Antiangiogenic Cancer Therapy: Monitoring with Molecular US and a Clinically Translatable Contrast Agent (BR55)¹

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Purpose:

To develop and test human kinase insert domain receptor (KDR)-targeted microbubbles (MBs) (MB_{KDR}) for imaging KDR at the molecular level and for monitoring antiangiogenic therapy in a human colon cancer xenograft tumor model in mice.

Materials and Methods:

Animal studies were approved by the Institutional Administrative Panel on Laboratory Animal Care. A heterodimeric peptide that binds to human KDR with low nanomolar affinity ($K_D = 0.5$ nmol/L) was coupled onto the surface of perfluorobutane-containing lipid-shelled MBs (MB_{KDR}). Binding specificity of MB_{KDR} to human KDR and cross-reactivity with murine vascular endothelial growth factor (VEGF) receptor 2 (VEGFR2) were tested in cell culture under flow shear stress conditions (at 100 sec⁻¹). In vivo binding specificity of MB_{KDR} to VEGFR2 was tested in human LS174T colon cancer xenografts in mice with a 40-MHz ultrasonographic (US) transducer. Targeted contrast material-enhanced US imaging signal by using MB_{KDR} was longitudinally measured during 6 days in tumors with ($n = 6$) and without ($n = 6$) antiangiogenic treatment (anti-VEGF antibody). Ex vivo VEGFR2 staining and microvessel density analysis were performed. Significant differences were evaluated (t , Mann-Whitney, or Wilcoxon test).

Results:

Cell culture experiments showed four times greater binding specificity of MB_{KDR} to human KDR and cross-reactivity to murine VEGFR2 ($P \leq .01$). In vivo imaging signal was more than three times higher ($P = .01$) with MB_{KDR} compared with control MBs and decreased significantly (approximately fourfold lower, $P = .03$) following in vivo receptor blocking with anti-VEGFR2 antibody. One day after initiation of antiangiogenic therapy, imaging signal was significantly decreased (approximately 46% lower, $P = .02$) in treated versus untreated tumors; it remained significantly lower (range, 46%–84% decreased; $P = .038$) during the following 5 days. Microvessel density was significantly reduced ($P = .04$) in treated (mean, 7.3 microvessels per square millimeter $\pm$ 4.7 [standard deviation]) versus untreated tumors (mean, 22.0 microvessels per square millimeter $\pm$ 9.4); VEGFR2 expression was significantly decreased (>50% lower, $P = .03$) in treated tumors.

Conclusion:

Human MB_{KDR} allow in vivo imaging and longitudinal monitoring of VEGFR2 expression in human colon cancer xenografts.

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Antiangiogenic treatment holds great promise for cancer therapy in patients with different cancer types, including colorectal cancer. In 2004, the combination of bevacizumab (Avastin; Genentech, South San Francisco, Calif), a humanized anti-vascular endothelial growth factor (VEGF) antibody, with chemotherapy was the first antiangiogenic treatment approved by the Food and Drug Administration in patients with metastasized colorectal cancer (1). Since then, five clinical trials involving 3085 patients have shown substantial improvement in terms of progression-free survival and overall survival of patients with metastasized colorectal cancer by using antiangiogenic treatment in combination with chemotherapy (2). Various types of antiangiogenic drugs are currently in preclinical development or are being tested in clinical trials. However, to our knowledge, there are no existing validated biomarkers or imaging strategies available as surrogate end points to predict early response and outcome in antiangiogenic cancer treatment. Traditional anatomic oncological imaging approaches for which treatment success is

based on tumor size criteria have been shown to be insufficient in evaluation of molecularly targeted therapies (3,4). Therefore, imaging strategies that allow direct assessment of treatment effects at the molecular level during antiangiogenic therapy are needed.

Ultrasonography (US) with the use of contrast microbubbles (MBs) targeted at molecular markers (molecular US imaging) is a promising technology that allows noninvasive imaging of tumor angiogenesis at the molecular level (5,6). US contrast agents are gas-filled, echogenic MBs, which can be functionalized to bind molecular markers of tumor angiogenesis by attaching specific ligands onto the MB shell (5). Following intravenous injection, these targeted MBs attach at tumor vascular endothelial cells, overexpressing molecular markers of angiogenesis. This accumulation of attached MBs results in an increase of the US imaging signal (7–9). Because contrast MBs stay predominantly within the vascular system owing to their size of several micrometers, molecular markers on tumor vascular endothelial cells can exclusively be visualized with molecular US, and the imaging signal is not confounded by eventual coexpression of markers outside the vasculature (eg, on cancer cells).

