Published in final edited form as:

Invest Radiol. 2011 March ; 46(3): 187–195. doi:10.1097/RLI.0b013e3181f9202d.

Assessment and Monitoring Tumor Vascularity With Contrast-Enhanced Ultrasound Maximum Intensity Persistence Imaging

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Abstract

Objectives—Contrast-enhanced ultrasound imaging is increasingly being used in the clinic for assessment of tissue vascularity. The purpose of our study was to evaluate the effect of different contrast administration parameters on the in vivo ultrasound imaging signal in tumor-bearing mice using a maximum intensity persistence (MIP) algorithm and to evaluate the reliability of in vivo MIP imaging in assessing tumor vascularity. The potential of in vivo MIP imaging for monitoring tumor vascularity during antiangiogenic cancer treatment was further evaluated.

Materials and Methods—In intraindividual experiments, varying contrast microbubble concentrations (5×10^5 , 5×10^6 , 5×10^7 , 5×10^8 microbubbles in 100 μ L saline) and contrast injection rates (0.6, 1.2, and 2.4 mL/min) in subcutaneous tumor-bearing mice were applied and their effects on in vivo contrast-enhanced ultrasound MIP imaging plateau values were obtained using a dedicated small animal ultrasound imaging system (40 MHz). Reliability of MIP ultrasound imaging was tested following 2 injections of the same micro-bubble concentration (5×10^7 microbubbles at 1.2 mL/min) in the same tumors. In mice with subcutaneous human colon cancer xenografts, longitudinal contrast-enhanced ultrasound MIP imaging plateau values (baseline and at 48 hours) were compared between mice with and without antiangiogenic treatment (anti-vascular endothelial growth factor antibody). Ex vivo CD31 immunostaining of tumor tissue was used to correlate in vivo MIP imaging plateau values with microvessel density analysis.

Results—In vivo MIP imaging plateau values correlated significantly ($P = 0.001$) with contrast microbubble doses. At 3 different injection rates of 0.6, 1.2, and 2.4 mL/min, MIP imaging plateau values did not change significantly ($P = 0.61$). Following 2 injections with the same microbubble dose and injection rate, MIP imaging plateau values were obtained with high reliability with an intraclass correlation coefficient of 0.82 (95% confidence interval: 0.64, 0.94). In addition, in vivo

MIP imaging plateau values significantly correlated ($P = 0.01$; $R^2 = 0.77$) with ex vivo microvessel density analysis. Tumor volumes in treated and nontreated mice did not change significantly ($P = 0.22$) within 48 hours. In contrast, the change of in vivo MIP imaging plateau values from baseline to 48 hours was significantly different ($P = 0.01$) in treated versus nontreated mice.

Conclusions—Contrast-enhanced ultrasound MIP imaging allows reliable assessment of tumor vascularity and monitoring of antiangiogenic cancer therapy in vivo, provided that a constant microbubble dose is administered.

Keywords

maximum intensity persistence; colon cancer; ultrasound; microbubbles; antiangiogenic treatment

With the advent of novel antiangiogenic drugs, there is an increasing need to noninvasively image the tumor vasculature in cancer patients. Traditional morphologic-anatomic imaging strategies often fail in monitoring treatment effects because novel antiangiogenic drugs change tumor vascularity before differences in tumor sizes become visible.¹ Because antiangiogenic drugs are expensive and can be accompanied with substantial side effects decreasing the quality of life of patients, noninvasive imaging strategies that allow quantification of antiangiogenic drug treatment effects early on before overt morphologic-anatomic changes of the tumors become visible are highly desirable to aid in development of new drugs and to better individualize treatment plans in cancer patients.^{2,3}

