Published in final edited form as:

J Ultrasound Med. 2011 July ; 30(7): 921–931.

A Triple-Targeted Ultrasound Contrast Agent Provides Improved Localization to Tumor Vasculature

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Abstract

Objectives—Actively targeting ultrasound contrast agents to tumor vasculature improves contrast-enhanced sonography of tumor angiogenesis. This report summarizes an evaluation of multitargeted microbubbles, comparing single-, dual-, and triple-targeted motifs.

Methods—Microbubbles were avidin-biotin linked to antibodies against mouse $\alpha_v\beta$ -integrin, P-selectin, and vascular endothelial growth factor receptor 2. These receptors are constitutively overexpressed in tumor vasculature. Binding comparisons between targeted microbubble groups were evaluated on mouse **SVR** angiosarcoma endothelial cells. Levels of the targeted receptors were characterized with flow cytometry. Targeted microbubble groups were administered to human MDA-MB-231 breast cancer tumor-bearing mice ($n = 3$) followed by contrast-enhanced sonography in a microbubble-sensitive harmonic imaging mode implemented on an **ultrasound scanner** equipped with a linear array transducer (5 MHz transmit and 10 MHz receive) to evaluate differences in microbubble accumulation in the tumor vasculature.

Results—In vitro analysis showed a 50% increase ($P < .001$) in triple-targeted microbubble binding over dual-targeted microbubble groups in mouse SVR cells. Mice bearing MDA-MB-231 tumors showed a 40% increase in tumor image intensity after dosing with triple-targeted microbubbles compared with single- and dual-targeted microbubbles ($P = .006$). Histologic staining confirmed the presence of $\alpha_v\beta$ -integrin, P-selectin, and vascular endothelial growth factor receptor 2 in the tumors.

Conclusions—Microbubble accumulation in the tumor vasculature was improved using a triple-targeted microbubble approach.

Keywords

$\alpha_v\beta_3$ -integrin; contrast-enhanced sonography; P-selectin; triple-targeted microbubbles; tumor vasculature; vascular endothelial growth factor receptor 2

The emergence of combination antiangiogenic cancer therapy has led to an increased demand for noninvasive modalities to monitor and evaluate tumor vasculature for determining an early treatment response. Recently, contrast-enhanced sonography was validated as a powerful tool for monitoring antiangiogenic therapy in cancer.^{1,2} In addition, clinical trials are ongoing to determine the importance of contrast-enhanced sonography for evaluating tumor vasculature changes during presurgical therapy.³ The evolution of contrast-

enhanced sonography has led to actively targeting microbubbles to tumor vasculature to provide improved visualization of angiogenesis and vessel assessment.⁴

Microbubbles are gas-filled colloidal particles surrounded by a flexible outer core composed of surfactant, polymer, or lipid molecules. Originally designed to improve ultrasound visualization during cardiac shunt evaluation,⁵ current **microbubbles** are stable in the circulation with a systemic half-life of several minutes. Their small size (1–10 μm) permits pulmonary passage, yet they are large enough to resist extravasation.⁶ During ultrasound exposure, microbubbles expand and contract in rapid oscillation and generate a nonlinear backscattered signal that can be isolated to improve contrast relative to surrounding soft tissue. These strategies ultimately improve vasculature visualization, making microbubbles useful ultrasound contrast agents.

To improve contrast-enhanced visualization of circulation and angiogenesis, targeted microbubbles have been generated that actively bind molecular markers expressed on the vessel lumen. These microbubbles are conjugated to proteins through either covalent linkage or avidin-biotin interactions.⁷ Although the use of avidin-biotin linkage is limited to preclinical conditions because of avidin immunogenicity, the application remains useful for investigators to explore the wide range of receptors available to improve targeting. Microbubbles conjugated with receptor-targeting ligands bind to receptors expressed on the vessel lumen in the tissue of interest. The accumulation of microbubbles through vessel wall interaction ultimately improves contrast-enhanced sonography and the visualization of angiogenesis.⁶

For specific targeting of microbubbles to the tumor vasculature, overexpressed receptors involved in survival mechanisms such as angiogenesis and adhesion are commonly used. Endothelial receptors involved in angiogenesis are routinely shown to be overexpressed in cancer and contribute to overall cancer progression.^{8–11} Vascular endothelial growth factor (VEGF) is a chemical ligand produced by cells undergoing hypoxia. It generates neovascularization by forming a chemotactic gradient for the recruitment of bone marrow-derived endothelial cells and through binding of vascular endothelial growth factor receptor 2 (VEGFR2) on preexisting endothelial cells, stimulating growth and proliferation.¹² Vascular endothelial growth factor receptor 2 mediates all cellular responses to VEGF, and targeting overexpression of the receptor has led to successful strategies in antiangiogenic therapy.^{13–15} The targeting of microbubbles to this receptor through antibody-microbubble coupling has been shown to improve contrast-enhanced sonography.^{16,17}

