Magnetic Resonance Imaging of Response to Chemotherapy in Orthotopic Xenografts of Human Bladder Cancer

Richard Mazurchuk,1 Dorothy Glaves, and Derek Raghavan
Department of Biophysics [R. M.] and Division of Medicine [D. G., D. R.], Roswell Park Cancer Institute, Buffalo, New York 14263

ABSTRACT
Although orthotopic xenografts offer the potential for improved modeling of tumor development and response to therapy, noninvasive methods are not readily available to serially monitor tumors growing at internal sites in small rodents. In this study, magnetic resonance (MR) imaging techniques were developed using a clinical 1.5 T whole body scanner to routinely monitor tumor growth kinetics of orthotopic UCRU-BL13 human bladder cancer xenografts after systemic chemotherapy. As a vehicle to test the system, comparisons were made between a standard agent, doxorubicin (DOX), and a novel formulation of liposome-encapsulated doxorubicin (DOXIL) to determine whether liposome encapsulation would alter chemosensitivity of BL13 in an orthotopic model. High resolution MR images acquired using direct three-dimensional data acquisitions yielded accurate volumetric measurements and detected tumors calculated to be ≤10 mm³. MR volume measurements were comparable with tumor volumes calculated from direct caliper measurements. Both DOX and DOXIL demonstrated statistically significant therapeutic effect against orthotopic tumors; DOXIL reduced tumor volumes by approximately 33% (P < 0.005), whereas treatment with free DOX resulted in 70% reduction in tumor volume at termination (P < 0.002). We show that noninvasive MR assessments can be performed longitudinally to construct classical tumor growth curves, which can be used to evaluate therapeutic response in animal models. Translation of the MR techniques used in this study into clinical diagnostic evaluation would allow optimization of individual treatment schedules and facilitate assessment of therapeutic response.

INTRODUCTION
Cytotoxic chemotherapy plays a substantial role in the management of bladder cancer, both as systemic treatment for advanced disease and as intravesical therapy for superficial tumors (1, 2). Although progress has been made in developing effective therapy for metastatic disease (3), the proportion of cured patients is small (4), and there is a need for more effective systemic agents and treatment regimens. The selection of new agents or treatment schedules for clinical trials is based upon data from experimental animal models of disease. Typically, these models involve screening agents in rodents against s.c. tumor transplants, which are easily monitored by caliper measurements to generate tumor growth kinetics. However, the microenvironment at the implantation site of the host organ can influence both the natural history of tumor development (5, 6) and its sensitivity to cytotoxic agents (7). For example, murine fibrosarcomas (8) and colon carcinomas (9, 10), as well as human ovarian xenografts (11), growing at different anatomical sites have shown different therapeutic responses to chemotherapy.

These studies emphasize the need to evaluate cancer treatments using tumors growing in their organ of origin where tumor vasculature, physiology, and growth patterns more closely mimic clinical disease processes. In the case of bladder cancer, the use of intravesical chemotherapy in clinical practice provides another reason for developing a model of local treatment. Although orthotopic xenografts offer the potential for improved modeling of tumor development and therapeutic response, noninvasive methods have not readily been available to monitor tumor growth serially at internal sites in small rodents. Current assessment methods generally use caliper measurements of tumors growing at accessible s.c. sites or caliper measurements/tumor weights made at necropsy.

We propose that the development of an easily implemented noninvasive approach using MR² imaging to monitor serially the longitudinal changes in individual tumor masses growing at internal sites would be a significant advance for the evaluation of experimental therapeutic agents. This approach would provide quantitative growth rates, generate tumor growth delay data, and reduce the numbers of animals needed for stringent evaluation of anticancer drug schedules directed against tumors growing in anatomically and physiologically relevant sites. We have examined this hypothesis using an orthotopic xenograft model of human bladder cancer treated with Adriamycin (doxorubicin), a standard agent used in the treatment of bladder cancer that has activity when administered intravesically and i.v. as a single agent and is a component of the only proven combination chemotherapy regimen (1–3). A novel formulation of interest (liposome-encapsulated doxorubicin) for which there is extensive preclinical experience was included that would allow validation in this model.

Received 11/15/96; revised 5/20/97; accepted 6/5/97.