Several imaging approaches have been proposed to assess tumor vascularity and perfusion, including dynamic magnetic resonance imaging (MRI),^{4–6} perfusion computed tomography (CT) imaging,^{5,7–9} and contrast-enhanced ultrasound imaging.^{5,7,10–14} Although ultrasound is not a whole-body imaging approach and is limited by a rather small field-of-view compared with CT or MRI, it has a great potential as a relatively inexpensive, high-throughput, and noninvasive imaging approach for screening the effect of new antiangiogenic drugs in preclinical animal studies.³ Furthermore, when focusing on ultrasonographically accessible target lesions such as liver metastases, quantification of tumor vascularity with ultrasound may be used for monitoring antiangiogenic therapy in the clinic without the need of ionizing irradiation and using ultrasound contrast agents that can be administered in patients with renal insufficiency.¹⁵

In contrast-enhanced ultrasound maximum intensity persistence (MIP) imaging following intravenous administration of contrast microbubbles, microbubble trajectories are mapped by displaying image pixels at their maximum brightness, creating a detailed visual map of the tumor vasculature. Once the contrast reaches equilibrium in the blood, the ultrasound signal plateaus to a maximum signal intensity value (henceforth, this is called as MIP imaging plateau values), which is used to assess tumor vascularity.¹⁶ Although there are some initial reports on using MIP to qualitatively assess tumor vascularity,^{16,17} the reliability of MIP imaging to quantify antiangiogenic treatment response is unknown; as is the relation between the imaging signal intensity and the concentration and injection rate of the ultrasound contrast agent.

The purpose of this study was to assess the effect of both concentration and injection rates of ultrasound contrast micro-bubbles on the in vivo ultrasound MIP imaging plateau values in tumor-bearing mice and to evaluate the reliability of in vivo MIP imaging in assessing tumor vascularity. Furthermore, the potential of in vivo MIP imaging for monitoring tumor vascularity during antiangiogenic tumor treatment was assessed in a subcutaneous human colon cancer xenograft tumor model.

MATERIALS AND METHODS

Murine Subcutaneous Tumor Models

The Institutional Administrative Panel on Laboratory Animal Care approved all experimental procedures on laboratory animals. Mouse SVR angiosarcoma cells (American Tissue Culture Collection [ATCC], Manassas, VA) were cultured in Dulbecco Modified Eagle Medium supplemented with 10% fetal bovine serum and 100 U/mL each of penicillin/streptomycin (Invitrogen, Carlsbad, CA). Human LS174T colon carcinoma cells (ATCC) were cultured in minimum essential medium supplemented with 10% fetal bovine serum. Both cell lines were grown to 70% to 80% confluency, after which they were trypsinized, counted in a hemocytometer, and resuspended in 50 μ L of matrigel (BD Biosciences, San Jose, CA). SVR (1.5×10^6) or LS174T (3×10^6) cells in matrigel were then injected subcutaneously on the right hind limbs of nude mice (6–8 weeks old, Charles River, Wilmington, MA) and allowed to grow for 7 days. The well-vascularized subcutaneous angiosarcoma tumors ($n = 5$) were used to assess the effects of microbubble concentrations and injection rates on MIP imaging plateau values. Because antiangiogenic treatment proved not to affect those angiosarcoma tumors (data not shown), we used the clinically relevant human colon cancer xenograft tumor model ($n = 16$) in the second part of the study to longitudinally assess MIP imaging plateau values in mice with and without antiangiogenic therapy.

In Vivo Small Animal Contrast-Enhanced Ultrasound MIP Imaging

Ultrasound imaging was performed using a 40-MHz high frequency transducer (real-time microvisualization scan head 704, VisualSonics, Toronto, Canada; resolution: 100 μ m (lateral) and 40 μ m (axial); focal length: 6 mm; transmit power: 50%; mechanical index: 0.14; dynamic range: 52 dB) and a dedicated small animal ultrasound imaging system (Vevo 770; VisualSonics). The transducer was fixed in a rail system and aligned to the center plane of the subcutaneous tumors using a micromanipulator measurement system. Tumor height, width and length were measured on B-mode ultrasound images using an electronic caliper and the volume was calculated using the formula for ellipsoid volume ($\text{Volume} = 1/6 \pi \times \text{width} \times \text{height} \times \text{length}$). During ultrasound scanning, all mice were anesthetized using 2% isoflurane and air (2 L/min).