Another commonly overexpressed surface molecule in tumor endothelial cells is the cell adhesion molecule P-selectin,¹⁵ which is expressed on stimulated endothelial cells and activated platelets. It participates in the recruitment of leukocytes to areas of inflammation, which are common in the tumor vasculature.^{18,19} In addition, the presence of P-selectin permits the adhesion of platelets and cancer cells to the tumor endothelium. Recruitment of activated platelets has been shown to induce localized production of VEGF, thereby stimulating angiogenesis and overall tumor enrichment.²⁰ Techniques in contrast-enhanced sonography have used the expression of P-selectin in echocardiography and atherosclerotic plaque detection with P-selectin-targeted microbubbles.^{21,22} The overexpression of P-selectin in the tumor vasculature by stimulated endothelial cells makes it a viable target for intravascular microbubble binding.

Integrins mediate attachment and interactions between cells and their surrounding tissues, which include neighboring cells and the extracellular matrix.²³ $\alpha_V\beta_3$ -Integrin is commonly overexpressed in the vasculature of breast cancer.²⁴ The $\alpha_V\beta_3$ -Integrins are involved in leukocyte recruitment, tumor progression, and angiogenesis and have been targeted in many

cancer therapies.²⁵⁻²⁷ Targeting by antibody- or binding peptide-labeled microbubbles has been shown to improve contrast-enhanced sonography of the tumor vasculature.^{17,28}

Improvements in contrast-enhanced sonography have been driven in part by the need for sensitive detection and characterization of vascular abnormalities. First-generation targeted microbubbles were single-targeted microbubbles to enhance visualization of inflammation and tumor angiogenesis.^{7,29} Soon thereafter, dual-targeted microbubbles emerged as superior agents for targeted contrast-enhanced sonography. For tumor vasculature imaging, dual combinations of VEGFR2 and $\alpha_v\beta_3$ -integrin,¹⁷ P-selectin and vascular cell adhesion molecule 1,²¹ and intercellular adhesion molecule 1 and sialyl Lewisx³⁰ were all shown to improve visualization over their single-targeted counterparts. Triple-targeted microbubbles may further improve contrast-enhanced sonography. In this report, microbubbles targeted to the commonly overexpressed vasculature receptors $\alpha_v\beta_3$ -integrin, P-selectin, and VEGFR2 were used in a triple-targeted strategy to enhance microbubble binding and overall visualization of tumor vasculature.

Materials and Methods

Cell Lines and Culture Methods

Human MDA-MB-231 breast cancer and mouse SVR angiosarcoma endothelial cell lines were purchased from the American Type Culture Collection (Manassas, VA). The MDA-MB-231 cell line was maintained in Dulbecco's modified Eagle's medium, 10% fetal bovine serum, and 1% L-glutamine. The mouse SVR cell line was maintained in Dulbecco's modified Eagle's medium, 5% fetal bovine serum, and 1% L-glutamine. All cells were cultured to 70% to 90% confluency before passaging. Cell lines were grown at 37°C in 5% carbon dioxide. Cell numbers for in vitro assays were determined with a hemocytometer and trypan dye exclusion.

Antibody-Microbubble Conjugation

Streptavidin-coated microbubbles (Targestar-SA) were obtained from Targeson (San Diego, CA). Biotinylated rat immunoglobulin G (IgG) antibodies against mouse $\alpha_v\beta_3$ -integrin (13-0512; eBioscience, San Diego, CA), mouse P-selectin (553743; BD Pharmingen, San Diego, CA), and mouse VEGFR2 (13-5821; eBioscience) were obtained. Microbubbles were conjugated to the antibodies by means of biotin-streptavidin chemistry. Briefly, streptavidin-bound microbubbles were incubated with the respective antibodies for 20 minutes, followed by 3 centrifuge washes (400 times for 3 minutes) to wash out unbound particles. Single-targeted microbubbles were prepared using 40 μ g of the antibodies. Dual- and triple-targeted microbubbles were prepared with equal amounts of the appropriate antibodies totaling 40 μ g. For the control group, 40 μ g of a biotinylated rat IgG antibody (Southern Biotech, Birmingham, AL) was used to generate isotype-targeted microbubbles. After each conjugation, the targeted microbubble concentration was determined via a hemocytometer. The amount of the antibody used during conjugation served to saturate the available streptavidin on the microbubble. The unconjugated antibody was removed from the targeted microbubble solution by centrifuge washing. All targeted microbubble groups prepared are shown in Table 1.

