Ultrasonic Imaging of Tumor Angiogenesis Using Contrast Microbubbles Targeted via the Tumor-Binding Peptide Arginine-Arginine-Leucine


Introduction

Angiogenesis is a requirement for the progression of solid tumors to a malignant, metastatically competent state (1). Specific endothelial molecular markers of angiogenesis are expressed in tumor vasculature, a fact that has several important clinical implications. To the extent that these markers correlate with tumor size and metastatic potential (2, 3), their identification in vivo can provide diagnostic and prognostic information, a mechanism for tumor-specific treatment, and a basis for tracking treatment response. Agents that bind to tumor angiogenesis–specific markers could thus provide a basis for molecular imaging and drug delivery. For example, the integrin αvβ3, which is selectively expressed on angiogenic endothelium (4), has been targeted for the purposes of imaging and drug delivery using magnetic liposomes (5) or echogenic microbubbles (MB; ref. 6) and particles loaded with therapeutic agent (7).

Numerous small peptides have been identified that specifically interact in vivo with various types of endothelial cells (EC), including angiogenic endothelium (8, 9). Tumor vasculature in particular has been targeted using angiogenesis-specific peptides containing asparagine-glycine-arginine (NGR), arginine-glycine-aspartate (RGD), or histidine-tryptophan-glycine-phenylalanine (HWGF; refs. 10–12). Using a peptide display library, Brown et al. identified various tumor vasculature–specific binding sequences, including the tripeptide arginine-arginine-leucine (RRL; ref. 13).

Contrast-enhanced ultrasound is a recently developed imaging technique that utilizes i.v. injected, gas-filled, acoustically active MBs that transit unimpeded through the microcirculation, acting as RBC tracers (14, 15) during ultrasound imaging. Recently, these MBs have been modified to adhere to specific endothelial surface epitopes, allowing for the ultrasonic detection of these molecular epitopes. For example, our group has showed that MBs targeted to the endothelial inflammatory marker intercellular adhesion molecule 1 (ICAM-1) selectively bind to cultured endothelial cells overexpressing that marker (16), and that these MBs can be used to echocardiographically detect acute cardiac allograft rejection in vivo (17).

Based on the above considerations, in the current study we hypothesized that ultrasound contrast MBs targeted to tumor vasculature via conjugation with the tumor-binding peptide RRL would preferentially adhere in vitro to tumor-derived versus normal endothelium, and that this selective binding phenomenon would result in increased contrast enhancement during in vivo ultrasound imaging of various solid tumors as compared with normal control myocardium in a murine model.

Materials and Methods

RRL Tumor-Binding Peptide

The tripeptide sequence RRL is a tumor EC-specific binding peptide previously identified using an in vitro bacterial peptide display library panned against tumor cells derived from SCC-VII murine squamous cell carcinomas (13).

Custom-synthesized 9-mer cyclic peptides contained either the RRL sequence or a glycine control sequence (Genemed Synthesis, South San
Francisco, CA). The RRL-containing cyclic peptide comprised the RRL sequence bracketed by glycines and terminated on both ends with cysteine residues (CGGRRLLGCC). The control peptide was identical except with the substitution of glycines for the RRL sequence (CGGGGGGGGC). Disulfide bonds between the terminal cysteines on each peptide maintain the cyclic structure. Both cyclic peptides were biotinylated at the amino-terminal cysteine.

**Microbubble Preparation**

Phospholipid-based ultrasound contrast MBs were prepared as previously described (18, 19). Briefly, perfluorobutane gas was dispersed by sonication in a solution of phosphatidyl choline, polyethylene glycol stearate, and biotinylated phosphatidylethanolamine. The resulting product comprised a perfluorobutane gas bubble encapsulated by a biotin-containing phospholipid shell.

The MBs were conjugated to the cyclic peptides via avidin/biotin bridging chemistry. Biotinylated MBs were incubated in a saturating solution of streptavidin (Molecular Probes, Eugene, OR). Unbound streptavidin was removed by washing and centrifugation. The MBs were then incubated in a saturating solution of cyclic peptide (100 μg/mL; either RRL or control) and washed again.