Maximum Intensity Persistence Algorithm

On the dedicated small animal ultrasound imaging system, the MIP algorithm is applied to a time series (t) of contrast ultrasound image frames. The series of contrast images are defined as $I(x,y,t)$, where x and y represent the lateral and vertical dimensions of the image, respectively. A time series of MIP images can then be defined as $I_{MIP}(x,y,t)$. A pixel value

in I_{MIP} at pixel location (a,b), at a given time point, n, (ie, image frame number) is defined as:

$$I_{MIP}(a, b, n) = \max\{I(a, b, t) | t \in (0, n)\} \quad (1)$$

where $t \in (0, n)$ is defined as “ t is within the range of zero to n .”

By using Eq. (1) a new time series (ie, $I_{MIP}(x, y, t)$) is generated automatically by the ultrasound software, which tracked the most intense pixel values that occurred over the time series of original contrast images (ie, $I(x, y, t)$; Fig. 1). In our study, this algorithm was applied in the Vevo770 Contrast Mode (800 frame cine loops, frame rate = 17/s; resulting in a total acquisition time per loop of 47 seconds) using a retrospective approach, which included the acquisition of a B-Mode image sequence with contrast agent (contrast set) and comparing each image frame to a set of B-Mode image frames without contrast agent (the reference set) using the sum of absolute differences algorithm.¹⁸ The pairing of the current contrast set image with the reference set image that generated the lowest sum of absolute differences value was saved and the process repeated for the next contrast set image. Finally, each reference set image was subtracted from its corresponding contrast set image, generating the final contrast images, $I(x, y, t)$ (displayed in green on the ultrasound scanner). Those contrast images were ultimately passed to the MIP algorithm described in Eq. (1) resulting in a series of MIP images for analysis. Because the subcutaneous tumors were placed at the level of the hind limbs in our mice, motion artifacts from breathing of the animals was not observed in our study.

Characterization of In Vivo Contrast-Enhanced Ultrasound MIP Imaging in Subcutaneous Tumors

In the first part of this study, the effects of increasing ultrasound contrast doses and injection rates on in vivo MIP imaging plateau values were assessed. The following doses of contrast microbubbles (Micromarkers, VisualSonics; microbubbles with a perfluorobutane gas core encapsulated by a phospholipid shell), suspended in 0.9% saline (injection volume, 100 μ L) were administered in the same mice and in the same imaging sessions with the transducer kept fixed in the same imaging plane: (a) 5×10^5 microbubbles (administered at an injection rate of 1.2 mL/min); (b) 5×10^6 microbubbles (at 1.2 mL/min); (c) 5×10^7 (at 1.2 mL/min); (d) A second injection of 5×10^7 (at 1.2 mL/min); (e) 5×10^8 (at 1.2 mL/min); (f) 5×10^7 (at 0.6 mL/min); and (g) 5×10^7 (at 2.4 mL/min). Each dose and ultrasound imaging experiment were separated by a period of 30 minutes to let microbubbles clear from the circulation based on our experience.^{19,20} To ensure a controlled, steady intravenous injection of contrast agent, a catheter was placed into a tail vein of the mice (12 cm polyurethane tubing connected to 27G butterfly needle), and the micro-bubbles were injected via an injection pump (GeniePlus; Kent Scientific, Torrington, CT).

Longitudinal In Vivo Antiangiogenic Treatment Monitoring Using Contrast-Enhanced Ultrasound MIP Imaging

In the second part of this study, the potential of MIP imaging to quantitatively monitor tumor vascularity during antiangiogenic treatment was evaluated. Figure 2 shows the

experimental timeline of the ultrasound imaging and antiangiogenic treatment plan. First, baseline contrast-enhanced ultrasound MIP imaging in 16 human colon cancer-bearing mice was performed. Of those 16 mice, 6 were then randomly selected after baseline imaging, and the tumors were excised to demonstrate correlation between in vivo MIP imaging plateau values and ex vivo microvessel density (MVD) analysis (as mentioned later in the text). The remaining 10 mice were randomly divided into following 2 groups: Group 1 (n = 5) received a single intraperitoneal (i.p.) saline injection (100 μ L), and group 2 (n = 5) received a single i.p. dose of an antiangiogenic antibody (B20-4.1.1, henceforth abbreviated as B20; Genentech, South San Francisco, CA) targeting both mouse and human vascular endothelial growth factor (VEGF).^{9,19,21,22} The antibody was administered at a concentration of 5 mg/kg body weight (100 μ L). After 48 hours, all 10 mice were reimaged with contrast-enhanced ultrasound MIP imaging using the exact same imaging protocol and scanning parameters as described above. After scanning, the mice were killed, and tumors were removed for ex vivo MVD analyses (Fig. 2).