Tumor angiogenesis is a complex process involving several signal transduction pathways (10–13) and VEGF receptor 2 (VEGFR2); in humans, this receptor is called kinase insert domain receptor (KDR), an endothelium-specific receptor tyrosine kinase, which is one of the best-characterized molecular markers of tumor angiogenesis (10). VEGFR2 is considered a major factor of tumor angiogenesis, and it is overexpressed on angiogenic vessels in several cancers, including colon cancer (14–17). Because many antiangiogenic drugs directly bind to the extracellular or intracellular domain of VEGFR2 or block its natural ligand VEGF, imaging

of VEGFR2 and/or KDR expression at the molecular level in tumor angiogenesis is very attractive for monitoring antiangiogenic cancer treatment.

In the past few years, several preclinical studies have shown feasibility of molecular US imaging for assessment of VEGFR2 expression in murine tumor models with contrast MBs targeted at VEGFR2 (18–24). In those studies, biotinylated antimouse VEGFR2 monoclonal antibodies were attached to streptavidin-containing MB shells through biotin-streptavidin interactions. Because streptavidin is immunogenic and can cause severe allergic reactions in patients (25–27), next-generation contrast agents with alternative binding chemistries are needed for future clinical translation of molecular US imaging in patients.

Therefore, the purpose of this study was to develop and test human KDR-targeted MBs (MB_{KDR}) for imaging KDR and/or VEGFR2 at the molecular level and for monitoring antiangiogenic therapy in a human colon cancer xenograft model in mice.

Advances in Knowledge

- Human kinase insert domain receptor (KDR)-targeted microbubbles (MBs) (MB_{KDR}) can be designed by integrating a high-affinity, heterodimeric small peptide into the MB shell.
- MB_{KDR} bind to human KDR and cross-react with murine vascular endothelial growth factor receptor 2 (VEGFR2) under flow shear stress conditions in a parallel plate flow chamber.
- Both in cell culture and in human colon cancer xenografts in vivo, MB_{KDR} show binding specificity to KDR and/or VEGFR2.
- Targeted contrast material-enhanced US imaging (molecular US) allows longitudinal monitoring of antiangiogenic treatment in human colon cancer xenografts in vivo.

Implication for Patient Care

- This study lays the foundation for a future clinical translation of molecular US imaging of KDR in cancer patients.

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Abbreviations:

KDR = kinase insert domain receptor

MB = microbubble

MB_{KDR} = KDR-targeted MBs

MB_{NT} = nontargeted MBs

PAE = porcine aortic endothelial

VEGF = vascular endothelial growth factor

VEGFR2 = VEGF receptor 2

Author contributions:

Guarantors of integrity of entire study, M.A.P., J.K.W.; study concepts/study design or data acquisition or data analysis/interpretation, all authors; manuscript drafting or manuscript revision for important intellectual content, all authors; approval of final version of submitted manuscript, all authors; literature research, M.A.P., J.K.W.; experimental studies, M.A.P., K.F., J.K.W.; statistical analysis, J.R., J.K.W.; and manuscript editing, M.A.P., K.F., M.S., J.K.W.

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See Materials and Methods for pertinent disclosures.

See also Science to Practice in this issue.

Materials and Methods

One author (M.S.) is an employee of Bracco Research (Geneva, Switzerland), who provided the contrast agent used in this study. The authors who are not Bracco employees (M.A.P., K.F., J.R., S.S.G., J.K.W.) had control of inclusion of any data and information that might present a conflict of interest for that author (M.S.). One author (S.S.G.) serves on the scientific advisory board of VisualSonics (Toronto, Ontario, Canada), which develops small-animal US imaging equipment.

Cell Culture Experiments

Standard cell culture methods were used and are described in detail in Appendix E1 (online).