MBs linked to the RRL-containing peptide were designated MB_{RRL}, and those linked to the glycine control peptide were designated MB_{control}.

**Microbubble diameter as measured by electrozone sensing (Multisizer-II, Beckman-Coulter, Fullerton, CA) was 3.2 ± 0.1 μm.** The MB synthesis protocol used excess quantities of RRL peptide that ensured saturation of the MB surface with a maximum amount of RBL peptide, which, based on prior experience with MBs, is on the order of 60,000 peptides per MB (17).

The MBs show no significant toxicity, and animals are routinely recovered without difficulty following contrast ultrasound imaging (17, 20, 21). The agent does not initiate inflammation or microvascular obstruction. Biodistribution of the MBs follows the distribution of blood flow in vivo (14).

**In vitro Studies**

**Cell Culture.** Tumor-derived ECs selectively isolated by collagenase digestion from s.c. SCC-VII squamous cell carcinomas grown in C3H/Hej mice (22) (7th to 8th passage) and human coronary artery ECs (3rd to 5th passage; Cambrex Corp., East Rutherford, NJ) were grown to confluence on glass coverslips in endothelial basal medium (Cambrex Corp.) supplemented with 5% fetal bovine serum. The tumor-derived cells were chosen because they had been used for the original identification of RRL (13) and thus had known binding affinity with RRL. Human coronary artery ECs were chosen to represent normal endothelium; our laboratory has experience manipulating their inflammatory status and investigating targeted MB adhesion to these cells (16, 17, 19).

**In vitro Perfusion Protocol.** A previously described rectangular parallel plate perfusion chamber (19, 23) was used to examine adhesion of MBs to cultured tumor–derived and normal ECs. Coverslips of ECs (n=6–8 per condition) were briefly incubated with quinacrine dihydrochloride (50 μmol/L) as a fluorescent label, mounted in the chamber, fastened with a vacuum seal, and primed with culture medium at 37°C. The coverslip was then incubated in a saturating solution of biotinylated cyclic peptide (either RRL or the control sequence GGG; Sigma-Genosys, The Woodlands, TX) and washed again. Unbound biotin was removed by washing and centrifugation. The MBs were then incubated in a saturating solution of biotinylated streptavidin (Molecular Probes, Eugene, OR). Unbound streptavidin was removed by washing and centrifugation. The MBs were then incubated in a saturating solution of biotinylated cyclic peptide (100 μg/mL; either RRL or control) and washed again.

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To create solid tumors, 2 × 10⁶ PC3 or Clone C tumor cells were injected s.c. into the flanks of athymic nude mice and allowed to expand for 6 weeks until the tumors were ~0.5 cm³. PC3 cells are derived from metastatic human prostate carcinoma (CRL-1435, American Type Culture Collection, Rockville, MD; ref. 24). Clone C cells are originally derived from murine NIH3T3 stable transfectants, engineered to secrete high levels of fibroblast growth factor 1 (25).

**Histologic Confirmation of In vivo RRL Binding.** Previous in vitro pilot studies suggested that RRL becomes bound and internalized from the endothelial surface (data not shown). We wanted to extend these studies to show in vivo tumor localization of i.v. injected RRL, prior to i.v. MB perfusion imaging. Accordingly, NH₂-terminal fluoresceinated peptides containing either RRL or the control sequence GGG (CGGRRLLGCC and CCGGGGGGC; Sigma-Genosys, The Woodlands, TX) were injected via the tail vein (1 μg in 0.2 mL saline) at hourly intervals for a total of five injections into athymic mice bearing PC3 or Clone C tumors. Fifteen minutes after the last injection, the mice were sacrificed and their vasculature flushed with saline. The heart, lung, kidney, liver, spleen, intestine, and tumors were harvested and frozen in ornithine carbamyl transferase. Unstained cryosections were observed for fluorescein localization. Tumor blood vessels were identified as luminal structures containing blood cells visualized under phase contrast/differential interference contrast microscopy. Digital images were acquired and rendered under identical settings and conditions.

