Monitoring Response to Anticancer Therapy by Targeting Microbubbles to Tumor Vasculature

Grzegorz Korpanty,1 Juliet G. Carbon,1 Paul A. Grayburn,3 Jason B. Fleming,1 and Rolf A. Brekken1,2

Abstract Purpose: New strategies to detect tumor angiogenesis and monitor response of tumor vasculature to therapy are needed. Contrast ultrasound imaging using microbubbles targeted to tumor endothelium offers a noninvasive method for monitoring and quantifying vascular effects of antitumor therapy. We investigated the use of targeted microbubbles to follow vascular response of therapy in a mouse model of pancreatic adenocarcinoma.

Experimental Design: Microbubbles conjugated to monoclonal antibodies were used to image and quantify vascular effects of two different antitumor therapies in s.c. and orthotopic pancreatic tumors in mice. Tumor-bearing mice were treated with anti-vascular endothelial growth factor (VEGF) monoclonal antibodies and/or gemcitabine, and the localization of microbubbles to endoglin (CD105), VEGF receptor 2 (VEGFR2), or VEGF-activated blood vessels (the VEGF-VEGFR complex) was monitored by contrast ultrasound.

Results: Targeted microbubbles showed significant enhancement of tumor vasculature when compared with untargeted or control IgG–targeted microbubbles. Video intensity from targeted microbubbles correlated with the level of expression of the target (CD105, VEGFR2, or the VEGF-VEGFR complex) and with microvessel density in tumors under antiangiogenic or cytotoxic therapy.

Conclusions: We conclude that targeted microbubbles represent a novel and attractive tool for noninvasive, vascular-targeted molecular imaging of tumor angiogenesis and for monitoring vascular effects specific to antitumor therapy in vivo.

The use of antiangiogenic agents holds great promise for cancer therapy (1, 2); however, it is crucial to develop surrogate markers of pathologic angiogenesis that will permit monitoring of response to antiangiogenic therapy in cancer patients (3). Hence, noninvasive imaging of tumor vasculature could be clinically useful for both diagnosis and monitoring of tumor response to therapy. Other imaging modalities can be used to image tumor vasculature. However, their expense and ionizing radiation limit patient accessibility (3). In contrast, ultrasound is the most widely used imaging technique, is inexpensive, is portable, and provides noninvasive real-time imaging (4). Introduction of microbubbles as contrast agents for ultrasound has improved image quality and diagnostic value (5). Microbubbles are small, 1 to 4 μm in diameter, bubbles constructed of a lipid or albumin shell, and are filled with a biologically inert gas (e.g., perfluoropropane). They behave hemodynamically like RBCs. The unique properties of microbubbles as true intravascular tracers have enabled noninvasive measurements of microvascular perfusion in heart, brain, kidney, skeletal muscle, skin grafts, and solid tumors (6). Furthermore, microbubbles can be directed selectively to specific vascular beds by conjugating targeting ligands (peptides and antibodies) to their surface (5, 7). In this regard, molecular imaging using targeted microbubbles has been used in vivo to assess inflammation, angiogenesis, and thrombus formation (6).

Cancer of the exocrine pancreas is characterized by extensive local invasion and metastases to the liver, which translates into a 5-year survival rate of 1% to 4% for all patients diagnosed with pancreatic adenocarcinoma (8). The current best therapy, including surgery, radiation, and chemotherapy, has done little to alter cancer-related deaths of these patients (9), emphasizing the need for more effective therapy. The progressive growth and metastasis of pancreatic cancer and other solid tumors is dependent on angiogenesis, the development of new vasculature from preexisting blood vessels and/or circulating endothelial stem cells (10). Vascular endothelial growth factor (VEGF) is a primary stimulant of angiogenesis in tumors (11, 12). Blocking VEGF activity is an attractive strategy for therapy of pancreatic tumors because human pancreatic adenocarcinoma cells secrete high levels of VEGF in vitro and in vivo (13). Additionally, VEGF and its receptors (VEGFR1 and VEGFR2) are expressed at higher levels in pancreatic adenocarcinoma than in...
normal pancreatic tissue (14). Furthermore, high levels of VEGF expression within the primary pancreatic tumor correlate with decreased time to recurrence after curative resection, liver metastasis, and cancer-related death (15). Preclinical animal models of pancreatic cancer that evaluated different strategies to inhibit VEGF activity [DC101, a rat monoclonal antibody (mAb) specific for murine VEGFR2, and A.4.6.1, a mouse mAb specific for human VEGF] showed reductions in tumor growth (16, 17). However, recent phase II clinical trials with the humanized version of A.4.6.1, bevacizumab (Avastin), in combination with gemcitabine (Gemzar), a nucleoside analogue with known activity against pancreatic adenocarcinoma, have generated only modest therapeutic responses (18, 19). The disparity between the results of preclinical animal studies and early clinical trials is likely due to multiple tumor and patient factors. Improved therapeutic outcomes might result from a better strategy to monitor patient response to VEGF inhibition and/or gemcitabine.