Design of MB_{KDR}

A 5.5-kDa heterodimeric peptide that binds to KDR with high affinity (dissociation constant, $K_D = 0.5$ nmol/L) was combined with the phospholipid 1,2-distearoyl-*sn*-glycerol-3-phosphoethanolamine-*N*-[amino (polyethylene glycol)-2000] to form KDR-binding lipopeptides (28,29). Reaction products were purified by using high-performance liquid chromatography (>90% pure) and verified by using matrix-assisted laser desorption- and ionization-time-of-flight mass spectrometry. Purified KDR-binding phospholipid-heteropeptides were incorporated into the shell of MBs (MB_{KDR} consisting of a monolayer of phospholipids surrounding the gas perfluorobutane). The mean diameter of the MB_{KDR} as assessed by using a cell counter and sizer (Multisizer III Coulter Counter; Beckman Coulter, Fullerton, Calif) was $1.5 \mu\text{m} \pm 0.1$ (standard deviation), with a range of 1–3 μm . The mean number of heterodimeric peptides per square micrometer on the MB shell was 34200 ± 1300 (range, 31800–36600). The number of heterodimeric peptides per square micrometer was assessed by determining the total lipopeptide concentration by using high-performance liquid chromatography and the total MB surface area. Nontargeted MBs (MB_{NT}) not functionalized with the KDR-binding peptide were used as control MBs.

All MBs (BR55; Bracco) were produced under good manufacturing practice conditions.

Flow Chamber Cell Attachment Studies

Flow chamber experiments were performed to confirm binding of MB_{KDR} to KDR expressed on endothelial cells under flow shear stress conditions (flow rate of 0.6 mL/min, corresponding to a wall shear rate of 100 sec^{-1}), simulating flow conditions in tumor capillaries (30). For this purpose, 2 million KDR-expressing endothelial cells (porcine aortic endothelial [PAE]/KDR cells) or KDR-negative endothelial cells (PAE cells) were grown on coated (Sigmacote; Sigma, St Louis, Mo) glass microscope slides for 24 hours and mounted upside down (to allow MBs to float toward cells) on a parallel plate flow chamber (GlycoTech, Rockville, Md) (Fig 1a). The following solutions were passed over the cells by using a syringe infusion or withdrawal pump (Genie Plus; Kent Scientific, Torrington, Conn): (a) phosphate-buffered saline rinsing for 2 minutes, (b) 5×10^7 MB_{KDR} for 4 minutes, and (c) another 2-minute phosphate-buffered saline rinse. Slides were then removed from the flow chamber, dipped briefly in a beaker of phosphate-buffered saline to remove residual freely floating MB_{KDR}, and wet mounted with a coverslip for immediate imaging with a phase-contrast bright-field microscope at a magnification of $\times 100$ (Axiovert 25; Carl Zeiss, Thornwood, NY) and a camera (AxioCam; Carl Zeiss, Bernried, Germany).

To further test cross-reactivity of MB_{KDR} with murine VEGFR2 (a prerequisite for in vivo imaging in mice), flow chamber experiments were performed with murine endothelial cells expressing VEGFR2 and negative control murine cells. Furthermore, all cells were passed with negative control MB_{NT}. Finally, to test binding specificity of MB_{KDR} to human KDR and murine VEGFR2 receptors expressed on vascular endothelial cells, experiments were repeated after preblocking of KDR- and VEGFR2-expressing cells prior to mounting on the flow chamber apparatus for 30 minutes with an anti-VEGFR2 antibody

(Millipore, Billerica, Mass), 30 $\mu\text{g/mL}$; this antibody binds to both human KDR and murine VEGFR2. All experiments were performed in triplicate for each cell line and MB type. Ten fields of view per slide were imaged for subsequent quantification of bound MBs per cell (Fig 1b).

Murine Subcutaneous Tumor Xenograft Model

All procedures performed with the use of laboratory animals were approved by the Institutional Administrative Panel on Laboratory Animal Care. Tumors were established by means of subcutaneous injection of 3×10^6 human colon carcinoma LS174T cells dissolved in 50 μL of basement membrane matrix (Matrigel; BD Biosciences, San Jose, Calif) in the right hind limb of 21 6–8-week-old nude female mice (Charles River, Wilmington, Mass). Tumors were allowed to grow for 7 days after injection. The mean volume was 40 mm^3 , and the range was 14–81 mm^3 , as measured by using US imaging (see below) and by using the formula for a prolate ellipsoid, $\pi/6 \cdot (L \cdot W \cdot H)$, where L is length, W is width, and H is height.