**Contrast-Enhanced Ultrasound.** Ultrasound imaging used a HDI-5000 ultrasound system and broadband L12-5 transducer (Philips Medical, Bothell, WA) in pulse inversion mode. Images were acquired with a mechanical index (a measure of acoustic output energy) of 0.6, which induces the MB destruction that creates a measurable backscatter signal (26). A small plastic bag of water was positioned as an acoustic interface between the ultrasound probe and the mouse flank. Probe position, gain settings, and offset focus were initially optimized and maintained throughout each experiment. Time-triggered images (up to four frames) of the tumor or myocardium were acquired at baseline (preinjection), 120 seconds, and 135 seconds after injection of MBs. Images were acquired within the linear range of the ultrasound system and using a linear postprocessing map, such that image videointensity (brightness) was proportional to the number of MBs in the ultrasound field. Images were recorded digitally and analyzed offline, and average pixel intensity (videointensity) was measured in regions of interest encompassing either the tumor or the left ventricle. Background-subtracted images were color-coded using a map in shades of red, progressing to orange, yellow, then white, representing increasing videointensity change (greater MB adhesion).

The goal of the ultrasound image protocol and analysis was to differentiate between the acoustic signal due to adhered MBs and the signal due to MBs still freely circulating in the bloodstream. A previously described method was used to isolate the acoustic signal due to adhered MBs (17, 20, 26). In pharmacokinetic studies of MB contrast agents using intravital microscopy of rat cremaster muscle microvasculature,5 we have consistently observed that by 120 seconds after i.v. MB injection, <2% of the original contrast remains in the circulation. In addition, in pilot imaging studies of mice receiving equivalent doses of a standard, nontargeted contrast agent (Optison, Amersham Health, Princeton, NJ), tumor videointensity was no longer detectable by 120 seconds after injection, suggesting that minimal MBs remained freely circulating after this period. Because MB destruction is required to detect MB presence (acoustic signal), imaging was suspended for 120 seconds after injection to allow for accumulation of MB binding while awaiting washout of unbound MBs. The contrast enhancement in the ultrasound image at 120 seconds should thus derive predominantly from adherent MBs, whereas any contrast at 135 seconds should be attributable to any persistently circulating MBs replenishing the beam in the intervening 15 seconds. Thus, the "videointensity difference" between the 120-second frame (adhered + circulating MBs) and the 135-second frame (predominantly circulating MBs) was defined as

Targeted Ultrasound Imaging of Tumor Angiogenesis

Results

In vitro Targeted MB Binding. Figure 1 shows brightfield micrographs of coverslips of tumor-derived and normal ECs perfused with MB\textsubscript{RRL} and MB\textsubscript{Control}. MBs targeted via the RRL peptide adhered preferentially to tumor-derived ECs (Fig. 1A) versus normal (Fig. 1C) endothelium. Glycine control MBs adhered minimally to both cell types (Fig. 1B and D). The extent of MB adhesion is summarized in Fig. 2. Adherence of MB\textsubscript{Control} was minimal to both tumor-derived and control ECs (0.4 ± 0.2 and 0.3 ± 0.1 MBs/cell). Adherence of MB\textsubscript{RRL} was three to six times greater than MB\textsubscript{Control} (P < 0.01), and was significantly greater to tumor-derived than control ECs (2.4 ± 0.6 versus 0.8 ± 0.1 MBs/cell, P < 0.01).

Tumor Localization of Intravenously Injected RRL. Cryosections from mice given i.v. injections of fluoresceinated RRL peptide showed localization on the tumor vasculature for both Clone C and PC3 tumors (Fig. 3). In Clone C tumors, which are highly angiogenic, the fluorescent signal outlined the endothelial layer and the area immediately deep to it. PC3 tumors possess fewer vessels, and fluorescence was found almost exclusively on the endothelial surface. The control peptide did not show appreciable intratumor accumulation in either tumor type, even at digital camera sensitivity 3-5 times above that used for RRL.

With the exception of the kidneys, no definable structure-related fluorescence was observed in the heart, lung, liver, spleen, or gastrointestinal tract. Renal glomeruli and tubules showed fluorescence, consistent with the observation that the urine was similarly colored.

