancer. Seventy-five percent of all patients with metastatic breast and prostate cancer and 90% of multiple myeloma patients develop bone metastases [1–3]. Metastases occur when cancer spreads from a primary site to a distant location. For a cancer to spread to the bone, some tumor cells must detach from the primary site, travel through the circulation to the bone marrow, and then stimulate osteoclast activation which leads to destruction of cortical and trabecular bone. Despite this knowledge, molecular mechanisms by which skeletal metastases are established are unclear. Most of the clinical problems associated with bone metastases are a result of tumor-induced deregulation of physiologic bone turnover. Severe bone pain, pathologic fractures, nerve compression syndrome, and hypercalcemia are among the many problems that patients with bone metastases may endure. While a variety of clinical methods are available to treat bone metastases, including chemotherapy, hormonal therapy, surgery, external beam radiation, analgesics, and bisphosphonate therapy [4], the development of more targeted cancer therapeutics is challenged by the identification of imaging modalities that can provide early disease diagnosis and monitor therapeutic response. The continued development of treatments to reduce the incidence and lessen the morbidity of bone metastases is of great clinical and social significance for improving the life quality of metastatic cancer patients.

Metastasis in bone can be characterized as either osteoblastic or osteolytic, based on the presence or absence of net bone formation. Nuclear medicine bone scans are sensitive to detecting osteoblastic bone metastases, however, bone scans may miss small lesions (<1 cm) and lack specificity. Tracer accumulation may occur in any condition with increased bone turnover, e.g., trauma, infection, and arthropathy [5]. Evaluation of therapeutic response may be difficult since areas of necrosis may also mimic metastases [5]. Tumor response in bone is often associated with a “flare” response on bone scans from host cell osteoblastic reaction. The inability to distinguish response from progression can cause clinicians to prematurely terminate potentially effective anti-tumor therapy or erroneously continue ineffective therapy. Classic osteolytic lesions associated with significant bone destruction can be detected by radiography only at advanced stages. Metastases to the bone become apparent on X-ray only after the loss of more than 50% of the bone mineral content at the site of the disease [6]. Radiography also often underestimates the number and extent of bone metastases.

Magnetic resonance imaging (MRI) is a powerful imaging tool for non-invasive, *in vivo* characterization of tumor development. Clinically, MR imaging provides a sensitive method for detecting the presence and extent of bone marrow metastases. Several clinical studies have shown that the sensitivity of MRI for detecting bone marrow metastases equals or exceeds nuclear medicine bone scans or bone marrow biopsies [5,7–9]. Traditionally, endogenous contrast in MRI experiments results from differences in spin density, spin-lattice (T1) relaxation time and/or spin-spin (T2) relaxation time. Differences in the biophysical signatures of water in healthy and diseased tissues, as reflected in these parameters, provide a means for distinguishing normal and pathologic tissue. For example, T2 relaxation times are typically longer in tumors than in the surrounding normal tissues [10,11], and thus tumors appear bright on T2-weighted images. Measuring T1 and T2 relaxation times is essential to utilizing and developing methods that will provide the greatest contrast between healthy and diseased tissues.

Animal models are currently utilized to study the pathogenesis of skeletal metastases and to evaluate potential therapeutic agents. Imaging of bone metastases in small animals generally relies on the evaluation of end-stage metastases as determined by X-ray imaging, autopsy

and histologic analysis of bone and visceral lesions. Currently there are no reliable imaging modalities to detect early tumor invasion into the bone marrow in small animals. The lack of early radiographic detection of bone metastasis significantly limits our ability to perform trials of novel therapies targeted to bone and to perform experiments to understand the mechanisms of tumor metastasis to bone.