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1 To whom requests for reprints should be addressed, at Department of Biophysics, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, NY 14263. Phone: (716) 845-5839; Fax: (716) 845-8899; E-mail: maz@tachy.med.buffalo.edu.

2 The abbreviations used are: MR, magnetic resonance; DOX, doxorubicin; RF, radio frequency; SE, spin echo; SPGR, spoiled gradient echo; Gd-DTPA, gadolinium diethylenetriamine-pentaacetic acid.
MATERIALS AND METHODS

Mice. Swiss CD1 athymic female mice (Charles River, Wilmington, MA) were used for tumor implantation. Animals were housed in a barrier-maintained facility in micro-isolator cages; cages, bedding, food, and water were autoclaved.

Orthotopic Xenografts. The UCRU-BL13 (BL13) human bladder cancer tissue culture cell line was originally developed from a pretreatment biopsy of an invasive stage I3, grade II transitional cell carcinoma (12). To initiate orthotopic tumors, an incision was made in the skin, and the bladder was exteriorized through an incision in the abdominal wall. BL13 cells (10^5) suspended in a volume of 10 μl were injected through a 30-gauge needle into the bladder wall. Tumor volumes were calculated from MR images as described below and at necropsy from caliper measurements made in three perpendicular directions (a, b, and c) using the formula:

Tumor volume = (a × b × c) × 0.5 mm^3

In preliminary experiments to determine MR image characteristics of tumor and urine, 1-mm pieces of solid BL13 xenograft tissue were transplanted onto the outer wall of the bladder using published on-plant procedures (13). This produced tumors that grew progressively from the bladder wall, protruding into the peritoneal cavity without compromising the bladder lumen.

Chemotherapeutic Agents. Adriamycin (DOX) as the hydrochloride salt (Sigma Chemical Co., St. Louis, MO) was dissolved in PBS and injected i.v. at 6 mg/kg, a dose based on previous experience (14). Liposome-encapsulated DOX (DOXIL; Liposome Technology Incorporated, Menlo Park, CA) was suspended in PBS and injected i.v. at 6 mg/kg DOX equivalence. Drugs, or equivalent volumes of PBS, were injected i.v. into groups of six mice on days 6 and 10 after tumor implantation. Iatrogenic deaths occurred in three mice, and three mice were euthanized following >20% loss of body weight, before the experiment was terminated 14 days after tumor implantation.

MR Imaging. All mice were imaged 4–6 days prior to tumor implantation and then 1–2 days prior to initial chemotherapeutic plan. Groups of two mice from each treatment group were imaged on days 7, 8, and 9 postimplantation, before the second injection of drug or vehicle on day 10 postimplantation. Two mice from each group were re-imaged on days 11, 12, and 13, before termination of the experiment on day 14. Thus, each mouse was imaged before tumor implantation, before each drug treatment, and then twice more before the experiment was terminated. Immediately before MR imaging, mice were anesthetized with ketamine/xylazine given i.p. at 0.1:0.01 mg/g and placed in a thermo-plastic positioning/restraining device (Tru-Scan Imaging, Annapolis, MD) molded to fit the contours of a mouse. This permitted acquisition of identical slices in repeat examinations. A tube containing 5 mm CuSO_4 was placed within the field-of-view and used as an image intensity calibration standard to ensure reproducibility and quantitation of signal intensities. MR imaging was obtained using a GE Signa Advantage 1.5 T whole body clinical MR imaging system with 5.5 system operating software purchased from General Electric Medical Systems (Milwaukee, WI) interfaced to a custom-designed slotted tube resonator small animal RF transceiver coil (15, 16). Construction of the whole-body murine RF transceiver coil was required for these experiments because it produced images with homogeneous RF fields and increased signal:noise ratios compared with standard clinical RF coils. The RF transceiver coil was manually tuned for optimal performance for each animal before data acquisition. T1 and T2 relaxation time measurements were performed in preliminary experiments using research software obtained from General Electric Medical Systems (4.X operating system; Ref. 17). T1 and T2 relaxation time results were then used to optimize data acquisition sequences. For T1 calculations, six values of TR were independently obtained (TE = 20 msec; TR = 4000, 2000, 1000, 500, 250, 100 msec) from SE image data sets and used to obtain an estimate of χ^2. For T2 calculations, seven values of TE were independently obtained (TR = 1000 msec; TE = 20, 40, 80, 120, 160, 200, and 240 msec) from SE image data sets using a Carr-Purcell-Meiboom-Gill pulse sequence (18). The best fit was accomplished by: (a) stepping through the values of T1 or T2; and (b) for each value of T1 or T2, solving the SE signal intensity equations (19) that yield the values that minimized χ^2 for that value of T1 or T2. An optimized MR pulse sequence was then determined by solving SE and gradient echo signal intensity equations using T1 and T2 relaxation times derived above for specific anatomical tissues. To optimize tumor-to-urine contrast and in-plane spatial resolution, a SPGR T1-weighted pulse sequence (TE = 9, TR = 240 ms, flip angle = 90°, 16 kHz bandwidth, 8 cm FOV (field of view), 1 NEX (number of excitations), and 256 × 256 matrix size) was selected to prescribe direct three-dimensional data sets in the coronal plane, yielding in-plane resolutions of 0.3125 mm^2 with a slice thickness of 0.7 mm in <33 min/mouse/acquisition.