Flow Cytometry

Mouse SVR cells were aliquoted (1×10^5 cells per tube) and stained with primary antibodies against mouse $\alpha_v\beta_3$ -integrin, P-selectin, and VEGFR2 per the manufacturers' recommendations. Phycoerythrin-labeled anti-rat IgG (405406; Biolegend, San Diego, CA) was used as a secondary stain. Tubes with cells and the secondary stain alone were used as controls to establish the background. Cells were analyzed for fluorescent counts (event

minimum, 5×10^4) using an Accuri C6 flow cytometer (Accuri Cytometers, Inc, Ann Arbor, MI). For Figure 1b, targeted microbubble groups were prepared and aliquoted (1×10^5 microbubbles per tube), followed by addition of phycoerythrin-labeled anti-rat IgG. Individual targeted microbubble groups were then analyzed (event minimum, 5×10^4) using the Accuri C6 flow cytometer. All experimental groups were analyzed in triplicate.

In Vitro Assay

Mouse SVR cells were plated at 1×10^5 cells per well in 6-well plates 24 hours before the binding assay. Individually targeted microbubble groups were added (5×10^6 microbubbles per well), bringing the final concentration of targeted microbubbles to 5×10^6 /mL. Plates were incubated 30 minutes at room temperature while rocking. Wells were then washed 3 times with phosphate-buffered saline. After the final wash, 2.0 mL of phosphate-buffered saline was placed in each well to allow the viewer to differentiate between bound and unbound microbubbles as the latter floated to the surface. Plates were randomly viewed by a blinded observer using an Olympus IX70 microscope (Olympus America, Inc, Melville, NY), and the 3 densest regions of microbubble accumulation were subjectively identified for each well (original magnification $\times 40$). Consequently, both the total number of attached microbubbles (to the cell surface) and cells in each region were counted (original magnification $\times 200$). Data were recorded for each reading as microbubbles per cell.

In Vivo Studies

Athymic female nude mice were obtained from the Frederick Cancer Research Center (Frederick, MD). MDA-MB-231 cells (2×10^6) were implanted in the mammary fat pad of each mouse ($n = 3$). Three weeks after implantation (average tumor size, $188.3 \pm 15 \text{ mm}^3$), targeted microbubble groups were intravenously injected (1×10^8 microbubbles per mouse in a 0.1-mL dose consisting of 0.05 mL of microbubbles and 0.05 mL of saline) in the tail vein in the following order: isotype-targeted microbubbles, single-targeted microbubbles ($\alpha_V\beta_3$ -integrin, P-selectin, and VEGFR2), dual-targeted microbubbles ($\alpha_V\beta_3$ -integrin + P-selectin, $\alpha_V\beta_3$ -integrin + VEGFR2, and P-selectin + VEGFR2), and triple-targeted microbubbles ($\alpha_V\beta_3$ -integrin + P-selectin + VEGFR2). The order was chosen to eliminate potential biases associated with lingering antibodies bound to cell surface receptors that could alter the performance of the subsequent groups. A 1-hour recovery period was maintained between microbubble group imaging. Two minutes after injection, each mouse was imaged along the largest transverse tumor plane in a microbubble-sensitive harmonic imaging mode implemented on a Sonix RP scanner (Ultrasonix Medical Corp, Richmond, British Columbia, Canada) equipped with an L12-5 linear array transducer (5 MHz transmit and 10 MHz receive), which was stabilized during scanning with a positioning arm (CIVCO Medical Solutions, Kalona, IA). Scanner settings (image depth, 4.0 cm; gain, 90%; dynamic range, 70 dB; and mechanical index, 0.12) were maintained constant throughout the course of this study. Mice were then euthanized and tumors excised for histologic analysis. Quantitative region-of-interest analysis for pixel intensity in tumors was performed using ImageJ software,³¹ and representative sonograms were acquired 10 seconds after contrast-enhanced sonography began. Matching (25.4-mm^2) regions of interest were compared in all groups for mean pixel intensity. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