We recently presented a new method of linking microbubbles to targeting moieties by incorporating avidin into the microbubble shell and biotinylating the targeting agent (20). In the present study, we used contrast ultrasound with targeted microbubbles to evaluate vascular effects of antiangiogenic and cytotoxic therapy in a mouse model of pancreatic adenocarcinoma. The goal of this study is to show that contrast ultrasound with targeted microbubbles can be used to effectively monitor response to different therapeutic regimens in multiple mouse models of pancreatic cancer. To address this goal, we show that contrast signal using microbubbles targeted to endoglin (CD105), VEGFR2, or the VEGF-VEGFR complex correlates with immunohistochemically assessed vascular expression of these markers and with tumor microvessels density (MVD). Thus, we propose that ultrasonic molecular imaging using targeted microbubbles could be used for noninvasive detection of tumor angiogenesis and assessment of vascular expression of molecular markers in response to therapy.

Materials and Methods

Cell culture. The murine (Pan02) and human (MiaPaca-2) pancreatic adenocarcinoma cell lines were obtained from the Developmental Therapeutics Program (Division of Cancer Treatment and Diagnosis, National Cancer Institute, Frederick, MD) and the American Type Culture Collection (Manassas, VA), respectively, and maintained as described (21). The mAbs MJ7/18 (22), AFRC Mac 48 (Mac 48, a rat IgG2a specific for phytochrome; European Collection of Animal Cell Cultures, Salisbury, United Kingdom), 9G10 (23) C44, 2C3, and 100 g of 2C3 mAb, or 3.5 mg gemcitabine. Treatment was initiated 21 days after tumor cell injection with biweekly i.p. injections of C44 (control), 200 g of 2C3 mAb, or combination of 200 g of 2C3 mAb and 3.5 mg gemcitabine. Treatment was continued in all mice for 2 weeks. Final tumor weight was calculated in conjunction with the residual pancreas.

Mice were housed in pathogen-free facility, and all experiments were done according to a protocol approved by the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center (Dallas, TX).

Ultrasound imaging with targeted microbubbles. Ultrasound imaging was done in straight B-mode using a S15-6L probe with 15 to 6 MHz extended operating frequency (Sonos 5500, Philips Ultrasound, Bothell, WA). Mechanical index was kept at 0.1 throughout the study. All animals were imaged using the same instrument settings and conditions. Images were obtained 5 minutes after i.v. injection of 100 g of washed microbubbles conjugated with control IgG (Mac 48), anti-CD105, anti-VEGFR2, or anti-VEGF-VEGFR complex specific mAbs. There was a 1-hour time interval between microbubble injections. The order of injection of untargeted, control IgG–targeted, or targeted microbubbles was randomized. Targeted microbubbles did not show toxicity, and animals recovered after imaging without any detectable distress.

All images were acquired in time-triggered mode (600 ms) during which the imaging probe was moved over the surface of the tumor to acquire representative images for the entire tumor volume. To image s.c. tumors, the probe was placed perpendicular to the sagittal axis of the animal and a minimum of 10 images was obtained from each animal. To obtain images of orthotopic pancreatic tumors, the probe was placed over the anterior abdomen and the spleen and kidney were identified and subsequently the probe was adjusted to image the pancreas, which lies between them. Myocardial imaging was done with the same protocol; however, the probe was placed over the chest of the animal and the images of the midventricular short axis of the heart were obtained.

