Angiogenesis Model for Ultrasound Contrast Research: Exploratory Study

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Rationale and Objectives. To optimize an angiogenesis model for imaging research that is stable and can be imaged several times over the angiogenic time course.

Materials and Methods. Mice and rats received two injections of 0.4 mL of extract of basement membrane matrix (Matrigel; Becton Dickinson Labware, Bedford, MA) in the subcutaneous spaces on either side of the spine. One of the two Matrigel plugs in each animal had either 0.1 μg/mL of basic fibroblast growth factor (bFGF) (11 mice), 1.0 μg/mL of bFGF (12 mice, 5 rats), or 1.0 μg/mL of bFGF and 60 U/mL of heparin (11 mice). Three to 12 days after implantation, animals were imaged before and after the administration of up to four injections of 0.1 mL AF0150. Phase inversion imaging was used on a Siemens Elegra (Siemens ultrasound, Issaquah, WA) equipped with a 13 MHz VFX transducer. Three observers subjectively assessed the pattern of enhancement using a four-point scale. The Matrigel plugs were then removed and two observers graded the angiogenic response on a four-point scale. Ten Matrigel plugs, five with 1.0 μg/mL bFGF and five without, were evaluated histologically following immunohistochemical staining with anti-CD31.

Results. The angiogenic response was greater in Matrigel plugs with 1.0 than with 0.1 μg/mL of bFGF. Heparin did not increase the angiogenic response. Vessels were predominantly at the periphery of the plugs with variable central penetration. Plugs appeared anechoic and homogeneous on ultrasound. Contrast enhancement within the plug occurred in 44% of mice with an angiogenic response at or after day 6 and the enhancement increased with the angiogenic response. In the others, peripheral enhancement could not be distinguished from the enhancement of surrounding tissues that were also hyperemic. The thicker rat skin interfered with plug assessment.

Conclusion. A stable angiogenesis model without the complexity of tumors is described. This model offers the opportunity to image the development and/or inhibition of angiogenesis. Neovasculature in Matrigel was detectable using ultrasound contrast. Quantitative studies correlating the degree of enhancement to microvascular density will be determined in subsequent studies.

Key Words. Angiogenesis; ultrasound; microbubbles; model; animal.
Angiogenesis plays an essential role in many physiologic and pathological processes including wound healing, arthritis, psoriasis, and tumor growth (1). Continual growth of capillaries provides tumors access to nutrients and the opportunity to metastasize to distant sites (2). Noninvasive assessment and follow-up of the angiogenic process could be useful for diagnosis and for monitoring the effect of therapy. While tumors would be the best model, tumor angiogenesis is a complex process that involves many steps and interactions between tumor cells and matrix, as well as tumor and host that introduce variability even within the same tumor. The development and testing of diagnostic and/or therapeutic systems can be aided if the model is stable and more predictable among animals. For these reasons, a nontumor angiogenesis model has been extensively used in fundamental angiogenesis research (3–6). This model consists of an extract of basement membrane matrix (Matrigel; Becton Dickinson Labware, Bedford, MA) to which a growth factor can be added. Matrigel is a complex mixture of basement membrane proteins including laminin, type IV collagen, entactin/nitrogen, and proteoheparan sulfate from which growth factors have been removed (3,5). The unique property of Matrigel is that it is liquid at 4°C and gels at room temperature (RT). When angiogenic growth factors are added, sprouts from adjacent vessels penetrate into the gel within days to form a new capillary network (3–6). If the angiogenic process in such a system can be imaged, it would be an ideal tool to study angiogenesis in vivo and to allow follow-up after anti-angiogenic therapy.

Microbubble-based ultrasound (US) contrast agents are limited in their distribution to the intravascular space as these microbubbles are 2–3 μm in diameter. Because of the high spatial resolution of US that can be viewed in real-time, the high sensitivity of US that can detect a single microbubble, and the availability of contrast specific imaging techniques that suppress background signal, US contrast imaging has the potential to visualize and quantify the angiogenic process in vivo (7,8).

The main objective of this study was to determine whether the Matrigel model could be used for US imaging and to optimize the model for US contrast research.