Histologic Analysis

Serial sections of $5.0 \mu\text{m}$ thickness were cut from formalin-fixed, paraffin-embedded tissue blocks and floated onto charged glass slides (Super-Frost Plus; Fisher Scientific, Pittsburgh, PA) and dried overnight in a 60°C oven. A hematoxylin-eosin-stained section was obtained from each tissue block. All sections subject to immunohistochemistry were deparaffinized and hydrated with deionized water. The tissue sections were heat treated with buffer

containing 0.01-mol/L Tris and 1-mmol/L EDTA (pH 9) using a pressure cooker (CEPC 800; Cook's Essentials, Caitang, China) for 5 minutes at maximum pressure (15 lb/in²). After antigen retrieval, all sections were gently washed in deionized water and then transferred to a 0.05-mol/L Tris-based solution in 0.15-mol/L sodium chloride with 0.1% (vol/vol) Triton X-100 (pH 7.6). Endogenous peroxidase was blocked with 3% hydrogen peroxide for 10 minutes. To further reduce nonspecific background staining, slides were incubated with 3% normal goat or horse serum for 20 minutes (Sigma-Aldrich, St Louis, MO). All slides were then incubated at 4°C overnight with rabbit anti-CD51 (Enzo Life Sciences, Ply-mouth Meeting, PA), goat anti-CD62p (Santa Cruz Biotechnology, Santa Cruz, CA), or rabbit anti-VEGFR2 (Cell Signaling, Danvers, MA). Negative controls were achieved by eliminating the primary antibodies from the diluents. After washing with the Tris–sodium chloride–Triton X-100 solution, peroxidase-conjugated goat anti-rabbit IgG (for CD51 and VEGFR2) and rabbit anti-goat IgG (for CD62p; Jackson ImmunoResearch, West Grove, PA) were applied to the sections for 30 minutes at room temperature. Diaminobenzidine (ScyTek Laboratories, Logan, UT) was used as the chromagen and hematoxylin (7211; Richard-Allen Scientific, Kalamazoo, MI) as the counterstain.

Statistical Analysis

Data are reported as mean $\pm$ SE. Statistical comparisons were performed using the SAS software package (SAS Institute Inc, Cary, NC). Assessment of in vitro experiments within single-targeted microbubble groups 1, 2, and 3 was performed with an analysis of variance test of microbubbles per cell attachment. This procedure was then repeated for intra–dual-targeted microbubble groups 1, 2, and 3. Intra-group data were combined after no significant difference was found within each group. To assess the statistical differences between overall single-, dual-, and triple-targeted motifs, all intragroup data were combined, and an unpaired 2-sample *t* test was used to compare single- versus dual-targeted microbubble groups and then repeated for dual- versus triple-targeted microbubble groups. To gauge the effect of mean pixel intensity with counts per pixel during quantitative analysis of contrast-enhanced sonograms, an unpaired 2-sample *t* test was used to compare the combined dual-targeted groups to the triple-targeted group. An analysis of variance test was used within the dual-targeted groups to ensure no significant differences, permitting combination of all dual-targeted microbubbles to be compared to triple-targeted microbubbles. Microbubble characterization was assessed by performing an unpaired 2-sample *t* test between the mean fluorescence of $\alpha_v\beta_3$ -integrin and VEGFR2. Microbubble characterization between the combinations of all dual-targeted groups and the triple-targeted group was performed using an unpaired 2-sample *t* test. Evaluation of synergy in vivo was performed using a paired 2-sample *t* test between the cumulative singular components that constituted the multitargeted groups and the groups themselves. $P < .05$ was considered statistically significant.

Results

Receptor Expression

For the in vitro studies, mouse SVR angiosarcoma cells were used because of the neoplastic endothelial origin of this cell line. To quantify receptor expression in these cells, characterization was established with antibodies against mouse $\alpha_v\beta_3$ -integrin, P-selectin, and VEGFR2 using flow cytometric analysis. Figure 1a displays the normalized (background-subtracted) fluorescent counts from this study. Targeted receptor expression was found to be relatively similar, albeit not statistically equal ($P > .05$), between $\alpha_v\beta_3$ -integrin (184.2 ± 13.3 counts) and VEGFR2 (116.5 ± 8.1 counts) compared to the expression of P-selectin (907.6 ± 19.6 counts), which was found to be higher ($P < .01$) in these cells.