Small-animal MRI provides *in vivo* characterization of tumors using non-ionizing radiation, enabling longitudinal assessment of disease and responses to therapy. Unlike human studies, the results of small-animal MRI experiments can be routinely validated by obtaining true histologic correlates. MRI techniques developed for small animals can often be directly adapted for use on human MR scanners. The higher resolution required for imaging small animals compared with humans is achieved on dedicated, small-animal MR scanners through the use of higher strength magnetic-field gradients, produced by small-diameter (e.g., 10 cm) gradient sets. Similarly, higher static magnetic fields (e.g., 4.7–11.74 T) and the use of small, high-sensitivity RF coils help to achieve the necessary sensitivity for imaging animals. Small-animal MRI has recently been applied to the development of animal models of cancer in brain [12–14], lung [15,16], kidney [17], bladder [18], prostate [19,20], and pancreas [21], as well as primary central nervous system lymphoma [22]. In a similar manner, MRI can be used to monitor the effects of cancer therapy in a wide variety of animal tumor models. Recent applications include studies of chemotherapeutic treatment of models of brain [23,24], prostate [25,26], colon [27], bladder [28], and liver [29] cancer, the development of tumors at wound sites following laparoscopic surgery [30] and responses to gene therapy in models of glioma [31,32].

Our study goals were to establish parameters for optimal imaging of mouse tibia and femur (the primary target for bone metastasis in this mouse model), to define baseline relaxation parameters of healthy and diseased bone marrow, and to demonstrate the feasibility of MRI as a method for detecting bone metastases in mouse models *in vivo*. We first describe an MRI method that provides detailed characteristics of mouse femur, tibia, and knee joint that can be applied to studying mouse models of bone marrow metastasis. Optimal imaging parameters are defined to provide detailed anatomic characterization of mice legs. Using these techniques, we measured T1 and T2 relaxation times in young healthy mice to provide baseline values of normal bone marrow. These methods were then applied to early stage B16 melanoma tumor-bearing mice and compared and correlated with comprehensive histological studies. We found that T2 relaxation times in the bone marrow of tumor-bearing mice were significantly different from those in bone marrow of normal mice and could therefore be used as an early marker of bone metastases.

Materials and methods

Animals

All animal procedures were performed in accordance with guidelines of Washington University's Animal Studies Committee. Eight 6-week old C57BL/6 mice (Harlan Laboratories, Indianapolis, IN) were used for tumor studies and eight age-matched healthy C57BL/6 mice served as controls. The tumor model involves injection of B16-F10 mouse melanoma cells (a gift from Dr. David Fisher, Dana Farber Cancer Institute, Boston, MA) into the left cardiac ventricle, as described previously [33,34]. Briefly, melanoma cells were cultured in Dulbecco's Modification of Eagle's Medium (DMEM) with 10% Fetal Bovine Serum. After 0.1% trypsin/0.2% EDTA treatment, B16 cells were re-suspended in phosphate-buffered saline (PBS) prior to *in vivo* injection. Insulin syringes with 29-gauge ½ inch needles (Terumo Medical Corporation, Elkton, MD) were used to inject 1×10^5 B16 cells 100 µL PBS into the left cardiac ventricle.

Imaging

Ten to twelve days following tumor cell injection, MR images of the left femur, knee, and tibia were collected. MRI experiments were performed using a Varian NMR Systems (Palo Alto, CA) INOVA console equipped with an Oxford Instruments (Oxford, UK) 4.7 T, 33-cm clear bore magnet and Oxford actively shielded gradient coils (15 cm ID, 18 G/cm, 200 μ s rise-time) driven by high-performance ($\pm$ 300 V, 200 A), low-noise Tectron gradient power supplies. Prior to data collection, the mice were anesthetized with isoflurane/O₂ (5% v/v) and maintained on isoflurane/O₂ (1–2% v/v) throughout the experiment. Mice were positioned and secured on a plate heated with circulating warm water to maintain body temperature at 37 °C. The left leg of each mouse was positioned and immobilized on a horizontal platform for consistent slice localization. A 1.5 cm outer-diameter, 2-turn, circular surface coil was centered over the knee as the receiver of MR signal. A 9 cm inner-diameter, linear Helmholtz coil was used as the radiofrequency transmitter. In this dual-coil arrangement, we were able to couple the high sensitivity of a surface coil with the homogeneous excitation provided by a large volume coil.