MR Measurement of Tumor Volumes. MR image data were processed using a commercial medical image analysis program (Analyze, version 7.5.5; CNSoftware Ltd., West Sussex, United Kingdom). MR three-dimensional volumetric determinations were performed by approximating the tumor as a solid after manually defining tumor boundaries in each slice using a "seed growing" algorithm. Specifically, volumes were defined by: (a) selecting a pixel located within tumor; (b) connecting all adjacent pixels within a range of gray scale values visually observed to encompass the entire tumor; and (c) performing a pixel-by-pixel three-dimensional integration of voxels containing tumor. Data were processed using a SUN MVX 4/470 workstation.

Data Analysis. Tumor growth rates were determined from MR volumetric data as follows.

\[
\text{Tumor growth rate} = \frac{\text{Tumor volume on day } X}{\text{Pretreatment tumor volume}} \times 100
\]

Statistical significance of tumor growth rate differences between groups were analyzed using Student's t test.

RESULTS

T1 and T2 Relaxation Times. Fig. 1 illustrates the RF transceiver coil used for data acquisition of tumor-bearing animals. Table 1 summarizes the T1 and T2 relaxation times obtained for BL13 orthotopic xenografts, urine in the bladder, surrounding fat, and muscle. Relaxation times were used for pulse sequence optimization by maximizing tumor-to-urine con-
Fig. 1 Whole-body murine RF transceiver coil. A modified slot-ted-tube resonator coil was custom-designed and interfaced to a clinical whole-body MR scanner operating at 1.5 T.

Table 1  T1 and T2 relaxation times obtained for BL13 orthotopic xenografts and surrounding normal tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>T1 relaxation time* (msec)</th>
<th>T2 relaxation time* (msec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor</td>
<td>1416</td>
<td>92</td>
</tr>
<tr>
<td>Urine</td>
<td>2226</td>
<td>179</td>
</tr>
<tr>
<td>Fat</td>
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<td>49</td>
</tr>
<tr>
<td>Muscle</td>
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*Relaxation times were used to optimize tumor-to-urine conspicuity for tumor volume measurements.

In general, T1-weighted SE images yielded tumor hyperintense to urine, whereas T2-weighted images yielded tumor hypointense compared to urine. A T1-weighted SPGR pulse sequence was chosen because it permitted direct three-dimensional data acquisition, higher signal-to-noise per unit time, and increased spatial resolution compared with T2-weighted SE or gradient echo pulse sequences. Moreover, preliminary experiments with BL13 tumors transplanted onto the outer wall of the bladder (on-plant), using Gd-DTPA to improve tumor-to-urine contrast, yielded increased signal intensity of urine compared with tumor. However, Fig. 2 illustrates the nonuniform enhancement of urine observed due to concentration gradients of Gd-DTPA within the bladder (20).