Images were recorded digitally on an optical disc and analyzed offline. Pixel intensity (video intensity; ref. 28) was quantified in tumor regions using Yabko software (Yabko LLC 2001 University of Virginia Patent Foundation, Charlottesville, VA). Average video intensity was measured automatically by the software after the region of interest that lies between them. Myocardial imaging was done with the same protocol; however, the probe was placed over the chest of the animal and the images of the midventricular short axis of the heart were obtained.
Table 1. Summary of in vivo experiments

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Figure</th>
<th>n</th>
<th>Tumor location</th>
<th>Therapy</th>
<th>Vascular target</th>
</tr>
</thead>
<tbody>
<tr>
<td>MiaPaca-2</td>
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<td>5</td>
<td>S.c.</td>
<td>Anti-VEGF</td>
<td>VEGFR2</td>
</tr>
<tr>
<td>Pan02</td>
<td>3</td>
<td>5</td>
<td>S.c.</td>
<td>Gemcitabine</td>
<td>CD105 + VEGFR2</td>
</tr>
<tr>
<td>MiaPaca-2</td>
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<td>4</td>
<td>Orthotopic</td>
<td>Anti-VEGF ± Gemcitabine</td>
<td>VEGF-VEGFR complex</td>
</tr>
</tbody>
</table>

NOTE: We did three in vivo experiments with either MiaPaca-2 or Pan02 cells to show the use of targeted microbubbles in monitoring the response of s.c. or orthotopic pancreatic tumors to either anti-VEGF or gemcitabine therapy. In each experiment, there were four or five mice per treatment group (n) and endothelial cells were targeted by linking microbubbles to antibodies directed to VEGFR2, CD105, or the VEGF-VEGFR complex.

Results

Noninvasive evaluation of vascular VEGFR2 levels after antiangiogenic therapy. The three therapy experiments we did in tumor-bearing mice are summarized in Table 1. Because microbubbles are obligate intravascular tracers, we sought first to determine if antiangiogenic therapy affected the localization of microbubbles targeted to endothelial antigens known to be altered by antiangiogenic strategies. Blocking VEGF activity in solid tumors has been shown to decrease the expression of VEGFR2 on tumor endothelial cells (30, 31). To show that microbubbles targeted to VEGFR2 could be used to non-invasively visualize this change, s.c. human pancreatic adenocarcinoma (MiaPaca-2) tumors were treated with the anti-VEGF mAb 2C3 or with a control IgG. Inhibition of tumor-derived VEGF with 2C3 resulted in decreased tumor growth and MVD compared with control-treated mice (Fig. 1A and B). After 5 weeks of therapy, mice were imaged with contrast ultrasound using VEGFR2-targeted microbubbles. There was a significant decrease in contrast enhancement in mice treated with 2C3 compared with the control-treated group (Fig. 1C and D). Relative video intensity values for animals treated with 2C3 (8.6 ± 1.5) were significantly lower than in control-treated animals (14 ± 1.1; Fig. 1D). Furthermore, blocking VEGF from
binding to VEGFR2 decreased expression of VEGFR2 on vascular endothelial cells (Fig. 1E). As reported previously, tumor contrast enhancement was not uniform when using targeted microbubbles (28). The majority of signal enhancement, particularly in mice treated with anti-VEGF therapy, was found in the periphery of the tumor. Histologic analysis showed larger, smooth muscle actin–positive vessels present on the border of the tumor when compared with necrotic core, thus offering likely explanation for the heterogeneity of contrast enhancement (Fig. 2). It is important to note that the changes observed after anti-VEGF therapy are not specific for 2C3-based therapy but also occur after therapy with Avastin. Therapy with Avastin was similar to treatment with 2C3 in terms of effect on tumor size, MVD, and VEGFR2 expression as determined by contrast ultrasound with targeted microbubbles or by immunofluorescence (data not shown).