T1 maps were generated from a series of variable flip angle, 3-D T1-weighted images collected with the following parameters: FOV $3.0 \times 3.0 \times 2.0$ cm³, data matrix $128 \times 128 \times 64$, TR = 200 ms, TE = 35 ms, flip angles 2, 5, 10, 20, and 30 degrees. The variable flip-angle method [35] was utilized due to its ease of use and the relatively short time (~12 min) required to acquire a complete data set.

Multi-slice, T1-weighted gradient echo (GE) scout images were obtained for slice selection (FOV 1.5×1.5 cm² or 2.5×2.5 cm², TR = 170 ms, TE = 30 ms, 15 slices, slice thickness 0.3 mm, one transient; total scan time 21 sec; inplane resolution 117–195 μ m). Sagittal images of the knee were acquired to provide an optimal view of the bone structure of the leg. A multi-slice, multi-echo, spin-echo (SE) imaging sequence was employed to acquire T2-weighted images for T2 map calculation. Six contiguous slices were chosen from the T1 GE images that contained the bone marrow cavity. Data were collected with the following parameters: FOV 1.5×1.5 cm² or 2.5×2.5 cm², slice thickness 0.3 mm, data matrix 128×128 , TR = 3 s, TE = 20 ms, 3 echoes with echo spacing of 20 ms (20, 40 and 60 ms), four averages; total acquisition time 25 min.

T1 and T2 maps were generated using a linear regression curve fitting routine written in Matlab (MathWorks; Natick, MA). T1 and T2 maps were created in Varian's Image Browser format and mean T1 and T2 values were computed using Image Browser by drawing regions of interest (ROIs) over regions of bone marrow on selected slices of the maps. For each mouse, the slice with the greatest area of visible bone marrow was selected for ROI analysis. ROIs were drawn by a single observer and care was taken to minimize partial volume effects from cortical bone. Student *t*-test statistical analyses were performed using Microsoft Excel.

Histology

All animals were euthanized within 2 days of the completion of the MRI studies and mouse legs were harvested for histologic analysis. Mouse femurs and tibias were then excised, cleaned of soft tissue, fixed in 10% formalin in phosphate buffered saline (PBS) and decalcified in 14% EDTA. Long bones were embedded in paraffin and sliced in sagittal sections through the center of the bone. Histological sections were stained with hematoxylin and eosin (H&E) and with tartrate resistant acid phosphatase (TRAP) to identify osteoclasts. Comparison of the histology and imaging results served to validate the MR methods.

Results and discussion

T2 weighted SE MR images delineate bone/bone marrow architecture

Figure 1 shows a series of four contiguous, sagittal T2-weighted SE MR images from the femur and tibia of a normal mouse. The marrow cavities of the metaphyseal portions of the femur and tibia were readily distinguishable as outlined by black cortical bone. Other anatomic components of the leg were likewise visible in these images, including the connective cartilage forming the growth plate, fluid of the synovial cavity, muscle layers and fascial planes and subcutaneous fat. The dimensions of a normal mouse leg are approximately $2.0 \times 0.1 \times 0.05 \text{ cm}^3$ and these images demonstrate the exceptional anatomical resolution that can be obtained using MRI techniques. The challenge in imaging with MR, both clinically and in small animals, is to achieve adequate spatial resolution while maintaining the highest possible signal-to-noise ratio. As described above, a small surface coil placed directly over the mouse knee provided the high sensitivity required in this study.

T2 weighted SE MR images delineate tumor from normal marrow

Twelve days after tumor cell injection into the left cardiac ventricle, 70% of the bones had widespread B16 tumor growth, with histologic evidence of trabecular bone loss [34,36], but autoradiographs were indistinguishable from non-tumor bearing animals (Figure 2).