**MR Imaging.** Baseline T1-weighted MR image results obtained from all mice prior to tumor initiation demonstrated features typical of normal bladder. Delineation of normal bladder wall and adjacent muscle was not observed at the reported field strength, resolution, and pulse sequence. By day 4 postimplantation, localized thickening of the bladder wall was detectable and appeared homogeneously hyperintense on T1-weighted SPGR images, compared with urine within the bladder. Pretreatment tumor volumes ranged from 2–10 mm³ and increased to a mean volume of 474 ± 231 mm³ by day 14 postimplantation for untreated tumors.

Fig. 3 illustrates typical pretreatment MR images seen in coronal sections of an entire mouse (Fig. 3A) and magnified tumor showing thickening of bladder wall emanating radially from regions adjacent to the focal tumor mass on day 4 postimplantation (Fig. 3B). Generally, orthotopic tumors were observed as homogeneously hyperintense masses compared with urine and were relatively isointense compared with muscle. Tumors grew as focal lesions into the lumen of the bladder with concomitant thickening of the bladder wall in regions adjacent to the primary mass. Tumor growth continued until mice were euthanized, due to hematuria and loss of body weight, or termination of the experiment on day 14 postimplantation. Typical MR images are illustrated in Fig. 3C for control animals on days 4–13 postimplantation. In all cases, coronal sections obtained approximately midline through the tumor (largest diameter) are shown for comparison. T1-weighted images of tumor masses were unremarkable in appearance except for localized regions within individual tumors consistent with foci of necrotic tissue, pockets of urine, and/or hemorrhage that could not be differentiated in these experiments.

**Direct Measurement of Tumor Volume.** Final tumor volumes calculated from caliper measurements made at necropsy are illustrated in Fig. 4. Both DOX and DOXIL-treated tumors demonstrated significant therapeutic effect against orthotopic BL13 tumors. DOXIL treatment reduced tumor volumes by approximately 33% (P < 0.005 versus control), whereas treatment with free DOX was more than twice as effective as DOXIL (P < 0.002, DOX versus DOXIL), resulting in a 70% reduction in tumor volume (P < 0.005, DOX versus control).
Fig. 2  T1-weighted SPGR three-dimensional volume acquisition of a BL13 tumor 8 days after tumor on-plant shown in a coronal section both before (A) and after administration of a contrast-enhancing agent, Gd-DTPA. a, tumor protruding into the peritoneal cavity; b, bladder containing urine; and c, urine within bladder after Gd-DTPA administration. Clearly shown in B is “pseudolayering” of Gd-DTPA within the bladder, resulting in nonuniform enhancement of urine in bladder after Gd-DTPA administration (20).

Fig. 3  A, coronal section of three-dimensional MR data set representative of orthotopic bladder cancer xenografts shown 4 days postimplantation, prior to treatment; a, tumor; b, heart; c, lungs; d, liver. B, magnified image from Fig. 1A depicting high-resolution MR image with excellent tumor-to-urine/bladder lumen conspicuity. C, typical T1-weighted three-dimensional SPGR coronal MR images of tumor developing within urine-filled bladder as a function of time postimplantation (Day 4, Day 9, and Day 13).
Fig. 4 Comparison of tumor volumes calculated from caliper measurements made at necropsy with tumor volumes measured from MR images made on the same days (means) in mice treated with DOXIL, DOX, or PBS (Control); bars, SE.

<table>
<thead>
<tr>
<th>Data set</th>
<th>Observer 1</th>
<th>Observer 2</th>
<th>Observer 3</th>
<th>Mean</th>
<th>SD</th>
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</thead>
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<tr>
<td>Day 9</td>
<td>161</td>
<td>174</td>
<td>155</td>
<td>163</td>
<td>10</td>
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<tr>
<td>Day 13</td>
<td>528</td>
<td>605</td>
<td>580</td>
<td>571</td>
<td>39</td>
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<td>Day 14</td>
<td>803</td>
<td>783</td>
<td>760</td>
<td>782</td>
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</tbody>
</table>

Table 2 Interobserver variation in quantitation of tumor volumes from MR image data

MR data acquired for orthotopic control tumor.

Fig. 5 Individual orthotopic BL13 tumor volumes calculated from MR images taken at intervals before and after treatment on days 6 and 10 postimplantation (arrows).