In vivo Targeted Ultrasound Imaging. Of the 21 animals studied, 4 tumor-bearing mice were excluded from the study due to inadequate tumor perfusion as qualitatively visualized during the initial Optison imaging.

Figure 4 shows representative background-subtracted, ultrasound images of two different tumors in two mice. The topmost panels show images of a Clone C tumor obtained 120 seconds after i.v. injection of MB\textsubscript{RRL} (Fig. 4A) or MB\textsubscript{Control} (Fig. 4B). Corresponding images from a PC3 tumor in another mouse are shown for MB\textsubscript{RRL} (Fig. 4C) and MB\textsubscript{Control} (Fig. 4D). There was intense contrast enhancement (acoustic signal) in both tumors after injection with MB\textsubscript{RRL} (Fig. 4A and C). The corresponding images for MB\textsubscript{Control} (Fig. 4B and D) showed only mild contrast enhancement.

With the PC3 tumor, the opacification due to MB\textsubscript{RRL} (Fig. 4C) was concentrated in the periphery of the tumor, whereas the central tumor core was not enhanced. Histologic images of a sectioned PC3 tumor are shown in Fig. 4E-G, demonstrating that the density of tumor neovasculature was highest at the tumor periphery.

Representative ultrasound images of normal myocardial tissue in a third mouse are also shown in Fig. 4H and I. There was little difference in videointensity resulting from the two types of MBs, suggesting that there was only minimal adhesion of the RRL-bubble to normal microvasculature. For all image sets, there was no significant tissue opacification 15 seconds later in any of the injections (images not shown), confirming that there were few remaining circulating MBs, and indicating that the acoustic signal at 120 seconds was due predominantly to adhered MBs.

Quantitative videointensity data are shown in Fig. 5. Each line represents a single mouse, with the first point representing the videointensity value (indicative of the number of adhered bubbles) from the MB\textsubscript{Control} injection, and the second point
representing the MB<sub>RRL</sub> injection. There was little difference in myocardial signal intensity between MB<sub>Control</sub> and MB<sub>RRL</sub>. There was a substantial increase in signal in all but one of the tumor-bearing mice when using MB<sub>RRL</sub> versus MB<sub>Control</sub>, indicating specific retention of the tumor-targeted agent in the tumor microvasculature.

A summary of the mean Vi<sub>Targeted</sub> data (defined above) for all 17 mice is shown in Fig. 5. The mean Vi<sub>Targeted</sub> for normal myocardium was 0.5 ± 1 intensity units, which was not significantly higher than zero, whereas that for the tumors was 5 ± 1 intensity units, which was both significantly higher than zero and significantly higher than normal tissue (P = 0.001). These findings are consistent with the preferential adhesion of the RRL-targeted agent to tumor microvasculature versus normal myocardium.

**Discussion**

In this study we report the noninvasive identification of tumor angiogenic vasculature in vivo using targeted ultrasound contrast imaging. Our main finding was that ultrasound contrast MBs targeted via the tumor vasculature-binding peptide RRL preferentially adhered to and acoustically illuminated angiogenic tumor microvasculature versus normal tissue. Our data support the hypothesis that MB<sub>RRL</sub> selectively adhere to tumor-derived versus normal endothelium, and that this adhesion causes differential contrast enhancement of tumors during ultrasound imaging. Furthermore, the in vivo fluorescent RRL studies confirm the endothelial localization of the RRL peptide and explain the intravascular binding of i.v. injected MB<sub>RRL</sub>. These data have implications for the development of a contrast-enhanced ultrasound technique that may enable the noninvasive detection and characterization of tumor angiogenesis and the monitoring of antitumor and antiangiogenic therapy.

**Targeted MB Adhesion to Tumor Endothelium.** The in vitro experiments used a parallel plate perfusion chamber, which has been widely utilized to study cellular adhesive events under controlled shear conditions (27, 28). MBs targeted via RRL selectively bound to cultured tumor–derived but not normal ECs under physiologically relevant shear conditions. In comparison, MBs conjugated to a control glycine peptide (MB<sub>Control</sub>) showed minimal adherence to both cell types, suggesting specific binding interactions between the RRL sequence and its ligand on the endothelial surface. MB<sub>Control</sub> binding was nonetheless greater than zero, which may indicate a degree of nonspecific adhesion by the cyclic peptide. The magnitude of this effect, however, was small compared with the specific RRL adhesive interaction, suggesting that neither the lipid components of the MB shell nor presence of avidin and biotin moieties contributed significantly to the observed adhesion.