Antibody-Microbubble Conjugation

Potential improvements in the binding performance of multitargeted microbubbles are attributed to the synergistic contribution of the antibodies and not to the preferential binding of a single target. To show that these antibodies contribute uniformly to the enhanced binding of the multitargeted microbubbles, characterization of the conjugation was performed. Figure 1b displays the results of flow cytometric analysis of the individual targeted microbubble groups. In the single-targeted groups, group 1 counts (370.3 ± 33.6) were similar ($P > .71$) to group 3 counts (364.4 ± 16.2). However, the fluorescent count for group 2, which represented the P-selectin antibody-microbubble conjugation, was 2-fold less (179.8 ± 17.1). In the dual-targeted groups, group 2 ($\alpha_V\beta_3$ -integrin + VEGFR2) counts (390.7 ± 12.3) were found to be comparable to the single-targeted groups containing $\alpha_V\beta_3$ -integrin and VEGFR2 antibodies. In the dual-targeted groups containing P-selectin antibodies (groups 1 and 3), the antibody-microbubble characterization counts were less (73.1 ± 8.7 and 130.1 ± 3.3 , respectively). The triple-targeted microbubble counts (239.6 ± 22) were significantly ($P < .05$) lower than counts observed for all 3 single-targeted groups. It is hypothesized that free biotin molecules in the P-selectin solution blocked streptavidin interaction with the biotinylated antibodies, thereby reducing the antibody-microbubble conjugation efficiency in those groups involving biotinylated P-selectin antibodies (single-targeted group 2, dual-targeted groups 1 and 3, and the triple-targeted group). Importantly, the level of the antibody conjugated to the triple-targeted microbubbles (239.6 ± 22.0) was not significantly higher ($P > .25$) than the average level of the antibody conjugated to the dual-targeted microbubble groups (198.0 ± 8.1).

In Vitro Binding Assay

The performance of triple-targeted microbubbles was evaluated using an in vitro binding assay with mouse SVR cells. As shown in Figure 1a, these cells express the individual receptors proposed for targeting. To illustrate the advantage of using the triple-targeting motif, single- and dual-targeted microbubbles were analyzed for comparison. As shown in Figure 2a, there was a statistically significant difference in adherent microbubbles per cell between the single-targeted (2.1 ± 0.15 microbubbles per cell) and dual-targeted (3.2 ± 0.3 microbubbles per cell) groups ($P < .001$). No significant difference was found during intra-group comparison with either the single-targeted ($P = .84$) or dual-targeted ($P = .41$) groups. For intergroup comparison, the triple-targeted microbubbles had higher binding efficiency (4.8 ± 0.4 microbubbles per cell) over the dual-targeted microbubbles (3.2 ± 0.3 microbubbles per cell; $P < .001$). Representative images (original magnification $\times 400$) are shown in Figure 2b for each of the targeted microbubble groups bound to mouse SVR cells. The isotype-labeled (rat IgG) microbubble group had no affinity for the mouse SVR cells during the in vitro binding assay.

In Vivo Contrast-Enhanced Sonography

To evaluate the performance of triple-targeted microbubbles in an animal model, MDA-MB-231 breast cancer cells were implanted into the mammary fat pad of athymic female nude mice. In Figure 3, representative images from a single mouse acquired 10 seconds after beginning contrast-enhanced sonography are shown. These images show the progression from single- to triple-targeted microbubbles and the developing intratumoral enhancement of vascular targeting, with the triple-targeted microbubbles displaying the greatest intensity. Quantitative analysis of these images showed a significantly higher mean pixel intensity in the triple-targeted microbubble injection (45.0 ± 5.2) compared to the dual-targeted microbubble injection (32.3 ± 3.3 ; $P = .006$), further verifying the enhanced performance of the triple-targeted motif. Considering that the amounts of antibodies conjugated to the microbubbles in the triple-targeted groups were not significantly greater ($P > .25$) than those

in the dual-targeted groups, the overall improvement in triple-targeted microbubble binding is attributed to the synergistic benefit of targeting 3 receptors.

Analysis for Synergy

To describe the synergistic effects of multitargeted microbubble binding, dual- and triple-targeted microbubble performances were compared to their respective single-targeted constituents' performance in both the in vitro binding assay and the in vivo contrast-enhanced sonographic evaluation with the MDA-MB-231 breast cancer animal model. In Figure 4a, the in vitro performance of the dual-targeted groups was significantly greater ($P < .05$) than the cumulative performance of the single-targeted groups. In each evaluation, the dual-targeted microbubble groups were compared to their cumulative single-targeted microbubble performance. In Figure 4b, results from the in vivo evaluation of the dual-targeted microbubble groups are compared to their single-targeted microbubble constituents' ability to bind tumor vasculature in a breast cancer animal model. Although the dual-targeted groups enhanced vasculature imaging in these cases, the difference was not significant ($P > .05$). The triple-targeted groups performed significantly better than the collective single-targeted microbubble components in both the in vitro ($P < .05$) binding assay with mouse SVR cells (Figure 4c) and in vivo ($P < .05$) contrast-enhanced sonographic evaluation (Figure 4d). Importantly, all data were normalized by antibody amounts added during microbubble conjugation.