**MATERIALS AND METHODS**

**Matrigel Model**

Forty National Institute of Health (NIH) Swiss mice 6- to 7-week-old and five rats were used in the experiment. They were housed in microisolator cages with laboratory chow and water available ad libitum. Animals were anesthetized before all procedures by intra peritoneal injections of ketamine (50 mg/kg) and acepromazine (1 mg/kg) and observed until fully recovered. The protocol was reviewed and received approval by our institutional Animal Subject Committee in accordance with the United States Department of Agriculture, Department of Health and Human Services, and the NIH policies regarding the humane care and use of laboratory animals.

Using different volumes of Matrigel in six mice, we determined that 0.4 mL of Matrigel injected subcutaneously was large enough to form a plug that could be consistently visualized and was small enough to be practical. This volume was used for the remainder of the 34 mice and five rats studied. Matrigel at 4°C (liquid state) was mixed with 0.1 or 1.0 μg/mL of basic fibroblast growth factor (bFGF; R&D Systems, Minneapolis, MN). Because of reports suggesting that the angiogenic response could be maximized with heparin (6), 60 U/mL of heparin was added to 11 plugs. 0.4 mL of Matrigel with additives was injected subcutaneously in the right paraspinal space and 0.4 mL without additives was injected in the left paraspinal space as control (Table). The Matrigel had to be kept on ice until the time of injection and the needle and syringe had to be cooled to prevent gelling in the needle.

**Imaging Procedure**

Animals were anesthetized as before, their backs shaved and a 25-gauge butterfly needle inserted in the tail vein. A Styrofoam box was constructed to hold the animal with the head and tail exposed (Fig 1). Ultrasound gel was heated to 35°C and 300 μg of cellulose per 100 mL of gel was added as tissue mimicking material. The gel was then centrifuged to remove the air bubbles. The space in the Styrofoam box surrounding the animal was completely filled with the warmed gel to provide contact and to keep the animal warm during imaging session. A

<table>
<thead>
<tr>
<th>Number of Animals Injected with 0.4 mL of Matrigel to which Heparin and/or bFGF were Added</th>
<th>bFGF (μg/mL)</th>
<th>With Heparin (60 UI/mL)</th>
<th>Without Heparin</th>
</tr>
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<tbody>
<tr>
<td>Mice</td>
<td>0.1</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Mice</td>
<td>1.0</td>
<td>5</td>
<td>18</td>
</tr>
<tr>
<td>Rats</td>
<td>1.0</td>
<td>0</td>
<td>5</td>
</tr>
</tbody>
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US standoff pad was placed on top of the box ensuring that it was in full contact with the gel (Fig 1). Imaging was performed with a 13-MHz VFX linear transducer and phase-inversion technique as implemented on a Sonoline Elegra (Siemens ultrasound, Issaquah, WA). Once an optimal transverse imaging plane allowing the adequate visualization of both Matrigel plugs was selected, the transducer was mechanically fixed in place.

Intravenous bolus injection of 0.1 mL of a microbubble-based US contrast agent, AF0150 (Imagent; Perfluorohexane Lipid Microspheres, IMCOR Pharmaceuticals, San Diego, CA) which encapsulates perfluorohexane in a thin lipid shell (5–8 × 10^8 microbubbles/mL) was given through the tail vein, followed by a 0.1 mL saline flush. Injections were repeated up to four times with various imaging settings. At least one series was performed with a transmit frequency of 12 MHz and 16% of maximum power. Once the setting was chosen, all parameters were held constant from before contrast administration to the end of the observation period.

Several frames were acquired at baseline before contrast administration. Imaging was halted and then re-initiated 30 seconds after the saline flush to allow the contrast agent to reach equilibrium. The post-contrast frames were acquired in intermittent mode at one frame per second for 5–10 seconds. The entire scan session was recorded on S-VHS videotapes. The entire imaging procedure from anesthesia to the last imaging series lasted 20–30 minutes. Mice were imaged once between days 3 and 12 and rats imaged on days 3, 6, and 12 after Matrigel implantation.

**Post-Mortem Analysis**

Immediately following imaging, animals were killed with pentobarbital sodium, the plugs removed, placed on a white background, and photographed. Plugs were then placed in a cryomold and embedded in an optimal cutting temperature compound for 30 seconds. They were flash frozen in liquid nitrogen and stored at −80°C.