Image Analysis of Contrast-Enhanced Ultrasound MIP Imaging

Postprocessing of ultrasound imaging data sets for MIP analysis was performed offline with a dedicated ultrasound imaging software (Vevo 770 high-resolution microultrasound imaging software; VisualSonics). An independent reader performed a blinded analysis of all the imaging studies in random order, and was not informed of the experimental settings or conditions. The reader placed a region of interest around the subcutaneous tumor graft boundaries as determined on B-mode images. The average video intensities of the MIP images within the region of interest were plotted versus the image frame number. In all experiments, the MIP imaging values reached a plateau value. This plateau value was used as the metric of the MIP image sequence, and is referred to as the MIP imaging plateau value in our study.

Ex Vivo MVD Analysis

Excised LS174T tumor xenografts were placed and frozen in optimum cutting temperature (Tissue-Tek [Fisher Scientific, Pittsburgh, PA]) in an orientation similar to that of the ultrasound imaging plane. To obtain approximately the same plane as the ultrasound imaging plane, tissues were first sliced at 40 μ m thickness until the largest, central area of the tumor was observed, and six 10 μ m slices were then obtained and mounted on microscope slides for immunofluorescence staining. The sections were fixed in ice-cold acetone and immunostained for endothelial cells overnight (4°C) with 1:100 primary rat antimouse CD31 antibody (Abcam, Inc., Cambridge, MA). Secondary antibody (donkey antirat fluorescein isothiocyanate (FITC)-conjugated antibody [Jackson ImmunoResearch, West Grove, PA]) was applied at 1:300 dilution in phosphate-buffered saline (Invitrogen, Carlsbad, CA) for 30 minutes at room temperature. Coverslips were then mounted onto slides with antifading medium (Fisher Scientific, Pittsburgh, PA). Fluorescent micrographs (100 $\times$ magnification) were captured using a microscope (Axiovert 25; Carl Zeiss, Thornwood, NY) and a camera (AxioCam, Bernried, Germany). MVD analysis was performed using a standardized protocol.²³ The total number of vessels was summed for 10 fields of view (single field of view area = 0.14 μ m² for each 10 μ m tumor slice, and MVD was calculated as the average number of vessels per total field of view area.

Statistical Analysis

All continuous measurements were expressed as mean $\pm$ standard deviation (SD). To test the trending pattern observed between MIP imaging plateau values and microbubble concentration, a nonparametric trend test was performed. The null distribution was obtained by randomly permuting the MIP imaging plateau values for each individual mouse. A mixed effects regression analysis was also performed to examine the association between MIP imaging plateau values and microbubble concentration. In the mixed effect regression model, log-transformed MIP imaging plateau values and microbubble concentration were the response and independent variables, respectively, and a mouse-specific random intercept was used to characterize the intramouse correlation. To test the association between MIP imaging plateau values and microbubble injection rate, the mean ratios of MIP imaging plateau values were estimated at different infusion rates and the corresponding 95% confidence intervals (CI) were constructed using the nonparametric bootstrap method. A *P* value was also generated using analysis of variance. To test the agreement between 2 separate injections of 5×10^7 microbubbles at 1.2 mL/min in each individual mouse, an intraclass correlation coefficient (ICC) was calculated, and a 95% CI for ICC was obtained using the bootstrap method. The correlation between MIP imaging plateau values [arbitrary units]; independent variable) and MVD (average vessel number per total field of view area; response variable) was tested by linear regression analysis. The 48 hours-changes in tumor volume and MIP imaging plateau values in nontreated and antiangiogenic drug-treated animals were compared using a 2-sample Wilcoxon rank test. The baseline tumor volume and MIP imaging plateau value were also compared with a 2-sample Wilcoxon rank test. Last, the tumor volumes and MIP imaging plateau values between baseline and 48 hours were compared with a 1-sample Wilcoxon rank test for nontreated as well as treated groups. All statistical analyses were performed using R 2.7.2 software (<http://www.r-project.org/>). A *P* less than 0.05 was considered statistically significant.