T1-weighted MR images revealed little contrast between healthy and tumor-bearing marrow (Figure 3, a and b). Bone marrow consists of both hematopoietic (red) marrow and fatty (yellow) marrow, with the relative red/yellow composition varying due to anatomical site and age of subject. Clinically, metastatic lesions on the bone marrow are visible on T1-weighting in sites of predominantly fatty marrow (or yellow marrow) where the lesion is hypointense compared to the marrow. Lesions in hematopoietic marrow (red marrow) are less conspicuous on T1 imaging [37]. The bone marrow in the tibia and femur of young mice consists mainly of hematopoietic marrow containing very little fat (Figure 4, a and c). This likely explains the lack of contrast seen on T1-weighted images in the tumor-bearing mice in our study. This also explains why fat suppression techniques, used frequently in clinical MR to increase the contrast between normal marrow and infiltrating tumor [7, 8, 37, 38], were unnecessary in this work. As animals age, the bone marrow is gradually replaced by fat, thus fat suppression techniques may be required if studying adult rodents.

In contrast, T2-weighted MR images demonstrated marked differences between tumor bearing and normal mice, with tumor appearing hyperintense relative to normal bone marrow and surrounding tissues (Figure 3, c and d). The mice in this model develop diffuse visceral and bony metastatic disease by day ten after cell injection without significant clinical signs of disease or changes on radiographs. Tumor cells have a predilection for development of tumor in the metaphyseal regions of the long bones, primarily due to the increased vascularity and bone turnover in those regions, especially in young, growing mice [7, 8]. For this reason, the distal femur and proximal tibia are ideal for evaluation of bone metastases both histologically and by MR imaging. Histologic analyses of tibiae after MR imaging correlate T2 MR findings with areas of tumor (Figure 4, panels b and d).

T2 MR imaging demonstrates tumor associated bone destruction

Once the B16 tumor was established in the metaphyseal region, extension into the epiphyses, invasion into the soft tissue, and stimulation of physiologic mechanisms of cortical destruction occurred. B16 tumor cells secrete osteoclastogenic factors that recruit and activate osteoclastic bone resorption to sites of active tumor growth. In Figure 5, the B16 tumor cells invaded into the surrounding soft tissue with the cortex remaining intact in

the diseased mouse, demonstrating tumor invasion prior to cortical destruction (arrow). Standard autoradiographs typically detect bone metastasis only after greater than 50% of the cortical bone is destroyed [6]. MRI can thus provide a means of detecting early tumor bone invasion prior to its appearance on radiographs. Identifying disease at this stage is critical to testing novel therapies.

Quantitative measurements of mean T1 and T2 relaxation times for normal and diseased bone marrow (eight mice each) were calculated (Table 1). Mean T1 values for both normal and diseased bone marrow were 1.4 sec (SD 0.4), consistent with the absence of observed contrast between normal and diseased marrow in T1-weighted images. Mean T2 values for normal marrow were 28 ± 5 ms and for diseased bone marrow were $41 \text{ ms} \pm 3$ and are thus significantly different ($P < 0.0001$). T2-weighted images and quantitative T2 measurements can identify abnormal tissue at day ten and potentially be used to determine even earlier changes.

Use of exogenous contrast agents such as gadolinium diethylenetriamine pentaacetic acid (Gd-DTPA) is valuable in imaging of most cancers. Gd-DTPA shortens the T1 relaxation time in tumors, causing an increase in signal intensity in vascular regions versus necrotic regions and normal tissue [39]. However, Gd-DTPA is not routinely used for imaging of bone metastases and due to the additional scan time required, was not included in this study.

We are currently unaware of any reports in the literature demonstrating MR as a method for detecting bone metastases in small animals. Beckmann, et al. [40,41] described a technique to monitor progression of rheumatoid arthritis in the hind paws of rats *in vivo*. Faure, et al., used MR to monitor disease progression in a rat rheumatoid arthritis model by evaluating global thickening of the synovial region of the knee due to inflammation [42]. While changes in trabecular bone (bone resorption and damage and loss of bone density) were visualized by MRI, no quantitative T1 and T2 relaxation times were measured for normal or diseased bone or bone marrow in either study.