Ultrasound images of healthy myocardium obtained with untargeted, control IgG–targeted, and VEGFR2-targeted microbubbles showed no difference in contrast enhancement. Furthermore, quantification of video intensity values of the myocardium showed no significant differences in control- or 2C3-treated animals (Table 2). These results show that blocking VEGF activity in the tumor with a systemic anti-VEGF antibody does not affect blood vessels in the heart and presumably in other normal tissues.

Expression of VEGFR2 and CD105 decreases in pancreatic tumor treated with gemcitabine. To determine if cytotoxic therapy also alters the expression of endothelial antigens that can be targeted with microbubbles, mice bearing s.c. tumors were treated with gemcitabine and imaged with microbubbles targeted to either CD105 or VEGFR2 (Fig. 3). Mice bearing s.c. Pan02 tumors treated for 5 weeks with gemcitabine had significantly smaller tumors and decreased tumor MVD (141.9 ± 22.7 vessels/mm²) compared with control-treated animals (210.8 ± 51.9 vessels/mm²; Fig. 3A and B). Contrast-enhanced ultrasound examination of gemcitabine and control-treated mice at the end of the experiment showed only scant tumor enhancement after infusion of untargeted or control IgG–targeted microbubbles (Table 2). However, significant signal enhancement compared with control IgG–targeted microbubbles was observed after administration of microbubbles conjugated with either anti-CD105 or anti-VEGFR2 mAbs (Table 2).

Gemcitabine therapy significantly decreased video intensity and relative video intensity from targeted microbubbles compared with video intensity from tumors in control-treated animals (Table 2; Fig. 3C). Furthermore, the level of CD105 and VEGFR2 expression on tumor vascular endothelial cells determined by immunofluorescence (Fig. 3D) correlated with the noninvasive determination by video intensity for microbubbles targeted to each endothelial cell marker.

Noninvasive monitoring of VEGF-activated blood vessels in orthotopic pancreatic tumors corresponds to response to therapy. To extend the studies described above, we evaluated the effect of therapy on contrast ultrasound using targeted microbubbles in established orthotopic pancreatic tumors in nude mice. Three weeks after orthotopic pancreatic (MiaPaca-2) tumors were established in nude mice, the animals were treated with a control IgG or the anti-VEGF mAb 2C3 alone or in combination with gemcitabine. Therapy was continued for 2 weeks at which time the mice were sacrificed and tissue, including the tumor, was harvested. 2C3 alone or in combination with gemcitabine controlled the growth of the tumors compared with control-treated animals, resulting in tumors of 0.8 ± 0.51 g, 0.8 ± 0.3 g, and 1.46 ± 0.4 g, respectively. To determine if contrast ultrasound using targeted microbubbles could follow the response of these orthotopic tumors to therapy, animals from each group were imaged on days 0, 7, and 14 of therapy. We chose to follow the VEGF-VEGFR complex using mAb Gv39M as a marker of VEGF-induced angiogenesis. On day 0, there was no significant difference in relative video intensity of tumors in mice from each group. However, on day 7, after two rounds of therapy, there was a significant decrease in relative video intensity in mice treated with 2C3 alone (6.2 ± 2.0) or in combination with gemcitabine (7.6 ± 3.4) when compared with relative video intensity values at day 0 and with control-treated mice on day 7 (14.9 ± 4.6). Two weeks after initiation of treatment (day 14), relative video intensity in the tumor decreased significantly in mice treated with 2C3 alone (11.1 ± 2.8) and in combination with chemotherapy (9.3 ± 2.7) compared with control-treated mice (13.7 ± 5.1; Fig. 4A).
VEGF-VEGFR complex levels on tumor vessels quantified by immunofluorescence after mice were sacrificed (day 14) correlated with relative video intensity of tumor images obtained with microbubbles targeted to the VEGF-VEGFR complex (Fig. 4B). The level of expression of the VEGF-VEGFR complex decreased significantly as determined by immunofluorescence in mice treated with 2C3 alone and in combination with gemcitabine compared with control-treated animals. 2C3