**In vivo Localization of RRL.** The fluorescent RRL studies showed for the first time that this peptide preferentially adheres to tumor vasculature of both Clone C and PC3 tumors in vivo. Furthermore, tagged RRL localization was not associated with native organs, except for some binding to renal tubules and glomeruli. These histology data suggest that the target of RRL, albeit as yet unidentified, is not only tumor specific but also endothelial in location. This is key because the endoluminal location of the target is a requirement for the binding of targeted MBs, which remain exclusively within the intravascular space. In addition, the target of RRL is accessible by injecting the peptide through a peripheral vein, as would be the route of MB injection, and binds to its target under the physiologic shear conditions to which MB<sub>RRL</sub> would be exposed. These findings, together with the in vitro data, offered a basis for interpreting the ultrasound imaging data in terms of specific microbubble-endothelial interactions.

**Ultrasound Imaging of Tumor Angiogenesis.** MBs targeted via RRL generated enhanced ultrasound opacification of tumors relative to normal myocardial tissue, which parallels our in vitro data showing greater adhesion of MB<sub>RRL</sub> to tumor endothelium.
than to normal endothelium. There was slight contrast enhancement of MBControl to both tissue types (Fig. 4B, D, and I), which is consistent with our in vitro observation that some MBControl adhere to endothelial cells, possibly due to nonspecific interactions between the control peptide and the endothelium, as discussed above. Alternatively, attachment of MBControl may represent MB binding to activated, endothelium-adherent leukocytes, as has previously been shown in models of acute inflammation (21). Regardless of the mechanism, the magnitude of contrast enhancement resulting from nonspecific attachment was significantly lower than that generated by MBRRL attachment.

As seen in Fig. 4, the pattern of tumor opacification often showed higher contrast enhancement in the tumor periphery than the core. This spatial distribution could indicate that the tumor periphery is more angiogenic, expressing a higher density of the endothelial tumor-specific ligand for RRL than the tumor core. However, because the initial Optison images delineated a similar pattern of perfusion, we believe that the opacification is matching the anatomic pattern of the tumor vasculature, with a highly vascularized tumor periphery and a necrotic tumor core. This hypothesis was validated by histologic examination of harvested tumor specimens (Fig. 4E-G), which showed large vessels predominantly in the tumor periphery, with necrosis and poor vascularity in the tumor center.

Comparison to Previous Studies. Previously reported techniques for the noninvasive imaging of tumor angiogenesis rely on anatomic measurements of tumor size, blood flow, or vascular permeability (29–31), whereas others have used molecular imaging techniques to assess tumor endothelial-specific molecular markers (5, 32, 33). These functional imaging techniques most often utilize ligands (usually peptides) that bind to tumor- or angiogenesis-specific endothelial markers that are visualized by radiolabeling or conjugation to a contrast agent. Examples include magnetic resonance imaging using anti-αvβ3 antibody-bearing contrast agents (5), positron emission tomography using RGD-bearing 18F-labeled glycopeptides (32), and 99m technetium-labeled RGD peptide (33).

Contrast-enhanced ultrasound has some advantages over other imaging modalities for the functional assessment of vascular beds in vivo. Magnetic resonance imaging generally offers good spatial resolution but currently has a relatively poor signal-to-noise ratio, translating to low sensitivity (34). Scintigraphic methods generally yield high sensitivity but suboptimal spatial

![Figure 4](https://www.aacrjournals.org/)