RESULTS

Characterization of In Vivo MIP Imaging in Subcutaneous Tumors

The in vivo MIP imaging plateau values obtained from subcutaneous tumors significantly correlated with contrast micro-bubble doses ($P = 0.001$; Fig. 3). Furthermore, the agreement between the 2 repeated MIP imaging plateau values at the same microbubble concentrations (5×10^7 microbubbles in 100 μ L saline) was high (ICC = 0.82; [95% CI: 0.64, 0.94]). When the same microbubble concentration (5×10^7 microbubbles in 100 μ L saline) was administered at 3 different injection rates, the in vivo MIP imaging plateau values at the injection rates of 0.6 mL/min and 2.4 mL/min were not significantly different ($P = 0.61$) from that at the injection rate of 1.2 mL/min for the same mouse (Fig. 3). Specifically, MIP imaging plateau values at the injection rate of 0.6 mL/min were similar to those at the injection rate of 1.2 mL/min with a 95% CI of the mean ratio ranging between 0.89 and 1.14. The 95% CI of the mean plateau value ratio compared with the injection rate of 2.4 mL/min was between 0.52 and 1.00. This broader 95% CI was the result of 1 outlier mouse with an imaging plateau value ratio of 0.40 (the MIP imaging plateau value ratio of the remaining 4 mice was 1.01, 1.00, 1.02, and 0.88, respectively).

Correlation of In Vivo MIP Imaging Plateau Value With Ex Vivo MVD

In vivo contrast-enhanced ultrasound MIP imaging plateau values obtained in subcutaneous xenograft tumors (Fig. 4) significantly correlated with *ex vivo* MVD analysis ($P = 0.01$; $R^2 = 0.77$).

Monitoring Tumor Vascularity With MIP Imaging Following Anti-Angiogenic Treatment

Figure 5 summarizes the response as a measure of tumor volume and MIP imaging plateau value for tumor-bearing mice at baseline (0 hour) and 48 hours after initiation of antiangiogenic treatment. The baseline tumor volumes as well as the change in tumor volumes from baseline (0 hour) to 48 hours after treatment were not significantly different between treated and nontreated mice ($P = 0.55$ for baseline tumor volumes; $P = 0.22$ for change in tumor volumes). Furthermore, tumor volumes at 48 hours after treatment were not significantly different from baseline volumes in treated ($P = 0.44$) and nontreated ($P = 0.06$) mice. In contrast, the change in MIP imaging plateau values from baseline to 48 hours after treatment was significantly different ($P = 0.01$) in treated versus nontreated mice (MIP imaging plateau values decreased on average by 25% in treated mice and increased on average by 29% in nontreated mice; Figs. 5, 6). At baseline imaging, MIP imaging plateau values in treated versus nontreated mice were not significantly different ($P = 0.10$).