![Image](https://example.com/image.png)

**Table 2. Specificity of targeting tumor vasculature with microbubbles**

<table>
<thead>
<tr>
<th>Rx</th>
<th>Organ</th>
<th>Group</th>
<th>Video intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Untargeted</td>
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<tr>
<td>Anti-VEGF</td>
<td>Tumor</td>
<td>Control IgG</td>
<td>24.9 ± 4.4</td>
</tr>
<tr>
<td>Anti-VEGF</td>
<td>Tumor</td>
<td>2C3</td>
<td>24.4 ± 3.6</td>
</tr>
<tr>
<td>Anti-VEGF</td>
<td>Heart</td>
<td>Control IgG</td>
<td>12.0 ± 2.0</td>
</tr>
<tr>
<td>Anti-VEGF</td>
<td>Heart</td>
<td>2C3</td>
<td>11.7 ± 1.7</td>
</tr>
<tr>
<td>Gemcitabine</td>
<td>Tumor</td>
<td>Control IgG</td>
<td>23.9 ± 2.5</td>
</tr>
<tr>
<td>Gemcitabine</td>
<td>Tumor</td>
<td>Gemcitabine</td>
<td>23.0 ± 2.1</td>
</tr>
</tbody>
</table>

NOTE: Video intensity of contrast ultrasound images captured from the target organ displayed in s.c. tumor-bearing mice treated with either anti-VEGF therapy or gemcitabine. Microbubbles were either untargeted or linked to a control IgG (IgG), anti-CD105 (CD105), or anti-VEGFR2 (VEGFR2). Animals in the anti-VEGF therapy studies were treated with a control IgG or 2C3, whereas animals in the gemcitabine therapy studies were treated with vehicle only or gemcitabine. Targeting microbubbles to CD105 or VEGFR2 significantly increased video intensity in tumor tissue compared with untargeted or control-targeted (IgG) microbubbles. Therapy (anti-VEGF or gemcitabine) did not alter video intensity from untargeted or control IgG–targeted microbubbles but did significantly decrease video intensity in the tumor from targeted (CD105 or VEGFR2) microbubbles. There was no specific targeting of microbubbles to cardiac tissue, and therapy did not alter video intensity in the heart from any of the microbubble preparations. Each value represents the mean and SD from at least 10 images from the target organ from each animal (n = 4 animals/group).

Abbreviation: NA, not available.

*P < 0.005 versus control IgG–targeted microbubbles.

**P < 0.05 versus control-treated animals.

Fig. 3. Gemcitabine therapy reduces tumor growth, MVD, and CD105 and VEGFR2 expression on tumor endothelial cells. Mice bearing s.c. pancreatic adenocarcinomas were treated with vehicle (control; n = 5) or gemcitabine (n = 5). Therapy was initiated 7 days after tumor cell injection and continued twice weekly for 5 weeks. A, columns, mean tumor weight at the time of sacrifice; bars, SD. B, MVD assessed by Meca32 immunohistochemistry at the time of sacrifice. *, P < 0.05. C, relative video intensity of contrast-enhanced images obtained using microbubbles targeted to CD105 or VEGFR2 showed significant decrease in tumors grown in animals treated with gemcitabine. *, P < 0.05. D, the level of expression of CD105 and VEGFR2 in tumors from mice treated with control or gemcitabine was determined by quantitative immunofluorescence. Immunofluorescent signal was quantified using MetaMorph software, and specific signal is presented as percentage of pixels exceeding the background (% Threshold). *, P < 0.05; **, P < 0.005 versus control. Total magnification, ×200.
alone or in combination with chemotherapy also decreased MVD significantly compared with tumors in the control group (Fig. 4C).

**Discussion**

In the present study, we show that contrast-enhanced ultrasound targeted to tumor vasculature can be used as a molecular imaging tool to follow response to cytotoxic and antiangiogenic therapy in a mouse model of pancreatic cancer. We also show that targeted ultrasonic imaging can detect changes in expression of vascular markers on tumor vessels in vivo and that this noninvasive measure corresponds with immunohistologic analysis.