Molecular US in Animal Tumor Models

Two-dimensional fundamental brightness-mode targeted contrast-enhanced US imaging was performed by using a dedicated small-animal high-spatial-resolution imaging system (Vevo 770; VisualSonics). A 40-MHz high-frequency linear transducer, equipped with a real-time microvisualization scanhead (RMV-704; VisualSonics), lateral resolution of 100 μm , axial resolution of 40 μm , focal length of 6 mm, transmit power of 50%, mechanical index of 0.14, and dynamic range of 52 dB, was fixed on a railing system. The acoustic focus was centered at the level of the subcutaneous tumor xenografts with the imaging plane aligned in the center of the tumor. For longitudinal imaging studies in the same animals, previous-day US images were used as reference to obtain the same central imaging plane. The mice were anesthetized with 2% isoflurane in room air (administered at 2 L/min) during scanning. All imaging settings were kept constant throughout each imaging

Figure 1

Figure 1: Cell culture binding assay of MBs to human KDR and mouse VEGFR2 by using a parallel plate flow chamber. **(a)** Schematic diagram of flow chamber apparatus setup used to flow different types of MBs over cells grown on a microscope slide at a wall shear rate of 100 sec⁻¹. **(b)** Phase-contrast bright-field micrographs (scale bar = 20 μm) show binding of human MB_{KDR} (KDR-MB) and negative control MB_{NT} (NT-MB) seen as white spheres (arrows) to KDR- and/or VEGFR2-positive and KDR- and/or VEGFR2-negative cells. Binding of MB_{KDR} was substantially higher to both KDR- and VEGFR2-expressing cells compared with negative control cells and could be substantially blocked by incubation of cells beforehand with an anti-KDR and anti-VEGFR2 antibody. **(c)** Box and whisker plot of attached MBs per cell number counted from micrographs (as in **b**). Whiskers are standard deviations. SVR and 4T1 are cell lines. Means with standard deviations are listed in Table 1.

session, as well as for longitudinal imaging studies.

After injection of 5×10^7 MB_{KDR} via the tail vein, molecular US imaging was performed by using an imaging sequence for targeted imaging as previously described (23) and detailed in Appendix E1 (online).

Images representing the adherent MBs were displayed by the imaging system as green overlays on the brightness-mode anatomic images.

To demonstrate specific binding of MB_{KDR} to VEGFR2 in vivo, tumor-bearing mice ($n = 3$) were first imaged following administration of MB_{KDR} as described above. MB_{KDR} from this first bolus injection were then cleared by waiting for 30 minutes to let most freely circulating MBs clear and by applying five 10-MHz destruction pulses, with parameters as

described above (31). MB clearance was verified by performing targeted imaging as described above and ensuring that the differences in imaging signal between images obtained before and after destruction were zero. In vivo blocking of VEGFR2 binding was then obtained by allowing 125 μg of anti-VEGFR2 antibody (eBiosciences, San Diego, Calif) injected via the tail vein to circulate for 30 minutes. Thereafter, tumors were imaged a second time in the same imaging plane, and US imaging with MB_{KDR} was performed after antibody blocking of VEGFR2. US imaging signals following administration of control MB_{NT} and MB_{KDR} were also compared in an additional group of three tumor-bearing mice by performing subsequent injections of each type of MB in random order, with a 30-minute clearance period between

each injection. Finally, imaging signal in another three tumors versus contralateral nonangiogenic control hind-limb muscles was measured by using the same imaging protocol.

Longitudinal in Vivo Antiangiogenic Treatment Monitoring with Molecular US

To determine whether molecular US with MB_{KDR} allows monitoring of antiangiogenic treatment, a longitudinal imaging study was performed in mice bearing human colon cancer xenograft tumors (Fig 2). First, all tumor-bearing mice ($n = 12$) were imaged following injection of MB_{KDR} to obtain baseline US imaging signals by using the imaging protocol described above. After imaging, mice were randomly divided into two groups of six mice each: Group 1 ($n = 6$) did not receive antiangiogenic

Table 1

Summary of Flow Chamber Cell Culture Attachment Studies with MB_{KDR} and Control MB_{NT}