One of the most challenging aspects in the management of bone metastases is evaluation of therapeutic response. Currently there is no reliable clinical imaging modality for evaluation of response, which is typically determined by clinical signs and symptoms. This further demonstrates the need for advanced imaging technologies with correlative histological analysis, emphasizing the need for small animal MR imaging technology development with potential to translate to the clinic.

Early diagnosis and accurate monitoring of therapeutic response are fundamental to improving the outcome for patients with metastatic bone disease. We report that MRI studies can detect early stage metastatic bone disease in small animals. The experiments outlined in this report could be applied to the characterization of other models of metastatic bone disease. MRI can assist scientists studying the molecular mechanisms of bone metastases by providing *in vivo* characterization of early bone disease and can potentially be applied to and further developed for *in vivo* longitudinal studies to follow disease progression and the effects of therapy.

Abbreviations

DMEM	Dulbecco's Modification of Eagle's Medium
FOV	field of view
Gd-DTPA	gadolinium diethylenetriamine pentaacetic acid, standard MRI contrast agent

GE	gradient echo
H&E	hematoxylin and eosin
MR	magnetic resonance
MRI	magnetic resonance imaging
NMR	nuclear magnetic resonance
ROI	region of interest
SD	standard deviation
SE	spin echo
TE	echo time, imaging parameter
TR	time to relaxation, imaging parameter
TRAP	tartrate resistant acid phosphatase, histological stain to identify activated osteoclasts

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Figure 1. Contiguous, 0.3 mm sagittal slices, FOV $2.5 \times 2.5 \text{ cm}^2$ from a T2W image through the femur, knee joint and tibia of a normal bone. Anatomy is identified as: (1) femur, (2) tibia, (3) cortical bone, (4) growth plate, (5) synovial cavity fluid, (6) muscle layers and fascial planes, and (7) subcutaneous fat.

Figure 2.
Conventional radiographs of (a) a normal mouse and (b) a mouse with diffuse bone metastases following injection with B16 melanoma cells.

Figure 3. MR images of a normal (a, c) and a tumor-bearing (b, d) mouse. The images represent a single 0.3 mm slice with FOV $2.5 \times 2.5 \text{ cm}^2$ for the normal mouse and FOV $2.0 \times 2.0 \text{ cm}^2$ for the tumor-bearing mouse. The top panels of this figure are T1-weighted images; the bottom panels are T2-weighted images. Apparent differences in muscle mass between the normal and tumor-bearing mice are due primarily to minor differences in the positioning of the imaging plane relative to the leg.

Figure 4. Corresponding H&E slides for the MR images shown in Figure 3. (a) Femur and (c) tibia of normal mouse; (b) femur and (d) tibia of a tumor-bearing mouse. In the tumor bearing mouse, the tumor is obliterating the metaphyseal regions of both the tibia and femur.

Figure 5.

(a) T2-weighted MR image through the tibia of a tumor bearing mouse. Corresponding histology slides through the tumor are shown in (b) H&E and (c) TRAP stain, identifying osteoclasts (stained red). The tumor is seen extending into the soft tissue, without cortical destruction, on both the MR image and corresponding histology slides (arrows). The lesion extending outside the cortex measures 150 μm in diameter. This mouse represents one of six mice that demonstrated soft tissue extension without cortical destruction.

Table 1

T1 and T2 relaxation measurements for bone marrow in mice.

Mouse	<T1 (s)>		<T2 (ms)>	
	<i>N</i>	<i>T</i>	Normal	Tumor-bearing
1	1.35	1.25	28	43
2	1.10	1.37	26	41
3	0.97	1.00	37	38
4	1.10	1.15	34	45
5	1.88	1.72	27	40
6	1.96	2.00	28	39
7	1.84	1.78	23	44
8	1.30	1.10	24	39
Mean	1.44	1.42	28	41
SD	0.40	0.37	4.8	2.6
<i>P</i> value*	0.93		1.18E-05	

* Statistical significance of difference between mean relaxation values in normal and tumor-bearing marrow.