Histologic Results

To show that enhanced binding of the triple-targeted construct was not due to the preferential binding of a single receptor, histologic analysis was performed to qualify targeted receptor expression in vivo. In Figure 5, representative images show histologic staining using antibodies against $\alpha_V\beta_3$ -integrin (Figure 5a), P-selectin (Figure 5b), and VEGFR2 (Figure 5c). The staining of these adjacent histologic slices shows positive expression of $\alpha_V\beta_3$ -integrin, P-selectin, and VEGFR2 in the tumors. Figure 5d shows the hematoxylineosin stain, which confirms that receptor expression was detected along the viable tumor periphery.

Discussion

Reported here is an evaluation of triple-targeted microbubbles for improved contrast-enhanced sonography of tumor vasculature. Targeted microbubbles were prepared using avidin-biotin linkage of antibodies against mouse $\alpha_V\beta_3$ -integrin, P-selectin, and VEGFR2. In vitro analysis showed a 52% increase in dual-targeted binding over the single-targeted microbubble groups and a 50% increase in triple-targeted microbubble binding over the dual-targeted microbubble groups when tested on angiosarcoma endothelial cells. Taking into account the 6-fold increase in P-selectin expression in these cells relative to $\alpha_V\beta_3$ -integrin and VEGFR2 expression, one would expect to see enhanced performance in the P-selectin-targeted groups. However, there was no significant difference found during intragroup comparison of the single-targeted groups, which points to the synergistic effect of multitargeting.

When in vivo analysis was performed with MDA-MB-231 tumor-bearing mice comparing the microbubble groups, increased visualization of tumor vasculature was achieved with the triple-targeted microbubbles, generating a 40% increase in image intensity over the dual-targeted microbubbles. A previous study evaluating dual- versus single-targeted microbubbles performed consecutive group dosing in the same animals and randomized the order of administration.¹⁷ However, in our study, the order of administration was biased toward blocking the overall performance of the multitargeted groups. For each tumor-

bearing animal, single-targeted microbubbles were administered followed by dual- and finally triple-targeted microbubbles. Residual antibodies outstanding from previous microbubble groups could serve to block subsequent group binding, thereby decreasing the amount of targeted microbubbles sequestered in the tumor, altering the overall microbubble targeting potential. The fact that the triple-targeted microbubble group performed significantly better than the single- and dual-targeted groups, despite the administration bias, shows the superiority of the triple-targeted motif.

To characterize the relative amounts of conjugated antibodies in the targeted microbubble groups, flow cytometric analysis was performed. The results showed that targeted microbubbles involving anti-P-selectin contained on average 2-fold fewer conjugated antibodies than targeted microbubbles without P-selectin antibodies. It is hypothesized that these reduced numbers were due to an excess of free biotin in the P-selectin antibody solution resulting from a poor biotin-P-selectin antibody conjugation. The free biotin would essentially act to block available streptavidin molecules from biotinylated antibody binding. This decrease in relative amounts of antibodies in the targeted microbubble groups involving P-selectin would lead to a decrease in vasculature binding. However, it was also shown that P-selectin was expressed nearly 6-fold higher in mouse SVR cells compared to $\alpha_v\beta_3$ -integrin and VEGFR2 expression. Considering the decreased antibody conjugation of the microbubble groups involving P-selectin, it was shown that the triple-targeted microbubbles did not contain more antibodies compared to the single- and dual-targeted microbubble groups ($P > .25$). Given the fact that P-selectin receptors were 6-fold higher than the other 2 receptors, even with 2 times less P-selectin antibody on the microbubbles, one would expect the binding in the vasculature to be dominated by P-selectin. The fact that it was not dominated by P-selectin but significantly improved by targeting the other 2 receptors in combination validates the synergy of the triple-targeting approach. In view of these conclusions, the poor conjugation involving P-selectin antibodies served as an additional means of verification for the triple-targeted motif and was included in the results.