Type of MB	KDR-Negative Cells	KDR-Positive Cells	VEGFR2-Negative Cells	VEGFR2-Positive Cells
MB _{NT}	0.21 ± 0.09	0.07 ± 0.04	0.14 ± 0.02	0.02 ± 0.02
MB _{KDR}	0.58 ± 0.11*	4.08 ± 0.59†	0.51 ± 0.10*	3.29 ± 1.01†
MB _{KDR} after blocking	...	0.85 ± 0.69‡	...	1.17 ± 0.37‡

Note.—Values are mean number of attached MBs per cell number ± standard deviation. MB_{NT} are control MBs. Blocking was done by using preincubation of KDR-positive and VEGFR2-positive cells with an anti-KDR and anti-VEGFR2 antibody (30 µg/mL).

* $P < .001$, KDR-positive cells compared with KDR-negative cells and VEGFR2-positive cells compared with VEGFR2-negative cells.

† $P = .52$, no difference in binding between KDR- and VEGFR2-positive cells.

‡ $P = .01$, comparison of before blocking and after blocking.

Figure 2

Figure 2: Timeline of both longitudinal US scanning and antiangiogenic treatment schedule. Human colon cancer LS174T cells were injected subcutaneously in the right flank region of nude mice, and tumors were imaged 7 days later to obtain baseline molecular US imaging. Antiangiogenic treatment (anti-VEGF antibody [B20 treatment]) was initiated in a group of six tumor-bearing mice, whereas another group of six tumor-bearing mice remained untreated. Anti-VEGF therapy continued every day, and a final US scan was obtained at 144 hours, after which tumors were excised for ex vivo analysis.

treatment (intraperitoneal administration of saline only), and group 2 ($n = 6$) received antiangiogenic treatment every 24 hours with an antibody targeting both human and mouse VEGF (B20-4.1.1 [B20]; Genentech) administered by using intraperitoneal injection (5 mg/kg dose, 80 µL volume) (32). Molecular US imaging with MB_{KDR} was performed at 24-hour intervals after each treatment application, for a total of 72 hours, and a final US scan was obtained at 144 hours.

In Vivo Molecular US Imaging Analysis

After all imaging sessions, imaging frames were recorded digitally and analyzed offline in random order by one independent

reader (J.K.W.) with commercially available software (Vevo 770 software; VisualSonics). The reader was blinded to the types of MBs and whether mice received antiangiogenic treatment. Video intensities (representing adherent MBs) were measured in regions of interest drawn over the whole tumor.

Ex Vivo Analysis

Standard immunostaining protocols were used and are provided in detail in Appendix E1 (online).

Statistical Analysis

All values are expressed as mean ± standard deviation. Box and whisker plots

were generated by using software (SPSS, version 11.5; SPSS/IBM, Chicago, Ill). Specific statistical tests applied to each experiment are provided in Appendix E1 (online). A difference with a P value of less than .05 was considered significant.

Results

Assessment of Binding Specificity of MB_{KDR} to Human KDR and Mouse VEGFR2 in Flow Chamber Cell Attachment Studies

Binding of MB_{KDR} to KDR-positive cells was significantly higher ($P < .001$) than it was to KDR-negative cells (Fig 1b). In addition, binding of MB_{KDR} to VEGFR2-positive cells was significantly higher ($P < .001$) than it was to VEGFR2-negative cells (approximately four MB_{KDR} per VEGFR2- or KDR-positive cells compared with approximately 0.5 MB_{KDR} per VEGFR2- or KDR-negative cell (Table 1, Fig 1c). There was no significant difference ($P = .52$) in MB_{KDR} binding to KDR-positive versus VEGFR2-positive cells. Furthermore, when KDR- and VEGFR2-positive cells were blocked beforehand with an anti-KDR and anti-VEGFR2 antibody, the binding of MB_{KDR} was significantly ($P = .01$) reduced from approximately four to one MB_{KDR} per cell (Table 1, Fig 1c).