DISCUSSION

Several techniques have been proposed to assess tumor vascularity or perfusion with contrast-enhanced ultrasound imaging. A traditional approach includes the acquisition of time-intensity curves following intravenous administration of ultrasound contrast agents (microbubbles). These time-intensity curves usually show a typical early rise in imaging signal, a short maximum, and a slow decay after bolus injection of contrast agent.^{17,24} Such curves can be characterized by descriptive terms such as the time to peak enhancement, peak enhancement, upslope of the enhancement curve, and area under the curve.^{17,24,25} These parameters are well reproducible at the same imaging depths²⁶ and same dose administration,²⁷ but only indirect descriptors for perfusion or blood volume.²⁵ For absolute quantification, an arterial input function needs to be measured, which is challenging in ultrasound imaging due to the limited field of view, inability to covisualize large vessels (such as the aorta), or by the difficulty in delineating a defined feeding vessel of the imaged tumor. Another method to quantify absolute perfusion parameters or parameters that are proportional to the blood flow in the examined anatomic region includes replenishment kinetics.^{7,28} Here, a high-MI (mechanical index) pulse is used to destroy all microbubbles contained in the examined slice and an increase in imaging signal follows as freely circulating microbubbles enter the slice from the adjacent tissue.^{17,28} This replenishment kinetics relies on the assumption that the concentration of bubbles in the blood is constant, which can be achieved with a constant infusion of contrast agents^{17,28}; however, caution must be exercised to ensure steady flow rate and accurate dose administration.

In our current study, we explored whether contrast-enhanced ultrasound MIP imaging allows in vivo assessment and monitoring of tumor vascularity, and how different contrast administration parameters influence the in vivo MIP imaging plateau values in tumors. First,

we assessed the effect of increasing ultrasound contrast agent concentrations on the absolute MIP imaging plateau value in a well-vascularized angiosarcoma tumor model. In intraanimal experiments administering 4 different ultrasound contrast concentrations in the same mice, we found an expected increase of the MIP imaging plateau value with increasing contrast concentrations, suggesting that ultrasound contrast agent concentrations need to be kept constant for longitudinal and repetitive MIP imaging examinations. Since in clinical practice, ultrasound contrast agents are often manually administered intravenously with some variance of injection rates of the contrast agents, we also injected ultrasound contrast agents at 3 different injection rates and assessed the effect of contrast injection rates on the in vivo MIP imaging plateau value. Our results showed that the in vivo MIP imaging plateau value was independent of the injection rate, suggesting that MIP imaging is a robust tool to assess tumor vascularity. This is also reflected by the high reliability of the technique in assessing tumor vascularity with an intraclass coefficient of 0.82 following repetitive injections of ultrasound contrast agents in the same mice.

We then tested MIP imaging for in vivo assessment and monitoring tumor vascularity during antiangiogenic treatment in a clinically relevant human colon cancer tumor model. In animals with subcutaneous human colon cancer xenografts and different grades of tumor vascularization, there was a good correlation ($R^2 = 0.77$) between in vivo MIP imaging plateau values and the levels of tumor vascularity as assessed by ex vivo MVD analysis, suggesting that MIP imaging can be used for in vivo assessment of tumor vascularity. Furthermore, in vivo MIP imaging showed a substantial drop of tumor vascularity as early as 48 hours after a single dose of antiangiogenic therapy administration. In contrast, the MIP imaging plateau values remained substantially higher in the nontreated mice. Notably, tumor sizes were not significantly different in treated versus nontreated mice within 48 hours. This highlights the potential of MIP imaging to assess early antiangiogenic treatment effects before overt morphologic-anatomic changes of the tumor become visible.

There is limited experience in using MIP imaging of tumor vascularity in preclinical and clinical applications. Sehgal et al²⁹ compared a technique called delta-projection imaging (a method similar to MIP imaging) with contrast-enhanced power Doppler ultrasound imaging in 25 mice with subcutaneous K1735 melanoma tumors and showed a strong correlation between the 2 techniques for assessment of tumor vascularity. Another study compared plateau signal intensity values on MIP images with parameters obtained from time-intensity curves (including time-to-peak, peak intensity, and upslope) following intravenous administration of ultrasound contrast agents at 2 different injection rates (50 μ L microbubbles injected either within 2 seconds or 10 seconds) in 7 human epidermoid carcinoma xenografts.³⁰ The study demonstrated that the MIP plateau imaging signal was less dependent on contrast injection rates and showed treatment effect earlier following administration of the receptor tyrosine kinase inhibitor SU11248 than parameters obtained from time-intensity curves.³⁰ Wilson et al¹⁶ showed feasibility of MIP imaging in 65 patients with focal liver lesions including hepatocellular carcinoma, focal nodular hyperplasia, adenoma, and liver metastases. We add on these studies, first, by characterizing in vivo MIP imaging at varying contrast administration parameters including 4 different contrast agent concentrations and 3 different contrast injection rates, and, second, by testing

reliability of MIP imaging for quantification of tumor vascularity in intra-animal experiments. Furthermore, in a clinically relevant human colon cancer xenograft tumor model, we showed that MIP imaging allows early assessment of treatment effect following administration of the antibody B20, an anti-VEGF antibody that is similar to the therapies currently used in patients with metastasized colon cancer.^{21,31,32}