To quantify the synergy generated when targeting multiple receptors, the binding performance between dual- and triple-targeted groups was compared to the binding performance of their single-targeted microbubble components. Performance values used for comparison were normalized against conjugated antibody amounts. When dual-targeted microbubble in vitro binding performances were compared to the sum of their single-targeted microbubble constituents performances, there was a significant ($P < .05$ for all comparisons) increase in dual-targeted microbubble binding. This increase can be attributed to the enhanced performance of antibodies working in combination to achieve greater binding than antibodies working independently. When dual-targeted microbubble groups were compared to single-targeted microbubble components in vivo, there was an increase in dual-targeted microbubble performance in most cases; however, it was not significant. This result may arise from a limitation in the contrast-enhanced sonographic system to exclude unbound transient microbubbles. For the triple-targeted microbubble comparison to single-targeted microbubble constituents, there was a significant ($P < .05$) increase in the overall performance of the triple-targeted motif over the collective single-targeted components when both in vitro and in vivo analyses were performed.

Another advantage of targeting multiple receptors simultaneously is the improved potential for microbubble binding when endothelial receptor expression is unknown. The performance of single-targeted microbubbles depends on the ability of the targeted microbubble to bind a single receptor. Therefore, shear flow notwithstanding, the density of the receptor target on the endothelium determines the amount of potential microbubble binding that may occur. When targeting multiple receptors, the opportunity for microbubble binding is dependent on the receptor density of multiple target receptors whose expression and consequent

microbubble binding may be independent of one another. Targeting microbubbles to tissue endothelium using a triple-targeted strategy improves the probability of microbubble binding 3-fold.

One explanation for the improved affinity of triple-targeted microbubbles for the vessel lumen can be found in the biological mechanism of leukocyte rolling and adhesion. The VEGFR2 receptor binds the small VEGF peptide, which is released by cancer cells during hypoxia.³² The $\alpha_v\beta_3$ -integrin is a slow-acting but high-affinity molecular fastener, anchoring cells during leukocyte recruitment to areas of inflammation.³³ P-selectin is a fast-acting, highly specific cell adhesion molecule expressed on activated endothelial cells, which acts to sequester leukocytes and platelets from vascular circulation.¹⁹ These adhesion molecules work in tandem to recruit cells and are powerful components of the cell rolling and adhesion mechanism, which works against the shear forces and turbulent flow of circulation to bind and sequester an entire cell. To successfully complete this undertaking, nature has selected a mechanism that involves multiple selectins and integrins, which work in combination to facilitate cell extravasation. This cell-arresting method is analogous to the mechanism desired for successful targeted microbubble imaging of the vasculature.²¹ The multitargeting of ultrasound contrast agents uses the normal biological mechanism involved in cell adhesion, giving the triple-targeted microbubble an advantage in successful binding and overall vascular accumulation.

The microbubble binding of cells in culture using the triple-targeted motif was improved by 50% over the dual-targeted motif. This improvement equated to an additional binding of 1.6 microbubbles per cell using the triple-targeted microbubble design. Although this number may initially seem small, considering the potential number of endothelial cells contained within a tumor, the number of microbubbles bound using the triple-targeted construct grows substantially when the total cell number is taken into account. In a tumor containing 1×10^6 endothelial cells, the number of dual-targeted microbubbles potentially bound to the vessel wall would surmount 3.2×10^6 microbubbles. In the same tumor vasculature, administration of triple-targeted microbubbles would result in 4.8×10^6 bound microbubbles. This difference would provide added visualization of the tumor vasculature during contrast-enhanced sonography. In addition, greater microbubble accumulation in the tumor vasculature would also lead to greater drug delivery during contrast-enhanced sonographically assisted chemotherapy.

Reported here is an advanced method of actively targeting microbubbles using a triple-targeted strategy. The molecular targets used, $\alpha_v\beta_3$ -integrin, P-selectin, and VEGFR2, provided a method to assess the potential of a triple-targeted microbubble construct during contrast-enhanced sonography, and the use of these targets in permutation was shown to provide enhanced visualization of tumor vasculature. The improvement in vascular targeting reported here is due to the synergistic advantage of targeting 3 receptors in combination. It is proposed that substituting $\alpha_v\beta_3$ -integrin, P-selectin, and VEGFR2 with 3 new receptor targets that are overexpressed in the tumor vasculature would not alter the trend toward increased binding. In the future, actively targeting microbubbles to multiple receptors will provide added visualization for cancer detection, disease staging, and vessel assessment. The noninvasive, inexpensive use of sonography in combination with multi-receptor-targeted microbubbles allows the modality to be widely used in the evaluation of tumor vasculature for treatment evaluation and disease characterization.