Binding of MB_{KDR} to Murine VEGFR2 in Vivo

Average US imaging signal in human colon cancer xenografts (Fig 3) was significantly higher ($P = .01$) following MB_{KDR} injection (mean, 44.0 video intensity ± 14.9) compared with that of control MB_{NT} (mean, 13.3 video intensity ± 0.8). In experiments for evaluation of the specificity of MB_{KDR} with prior administration of a blocking anti-VEGFR2 antibody, US video intensities were significantly reduced (mean, 3.9-fold ± 1.2; $P = .03$) after VEGFR2 blockade compared with US video intensities before blocking. Furthermore, US imaging signal in control hind-limb muscle tissue (mean, 2.3 video intensity ± 1.5) was significantly lower ($P = .01$) compared with imaging signal in human colon cancer xenografts. Taken together, these results confirmed in vivo cross-reactivity of MB_{KDR} to murine VEGFR2.

Figure 3

Figure 3: Transverse US images of a subcutaneous human colon cancer xenograft (yellow arrows) imaged following intravenous administration of (a) control MB_{NT} and (b) MB_{KDR} in the same mouse. Targeted US imaging signal, shown as green signal (white arrows) overlaid on gray-scale brightness-mode image, was substantially higher with MB_{KDR} than with control MBs.

Longitudinal in Vivo Antiangiogenic Treatment Monitoring with Molecular US Imaging

Tumor sizes were not significantly different ($P = .52$) at the beginning of the drug treatment (mean, $46 \text{ mm}^3 \pm 24$ in the nontreatment group versus $34 \text{ mm}^3 \pm 13$ in the treatment group). Tumor growth over time relative to baseline was significantly different ($P = .005$) in treated versus untreated animals (Fig 4a), with an increase of tumor size in the untreated group from a mean of $46 \text{ mm}^3 \pm 24$ to $319 \text{ mm}^3 \pm 350$ and only small changes in tumor size in the treated group of mice from a mean of $34 \text{ mm}^3 \pm 13$ to $68 \text{ mm}^3 \pm 43$. Although tumor sizes of treated and untreated mice were not significantly different ($P = .11$) at 24 hours after initiation of antiangiogenic treatment, there was a significant difference in US imaging signal at 24 hours between treated and untreated tumors (relative to starting baseline signal, mean for untreated mice was 0.99 ± 0.39 and that for treated mice was 0.64 ± 0.28 ; $P = .02$) (Table 2, Fig 4b). Treated tumors demonstrated a significant decrease in the signal at 24 hours (46%, $P = .03$), and untreated tumors did not show any significant change in the imaging signal (0.01%, $P = .17$). Overall, the US imaging signal between animals undergoing antiangiogenic treatment versus those not undergoing treatment was significantly different ($P = .038$) (Table 2, Fig 4b).

Table 2

Summary of Longitudinal Time Course of in Vivo Molecular US Imaging Signal in Untreated and B20-treated Mice Bearing Human Colon Cancer Xenograft Tumors

Tumor-bearing Mice	0 hours, baseline	24 hours	48 hours	72 hours	144 hours
Untreated group ($n = 6$)	$1.00 \pm 0^*$	$1.37 \pm 0.57^\dagger$	2.17 ± 1.81	2.23 ± 2.21	1.77 ± 2.19
B20-treated group ($n = 6$)	$1.00 \pm 0^\ddagger$	0.59 ± 0.30	0.27 ± 0.25	0.25 ± 0.18	0.18 ± 0.11

Note.—Numbers are mean $\pm$ standard deviation of imaging signal and are expressed as relative values compared with imaging signals at baseline US imaging. B20-4.1.1 (B20), an antibody targeting both human and mouse VEGF, was administered daily by intraperitoneal injection (5 mg/kg). For comparison of untreated and B20-treated groups at 24, 48, 72, and 144 hours, $P = .038$.

* $P = .17$, comparison of untreated group at baseline and at 24 hours.

$^\dagger P = .02$, comparison of untreated and B20-treated groups at 24 hours.

$^\ddagger P = .03$, comparison of B20-treated group at baseline and at 24 hours.

Ex Vivo Analysis

Figure 4c shows representative tumor slices from treated and untreated mice. Microvessel density was significantly reduced in mice following antiangiogenic treatment (mean, 7.3 microvessels per square millimeter ± 4.7) compared with that in untreated mice (mean, 22.0 microvessels per square millimeter ± 9.4 ; $P = .04$). Moreover, untreated tumors demonstrated strong coexpression of CD31 and VEGFR2 (Fig 4c), whereas treated tumors demonstrated significantly ($P = .03$) reduced levels of VEGFR2 on remaining tumor microvessels.