**Figure 4.** A, background-subtracted, color-coded ultrasound image taken 120 seconds after injection of MBs conjugated to RRL (MBRRL) into a mouse bearing a Clone C tumor. Within the colored areas, gradations from red to orange to yellow to white denote greater signal enhancement by contrast material. Non-color coded portions are not background subtracted and do not influence the videointensity data. MBRRL resulted in greater contrast enhancement. B, corresponding image for MBs conjugated to a glycine control peptide (MBControl) in the same mouse as A. C and D, similar ultrasound images as in A and B, but from a mouse with a PC3 tumor. E, collage of high-resolution photomicrographs taken of a midline PC3 tumor section immunohistochemically stained for factor VIII, showing localization of the microvasculature predominantly to the periphery of the tumor. Cells are counterstained with hematoxylin. Some expected shrinkage has occurred secondary to formalin fixation. Original magnification ×20. F and G, high magnification of selected areas of image in E. H and I, similar ultrasound images as in A and B but of normal myocardium (short-axis view of left ventricle).
resolution (35). Both magnetic resonance imaging and positron emission tomography are hindered by their requirement for instrumentation typically limited to tertiary care centers. Conventional, noncontrast ultrasound can image only the larger vessels and has limited utility for assessing microvascular tumor angiogenesis (35). Conversely, contrast-enhanced ultrasound has good spatial resolution, is portable, widely available, offers real-time imaging, and uses contrast agents that remain exclusively intravascular, minimizing nonspecific signals from extravasated contrast.

Targeted contrast ultrasound has previously been utilized to detect vascular disease, including thrombus using fibrinogen-targeted MBs (36), inflammation using MBs targeted to P-selectin (37) or ICAM-1 (16), and acute cardiac transplant rejection by targeting ICAM-1 (17).

Contrast-enhanced ultrasound has only recently been investigated as a tool for imaging angiogenesis. Leong-Poi et al. reported that MBs bearing anti-integrin antibodies adhered to fibroblast growth factor–stimulated vessels overexpressing \(\alpha_v\) integrins \(\text{in vivo}\) (6). We advance upon this work, first, by using short cyclic peptide rather than animal-derived antibodies, which are costly and immunogenic, and second, by investigating tumor neovasculature rather than nontumor laboratory models of generalized angiogenesis. Our study is thus unique in that we targeted ultrasound contrast MBs to tumor vasculature using a clinically relevant \(\text{in vivo}\) model of neoplasia and a targeting moiety (RRL) raised specifically for tumor endothelium. Furthermore, we were able to show specific adherence of the targeted agent to tumor versus normal vasculature.

Limitations. Limitations of the parallel plate studies are those inherent to \(\text{in vitro}\) experiments in general. Cultured ECs are an imperfect model for \(\text{in situ}\) endothelium. Perfusion flow was nonpulsatile and included no blood components. Tumor-derived and normal ECs were of different species (murine versus human); however, we have observed that RRL does species cross-react with some human tumors, including sarcomas and prostate tumors such as PC3.\(^7\) RRL may show differential binding interactions between different tumor types (as seen in Fig. 4), and the RRL peptide may internalize beyond the endothelial lining, as the extravascular fluorescence in Fig. 3 may indicate. The molecular target for the tumor-binding peptide RRL is as yet unknown; the identification of the specific target is beyond the scope of this proof-of-principle study. Preliminary investigations into this issue indicate that RRL is likely not a standard endothelial inflammatory marker, as its binding to ECs \(\text{in vitro}\) does not increase with inflammatory stimulation (data not shown).

**Summary.** Our data show the ability of targeted contrast-enhanced ultrasound to noninvasively detect tumor angiogenesis \(\text{in vivo}\). Contrast MBs targeted to tumor vasculature via conjugation with the tumor-binding peptide RRL were shown to preferentially bind \(\text{in vitro}\) to tumor-derived cultured endothelium. Furthermore, we demonstrate the noninvasive, \(\text{in vivo}\) ultrasonic detection of angiogenic tumor vasculature in a tumor-bearing mouse model and showed that this technique could distinguish between normal tissue and tumor tissue.

Such ultrasonic molecular imaging of tumor angiogenesis may permit the functional assessment of tumor vasculature in the clinical setting and could thus offer noninvasive identification of tumors, assessment of malignant potential, and improved monitoring of tumor response to antiangiogenic therapies.

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