Acknowledgments

This research was supported in part by National Institutes of Health grant UL1RR025777 and National Cancer Institute grant CA13148-38.

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Abbreviations

IgG	immunoglobulin G
VEGF	vascular endothelial growth factor
VEGFR2	vascular endothelial growth factor receptor 2

Figure 1. $\alpha V\beta_3$ -Integrin, P-selectin, and vascular endothelial growth factor receptor 2 (VEGFR2) characterization. **a**, Relative expression of $\alpha V\beta_3$ -integrin, P-selectin, and VEGFR2 in mouse SVR cells as determined by flow cytometry. Normalized fluorescent counts represent total fluorescent counts minus control fluorescent counts. **b**, Characterization (flow cytometry) of biotinylated antibodies to streptavidin-coated microbubble conjugation with single-targeted microbubble groups (MB_{S1} , MB_{S2} , and MB_{S3}), dual-targeted microbubble groups (MB_{D1} , MB_{D2} , and MB_{D3}), and the triple-targeted microbubble group (MB_T). Data are reported as mean $\pm$ SD.

Figure 2.

In vitro evaluation of single-targeted, dual-targeted, and triple-targeted microbubbles in mouse SVR cells. **a**, Manual counts of adherent microbubbles per cell with single-targeted microbubble groups (MB_{S1}, MB_{S2}, and MB_{S3}), dual-targeted microbubble groups (MB_{D1}, MB_{D2}, and MB_{D3}), and the triple-targeted microbubble group (MB_T) using in vitro microscopy. **b**, Representative in vitro microscopic images (original magnification $\times 400$) of various targeted microbubble groups on mouse SVR cells. The MB_C group represents isotype-targeted microbubbles. Asterisks indicate statistical significance. Data are reported as mean $\pm$ SE.

Figure 3.

Contrast-enhanced sonograms of targeted microbubble groups in a tumor-bearing animal model. Shown are representative images from a single mouse taken over the study duration. Groups include isotype-targeted microbubbles (MB_C), single-targeted microbubbles (MB_{S1}, MB_{S2}, and MB_{S3}), dual-targeted microbubbles (MB_{D1}, MB_{D2}, and MB_{D3}), and triple-targeted microbubbles (MB_T).

Figure 4.

Comparison of targeted microbubble group performance showing the synergistic benefit of the triple-targeted configuration. **a** and **b**, Performance of dual-targeted microbubble groups (MB_{D1} , MB_{D2} , and MB_{D3}) compared to their respective single-targeted microbubble constituents' (MB_{S1} , MB_{S2} , and MB_{S3}) during the in vitro binding assay on mouse SVR cells (**a**) and contrast-enhanced sonography in an MDA-MB-231 breast cancer animal model (**b**). **c** and **d**, Performance of the triple-targeted microbubble group (MB_T) compared to the single-targeted constituents during the in vitro binding assay (**c**) and contrast-enhanced sonography in the breast cancer animal model (**d**). Normalized MBs/cell represents the binding performance of each group divided by the antibody amounts conjugated to the microbubble. Mean pixel intensity (arbitrary units [a.u.]) represents the mean pixel intensity as determined by region-of-interest analysis divided by the antibody amounts conjugated to the microbubble. Data are reported as mean $\pm$ SE.

Figure 5.

Histologic analysis of $\alpha_v\beta_3$ -integrin, P-selectin, and vascular endothelial growth factor receptor 2 (VEGFR2) expression in MDA-MB-231 breast cancer tumors. Representative images (original magnification $\times 100$) from a single tumor show $\alpha_v\beta_3$ -integrin expression (a), P-selectin expression (b), and VEGFR2 expression (c). d, Hematoxylineosin stain from the same tumor.

Table 1

Antibody-Microbubble Conjugation Groups

Group	Isotype	$\alpha_v\beta_3$ -Integrin	P-Selectin	VEGFR2
MB _C	√			
MB _{S1}		√		
MB _{S2}			√	
MB _{S3}				√
MB _{D1}		√	√	
MB _{D2}		√		√
MB _{D3}			√	√
MB _T		√	√	√

MB_C indicates isotype-targeted microbubble group; MB_D, dual-targeted microbubble group; MB_S, single-targeted microbubble group; and MB_T, triple-targeted microbubble group.