Discussion

The objective of this study was to develop and test MB_{KDR} as a next step toward

future clinical translation of molecular US imaging of KDR expression in cancer patients. The MB_{KDR} were designed by incorporating phospholipid-heteropeptides into lipid-shelled, perfluorobutane-filled MBs and were found to bind human KDR and mouse VEGFR2 in cell culture and in vivo. Moreover, KDR-targeted US imaging allowed in vivo monitoring of antiangiogenic treatment in human colon cancer tumor xenografts. The design of MB_{KDR} avoids the streptavidin and/or avidin-biotin binding chemistry commonly used in preclinical evaluation of targeting MBs with molecular US.

Because of its wide availability, high effective dissociation constant, and flexible use for preclinical experiments, streptavidin and/or avidin is commonly used for noncovalent attachment of

Figure 4

Figure 4: Longitudinal study monitoring antiangiogenic treatment with molecular US and MB_{KDR}. **(a)** Graph of tumor volumes measured over time in untreated and B20-treated mice. **(b)** Box and whisker plot of MB_{KDR} targeted US signal (video intensity normalized to that at baseline at 0 hours) measured in tumors of untreated and B20-treated mice. Whiskers are standard deviations. Average signals are listed in Table 2. **(c)** Representative micrographs (scale bar = 100 μ m) of tumor tissue slices from untreated tumor (top row) and treated tumor (bottom row). Slices were immunofluorescently stained for CD31 (green, left images) and VEGFR2 (red, middle images) expression. Merged images (right images) demonstrate colocalization of VEGFR2 expression (yellow) on tumor endothelial cells.

biotinylated ligands onto the shell of US contrast MBs and avoids the need to design MBs for each vascular molecular target under investigation. To our knowledge, in all recent studies demonstrating proof-of-principle molecular US imaging of VEGFR2 expression in different small-animal cancer models, including breast cancer, melanoma, angiosarcoma, glioblastoma, ovarian cancer, pancreatic cancer, and squamous cell carcinoma (19–24), biotin-streptavidin and/or avidin chemistry was used to attach VEGFR2-targeted monoclonal antibodies onto the shell of contrast MBs. A drawback of this approach is that streptavidin and/or avidin can cause immunogenic and allergic reactions in patients (25–27).

Another possible side effect of streptavidin and/or avidin is related to potential binding of streptavidin to physiologically available biotin, which is needed for fatty acid synthesis and gluconeogenesis in the human body (33). Therefore, current streptavidin- and/or avidin-containing MBs targeted at KDR are unlikely to be translated into cancer patients.

For this reason, alternative strategies that allow covalent binding of ligands to the MB shell have been introduced (34). One approach included the binding of activated carboxylic groups in the MB shell with amino groups from lysine residues of binding ligands (35). However, coupling yields of ligands to MBs were limited with this reaction,

and large excess amounts of binding ligands have to be added to yield reasonable ligand-MB coupling ratios (35,36). Also, since ligands often have more than one lysine residue that can be located close to the binding site of the ligand, a site-specific attachment of the ligand to the MB shell often cannot be controlled, which can interfere with the binding affinity of the ligand (34). In an alternative approach, maleimide can be introduced into the MB shell and can react with an activated thiol group on ligands to form stable thioether bonds (37). Because single thiol residues can be introduced into the nonfunctional parts of the ligand, for example by site-directed mutagenesis or by direct synthesis,

site-specific attachment of the binding ligand to the MB shell can be achieved, thereby reducing the risk of interfering with the binding affinity of the ligand (35).

A third approach of ligand attachment includes the coupling of ligands to components of the MB shell (eg, phospholipids), followed by the formation of MBs by using these functionalized components in a second procedure. In this approach, synthesis and purification of ligand-phospholipid complexes can be performed in a stepwise and controlled setting, providing high coupling yields and site specificity of the coupling process (35). In our study, we first attached KDR-binding heterodimeric peptides onto phospholipids to form lipopeptides. In a second step, these lipopeptides were integrated into the MB shell during the process of MB synthesis. The average coupling yield from this procedure was 34200 ligands per square micrometer of MB shell surface.