Significant advances in contrast ultrasound imaging during the past decade have expanded applications for microbubbles from being passive blood pool enhancement entities to molecular imaging agents specifically targeting endothelium (5, 6). We developed a method of conjugating targeting moieties to albumin-based microbubbles and have shown previously specific binding of targeted microbubbles to endothelial cells in vitro (20). Here, we report in vivo application of microbubbles targeted to distinct endothelial markers, VEGFR2, the VEGF-VEGFR complex, and CD105. S.c. pancreatic tumors were used to show that targeted microbubbles are efficacious for following the relative expression of CD105 or VEGFR2 on tumor endothelial cells after antiangiogenic or cytotoxic therapy. S.c. tumors offer advantages, such as accessibility and the ability to follow tumor size (volume) over time in response to therapy. However, it is clear that the microenvironment of s.c. tumors does not accurately reflect the environment in which pancreatic tumors develop naturally. For example, it has been shown that the orthotopic pancreas microenvironment enhances VEGF expression, which stimulates growth of human xenograft tumors compared with s.c. implants (32). About imaging, it is also much more challenging to image visceral tumors, such as orthotopic pancreatic tumors, than a s.c. implanted tumor. Therefore, we sought to show that targeted microbubbles could also be used in an orthotopic tumor setting.

Targeting microbubbles to vascular markers resulted in significant signal enhancement when compared with untargeted or control IgG–targeted microbubbles (Table 2). Furthermore, targeting microbubbles to vascular markers
allowed noninvasive detection of changes in vascular expression of the antigen as a result of therapy. These changes were specific to the tumor vasculature. Contrast ultrasound imaging of nontumor tissues, including myocardium, showed no significant difference in contrast enhancement using any of the microbubble constructs in the presence or absence of antitumor therapy. Quantitative video intensity analysis confirmed this observation, suggesting that CD015 and VEGFR2 are expressed at a higher level on tumor endothelium than normal endothelium and that this up-regulation is due in part to the tumor microenvironment. For example, blocking VEGF activity with 2C3 (Fig. 1) or Avastin (data not shown) resulted in a detectable change in the level of VEGFR2 on tumor endothelium, suggesting that microbubbles targeted to VEGFR2 could potentially be used as a noninvasive marker of anti-VEGF activity. These results are consistent with previous observations made by our group and others showing that the number of VEGFR2-positive blood vessels in tumors from animals treated with anti-VEGF therapy decreases (30, 31).

Gemcitabine therapy of s.c. tumors also resulted in a quantitative decrease in ultrasound signal enhancement after injection of microbubbles targeted to either CD105 or VEGFR2 (Table 2; Fig. 3C). Endoglin (CD105) is a promising diagnostic and prognostic marker of tumor vasculature in various human malignancies (33, 34) and has been previously shown to be modulated by chemotherapy (35). However, to our knowledge, gemcitabine-induced changes in tumor VEGFR2 levels have not been reported previously.

The VEGF-VEGFR complex is an attractive marker of tumor vessels (24, 36) for both diagnostic and therapeutic vascular targeting. Immunohistochemical reactivity of mAbS that bind to the VEGF-VEGFR complex has previously been used to show VEGF-induced angiogenesis in tumor tissue and to correlate with poor prognosis (36, 37). We show here that noninvasive detection of VEGF-activated blood vessels in orthotopic pancreatic tumors is an effective monitor of response of tumors to antiangiogenic and cytotoxic treatment.

Robust surrogate markers that predict response to antiangiogenic or cytotoxic chemotherapy are needed (38). The expressions of CD105, VEGFR2, and the VEGF-VEGFR complex are examples of potential targets, which can be monitored on tumor endothelial cells by contrast ultrasound using targeted microbubbles. However, this technology could be applied to virtually any cell surface target expressed by endothelium in the tumor microenvironment. The major finding of the present study is that microbubbles targeted to tumor vasculature can be used to noninvasively monitor vascular response to antitumor therapy. This strategy also allows noninvasive approximation of expression of markers on tumor vascular endothelium, variables currently used to predict response to therapy.

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References


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