The following limitations of our study need to be acknowledged. First, the dedicated small animal Vevo770 ultrasound system applied in our study uses B-mode imaging for contrast agent detection, and tissue-echo cancellation is accomplished by subtracting the baseline tissue images from images obtained after administration of contrast microbubbles. Therefore, in the case of small microbubble concentrations, only a marginal microbubble signal differential over the baseline images can be observed. Even if the detected microbubble signal is sufficient for imaging (as in our study), a fraction of the microbubbles might not have been detected by this ultrasound system. Second, the Vevo770 ultrasound system operates on B-Mode images generated after log-compression, resulting in a contrast signal that cannot be quantified in a linear manner. Recently, following the completion of this study, a linear array-based dedicated small animal ultrasound system has been released. This new system is capable of operating in a contrast-specific imaging mode with uncompressed image signals for quantification. The new imaging mode uses the nonlinear properties of microbubbles for segmenting them from tissue using a pulse-sequencing approach, resulting in improved detection of small quantities of micro-bubbles and image signals that are linearly proportional to microbubble concentration.³³ Future studies are warranted to evaluate contrast-enhanced MIP imaging for assessment and monitoring tumor vascularity using this next generation ultrasound imaging approach. Additionally, in our study contrast-enhanced ultrasound MIP imaging was only performed in the 2-dimensional plane of the beam elevation. Three-dimensional approaches are needed to adjust for morphologic tumor heterogeneity and different extent of vascularization within the 3D dimension of the tumors.³⁴ Furthermore, the MIP imaging method used in our study required postprocessing of cine loops and, therefore, is not a real-time imaging tool. Technical improvements are currently under way to make this imaging approach a real-time imaging tool to improve practicability in particular for clinical applications. Finally, the imaging technique used in our study with subtracting the baseline tissue images from contrast-enhanced images is prone to artifacts from motion. Although this was not an issue in our study because the subcutaneous tumor grafts were implanted at the level of the hind limbs where motion artifacts from breathing is minimal, implementation of motion correction techniques or respiratory gating techniques are needed to make contrast-enhanced MIP imaging a reliable tool in areas with potentially substantial motion artifacts (such as in tumors located in the upper abdomen).

In conclusion, our results suggest that contrast-enhanced ultrasound MIP imaging is a reliable tool that may be used to assess tumor vascularity in vivo. MIP imaging plateau values are independent of the contrast microbubble injection rates, which may be advantageous for clinical translation of quantitative MIP imaging. Furthermore, MIP imaging allows in vivo monitoring of tumor vascularity during antiangiogenic therapy in human colon cancer tumor xenografts in mice.

Acknowledgments

Funding source: ICMIC developmental grant NIH P50 CA114747-06.

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FIGURE 1.

Schematic representation of the MIP algorithm applied to consecutive imaging frames $I(x,y,t)$ captured at 4 time points ($n + 1$ through $n + 4$). Each imaging frame (I) is represented as a function of depth (y), width (x), and time (t) at time points n through $n + 4$ (shown in the upper row of the figure). Each imaging frame represents an idealized snapshot of a single microbubble coursing through the imaging plane and captured at a different spatial and temporal location. Through application of the MIP algorithm ($I_{MIP}(x,y,t)$, shown in the lower row of the Figure), the spatial location of the microbubble persists though time and can be tracked visually on the ultrasound monitor.

FIGURE 2.