To eventually translate MB_{KDR} into human clinical studies in cancer patients, binding specificity and in vivo US imaging signal following MB_{KDR} injection need to be tested in animal cancer models, such as murine xenograft tumor models. However, tumor angiogenesis in these models is regulated by the host, with expression of murine VEGFR2 and not human KDR on tumor vascular endothelial cells. Because murine VEGFR2 has about 85% homology with human KDR and plays a role in mouse physiology analogous to the role of KDR in humans (38,39), we hypothesized that the heterodimeric KDR-binding peptide cross-reacts with murine VEGFR2. Our results confirmed this hypothesis, showing increased MB_{KDR} binding to murine VEGFR2-positive cells compared with VEGFR2-negative cells. In addition, cross-reactivity of MB_{KDR} to murine VEGFR2 could be confirmed in cell culture-blocking experiments, with substantial decrease of MB_{KDR} binding following incubation beforehand of VEGFR2-expressing cells with anti-VEGFR2 antibodies.

Because MB_{KDR} were found to bind mouse VEGFR2 in cell culture experiments, we proceeded to test those MBs in an in vivo model of tumor angiogenesis

in colon cancer in mice. Our results showed substantially higher imaging signal in colon cancer xenografts than in control nonangiogenic muscle tissue following MB_{KDR} injection, and imaging signal in colon cancer xenografts was substantially lower following injection of control MB_{NT}, compared with MB_{KDR}. Furthermore, in vivo imaging signal after MB_{KDR} injection could be substantially inhibited by injecting blocking anti-VEGFR2 antibody. These experiments confirmed binding specificity of MB_{KDR} to murine VEGFR2 in vivo and indicated that MB_{KDR} can be tested in mouse models of tumor angiogenesis prior to translational studies in patients.

We further tested whether molecular US imaging with MB_{KDR} allowed in vivo monitoring of antiangiogenic tumor treatment in colon cancer. We used the anti-VEGF antibody, B20-4.1.1, which has been shown to bind to both human and murine VEGF, to maximize antiangiogenic treatment effects on human tumor xenografts in mice (32,40). As early as 24 hours after initiation of antiangiogenic tumor treatment, molecular US imaging signal with MB_{KDR} was substantially reduced compared with baseline imaging, whereas tumor size did not change significantly. This finding highlights the potential of molecular US imaging with MB_{KDR} for assessment of early antiangiogenic treatment effects before overt morphologic-anatomic changes of the tumor become visible. In untreated animals, the US imaging signal remained substantially higher than that in treated animals at all times in our study. We also observed substantial variability of the in vivo imaging signal in untreated animals, reflected by the high standard deviations of the imaging signals. This variability may be explained by differing patterns of tumor outgrowth in untreated animals and points to the limitations of subcutaneous tumor models, with substantial variability of the tumor biology among subcutaneous tumors (41). In fact, a recent study has shown that VEGFR2 expression in subcutaneous human U87MG xenografts in mice substantially varied depending on tumor size, as assessed by using ex vivo immunofluorescence and Western blotting, and that in

vivo VEGFR2-targeted positron emission tomographic tracer uptake varied in animals with different tumor sizes (varying between 3% and 15% injected dose per gram in small and large tumors) (42).

The following limitations of our study need to be addressed. VEGFR2 expression was assessed only in the limited volume of the ultrasound beam elevation. Although the imaging plane in the tumor center as chosen in our study may represent VEGFR2 expression in the entire tumor, three-dimensional approaches are needed to adjust for morphologic tumor heterogeneity and different expression levels on tumor endothelial cells in the same tumor (20). Furthermore, the dedicated small-animal US imaging system used in the current study only allowed imaging in the fundamental mode, and nonlinear scanning available on many clinical US scanners was not possible with this imaging system. Additional studies are warranted to test MB_{KDR} with US systems equipped with nonlinear imaging capabilities. Also, the in vivo imaging signals obtained by using a dedicated small-animal high-frequency US scanner in our study may be different compared with those obtained by using clinical US scanners operating at lower frequencies.

In conclusion, our results suggest that clinically translatable, human KDR-targeted MBs can be designed and successfully used for in vivo molecular US imaging of VEGFR2 in the tumor vasculature in mice. In addition, molecular US with MB_{KDR} allows longitudinal in vivo monitoring of antiangiogenic treatment in human colon cancer xenografts in mice. These results pave the way toward a future translation of molecular US imaging with MB_{KDR} in cancer patients.

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