**Tumor Volume by Noninvasive MR Measurement.**

Results of MR imaging data are compared with final tumor volumes determined from caliper measurements in Fig. 4. The data not only confirm the therapeutic efficacy of DOX and DOXIL but also show that MR volume measurements are comparable with direct physical measurements and, therefore, can be used to construct valid tumor growth curves.

Individual tumor volumes measured from MR data obtained throughout tumor growth are illustrated in Fig. 5. In general, tumor boundaries were readily identified due to the high tumor-to-urine contrast. Segmented tumor boundaries were visually assessed for accuracy on an image-by-image basis, and seed-growing parameters were modified as necessary for tumor definition using identical criteria. To determine reproducibility, independent measurements were made in a blinded fashion by three observers on each of three data sets from a control animal. The results in Table 2 show that interobserver differences were
not statistically different, with SD of 2.8, 6.0, and 6.9% for the mean tumor volumes calculated independently for the three data sets.

Data from Fig. 5 were used to calculate tumor growth rates for each treatment group, which are shown in Fig. 6. The results demonstrate that bladder tumors could be imaged 4 days after initiation. Also, a response to DOX therapy could be detected 2 days after the first treatment and by 1 day following the second DOX injection. MR measurements showed that the therapeutic response to DOXIL was delayed and less marked compared with free DOX. Furthermore, MR measurements were able to show that tumor regrowth began 2 days after the second treatment with both agents.

**DISCUSSION**

Orthotopic xenografts represent a useful model for preclinical studies of tumor development and response to treatment (5-11). Orthotopic implantation allows interaction of tumor cells with organ site-dependent microenvironmental factors, including host vasculature, stroma, and growth factors such as epidermal growth factor and vascular endothelial growth factor, which can modulate tumor growth patterns, progression, and metastatic potential (5, 6, 21, 22).

In common with tumors at other visceral sites, experimental bladder cancers are difficult to assess for noninvasive, serial monitoring of treatment response, unless approaches such as MR are used. In this study, MR imaging techniques were developed and used to monitor noninvasively and to longitudinally assess the growth kinetics of BL13 orthotopic xenografts. The natural history of bladder tumor growth seen in this study (thickening of the bladder wall, extension of the tumor into the bladder lumen, and muscle invasion) is typical of the clinical course of invasive human bladder cancers and also reflects that observed in diagnostic MR images of human bladder cancer. Moreover, BL13 tumors demonstrated therapeutic responses similar to those observed in the clinic (i.e., objective response in 10%-41% of cases) after systemic DOX administration. BL13 tumors also responded to liposome-encapsulated DOX, albeit to a lesser extent, a result we had not anticipated and which will be characterized in future studies that apply this technique, as well as in vitro models.

Although the numbers of “cases” treated in this study are small, the SD are tight and results are statistically significant. Where individual tumors were not all re-measured at each time point because of constraints imposed by machine time and repeated anesthesia, no likely biological variability would have been anticipated with dramatic changes within each time frame. Although microenvironmental factors appear to be important in tumor growth and progression, there is limited information about treatment response at various anatomical sites. Clinically, bladder cancers that have metastasized to different organs show site-associated differences in response to chemotherapy. For example, patients with liver and bone metastases respond less than those with lymph node or pulmonary metastases (3, 23). The BL13 xenograft is a useful model for studying this variability in response because s.c. BL13 xenografts are unresponsive to DOX (14), whereas we have now shown that orthotopic tumors respond well. The reasons for this difference in this model will be the subject of detailed future study.

The present study demonstrates that high resolution MR images can be used for accurate volumetric measurements and can detect orthotopic bladder cancer xenografts calculated to be ≤10 mm³. We have shown that noninvasive MR volumetric determinations can be performed longitudinally as a function of time to construct classical tumor growth curves. Therefore, MR can be used as a tool to evaluate therapeutic impact in terms of tumor growth rate and has significant potential to optimize treatment schedules. Translation of the MR techniques used in this study into clinical diagnostic evaluations would allow optimization of individual treatment schedules and facilitate assessment of therapeutic response.

**ACKNOWLEDGMENTS**

We acknowledge the technical assistance of Michael K. Murray in animal experimentation and MR image processing.
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