Timeline of MIP ultrasound imaging and antiangiogenic treatment experiments. Seven days following subcutaneous human colon cancer cell injections in 16 mice, baseline (0 hour) ultrasound MIP imaging of xenograft tumors was performed. Six mice were randomly selected for correlation of in vivo MIP imaging plateau value with ex vivo microvessel density analysis in excised tumors. Following baseline ultrasound MIP imaging, the remaining 10 mice were divided randomly into 2 groups receiving either a single i.p. B20 anti-VEGF antibody injection (B20-treated, n = 5) or a single i.p. saline injection (nontreated, n = 5). After 48 hours, ultrasound MIP imaging was repeated and tumors were excised for ex vivo analysis.

FIGURE 3.

Bar chart demonstrates that the average MIP imaging plateau values are dependent on microbubble concentration but not infusion rate. MIP imaging plateau values obtained at each microbubble concentration (5×10^5 , 5×10^6 , 5×10^7 [injection 1 and injection 2], and 5×10^8) and infusion rate (0.6 mL/min, 1.2 mL/min, and 2.4 mL/min) were normalized to MIP imaging plateau values obtained with 5×10^5 micro-bubbles for each mouse; bars are mean $\pm$ SD. Two injections (injection 1 and injection 2) of 5×10^7 were performed to assess reliability of MIP imaging measurements obtained from the same contrast agent concentration.

FIGURE 4.

Correlation between microvessel density (MVD) and MIP imaging plateau values. A, Linear graph of data points obtained for individual mice shows good correlation between MVD and in vivo MIP imaging ($R^2 = 0.77$; $P = 0.01$). B, Representative transverse in vivo ultrasound MIP images (left panel) and corresponding ex vivo CD31-stained microscopic images (100 $\times$; right panel) of 2 subcutaneous human colon cancer xenografts (yellow arrows) with different levels of tumor vascularity. Note that a low in vivo MIP imaging plateau value was associated with low ex vivo microvessel density (white arrows show CD31-positive tumor

vessels) and that a high in vivo MIP imaging plateau value was associated with high ex vivo microvessel density.

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FIGURE 5.

Bar charts shows average (A) and individual mouse (B) response in tumor volume (left y-axis; blue [0 hour] and red [48 hour] bars) and MIP imaging plateau values (right y-axis; square [0 hour] and triangle [48 hour] individual points) following antiangiogenic therapy with B20 antibody or sham treatment with saline (control, nontreated). A, Bars represent the mean $\pm$ SD of tumor volumes (mm^3) measured at 0 hour (blue; dark blue error bars) and 48 hours (red; pink error bars) for nontreated and B20-treated mice. Note that on average, starting tumor volumes (0 hour) were similar between nontreated and B20-treated mice;

however, tumor sizes were substantially smaller at 48 hours in the B20-treated mice than the nontreated mice. Data points represent the mean $\pm$ SD of relative MIP imaging plateau values. MIP imaging plateau values for each mouse at 48 hours are expressed relative to the MIP imaging plateau at 0 hour, and then averaged for each group (nontreated and B20-treated [black dotted error bars]); note that the relative MIP imaging plateau value at 0 hour is therefore 1. Notably, MIP imaging plateau values increased in nontreated mice, but significantly decreased in B20-treated mice. B, Bars represent the raw tumor volume measured at 0 hour (blue) and 48 hours (red) for each mouse in each group (nontreated and B20-treated). Data points represent the raw MIP imaging plateau values (arbitrary intensity units) for each mouse in each group (nontreated and B20-treated).

FIGURE 6.

Representative transverse in vivo ultrasound MIP images (left and middle panels) of a nontreated subcutaneous human colon cancer xenograft (yellow arrows; upper row) and of a B20-treated subcutaneous human colon cancer xenograft (yellow arrow; lower row), both imaged at baseline (0 hour) and 48 hours after a single i.p. injection of B20 anti-VEGF antibody. The in vivo MIP ultrasound imaging plateau value increased in the nontreated tumor, whereas the MIP ultrasound imaging plateau value substantially dropped in the treated tumor after 48 hours. Ex vivo CD31-immunostained micrographs (100 $\times$) obtained from the 2 tumors (right panel) confirmed higher tumor vascularity in nontreated versus B20-treated tumors.