**Qualitative Grading of Ultrasound Contrast Enhancement**

Because acquisition was halted until 30 seconds after the contrast injection, the first image exhibits the highest enhancement because the capillary bed is filled with microbubbles before starting the enhanced imaging sequence. Hence, contrast enhancement of each Matrigel plug was assessed by comparing the last pre-contrast frame with the first post-contrast frame of the same series. Because the aim of this preliminary study was to appreciate the ability to visualize (and not to quantify) the angiogenic activity induced in a Matrigel plug using contrast-enhanced US, degree of contrast enhancement within each plug was qualitatively assessed and graded using a
Macroscopic Grading of Angiogenic Response

Two observers (O.L., Y.K.) reviewed the photographs of each plug, which were not identified, and graded by consensus the angiogenic response using a four-point scale as detailed in Figure 3.

When possible, differences in the fraction of plugs were compared among groups for statistical significance using Fisher’s exact test due to the small number of mice. All P values were two-tailed and considered significant when they were less than .05.

Immunohistochemistry

Ten Matrigel plugs extracted from mice, five impregnated with bFGF and assigned an angiogenic response greater than grade II and their respective control plugs, were evaluated histologically. Five-micrometer sections were fixed in acetone for 5 minutes at RT followed by triple washes with a phosphate-buffered solution. Endogenous biotin was removed by incubating the sections with 0.1% avidin for 15 minutes followed by incubation with 0.01% biotin for 15 minutes at RT. Sections were then incubated with rat anti-mouse CD31 (PharMingen/BD, San Diego, CA) antibody in 1% bovine serum albumin/phosphate-buffered saline for 30 minutes at RT. They were then incubated with biotin-labeled goat anti-rat IgG (PharMingen/BD) for 30 minutes at RT followed by incubation with horseradish peroxidase streptavidin (Jackson ImmunoResearch, West Grove, PA). Triple washes with phosphate-buffered saline were performed between all the steps detailed above. Sections were developed with Vector VIP substrate kit for peroxidase (Vector Labs, Burlingame, CA) for 2–6 minutes. Isotype control staining was carried out for all specimens. Sections were counterstained with hematoxylin. Three serial sections were taken from each plug and observed with a light microscope at 20× magnification by one observer (T.N.) to determine the neovascular pattern.

RESULTS

The injected Matrigel rapidly formed a single, solid gel plug under the skin. It remained intact and fixed at the initial site of injection for the 12-day observation period. In mice, macroscopically, Matrigel plugs were transparent and yellow-orange in color with reddish tubular structures
located more densely at the periphery than the center, easily seen on a white background. The macroscopic angiogenic grading of the 34 control plugs (left side) was 0 (n = 17/34, 50%) or I (n = 17/34, 50%). In the 34 bFGF added plugs the angiogenic response was 0 in four of 34 (12%), I in eight of 34 (23%), II in 15 of 34 (44%), and III in seven of 34 (21%). Because 50% of the control plugs were graded I, we only considered grades II and III as a significant angiogenic response. The effects of bFGF dose and heparin on the angiogenic response are shown in Figure 4. Regardless of heparin, Matrigel plugs impregnated with 1 μg and 0.1 μg of bFGF/mL exhibited an angiogenic response in 19 of 23 (83%) and two of 11 (18%) of cases (P < .001), respectively. The addition of heparin did not significantly affect the angiogenic response (P = .53). The angiogenic response gradually increased from day 3 after implantation and reached a plateau on day 7 to 9 (Fig 5); however, the number of mice

<table>
<thead>
<tr>
<th>Grade</th>
<th>Definition</th>
<th>Example</th>
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<tr>
<td>0</td>
<td>No vessel</td>
<td><img src="image1" alt="Example" /></td>
</tr>
<tr>
<td>I</td>
<td>Few tiny peripheral vessels</td>
<td><img src="image2" alt="Example" /></td>
</tr>
<tr>
<td>II</td>
<td>Larger vessels with shallow penetration</td>
<td><img src="image3" alt="Example" /></td>
</tr>
<tr>
<td>III</td>
<td>Several large vessels with deep penetration</td>
<td><img src="image4" alt="Example" /></td>
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Figure 3. Assignment of macroscopic angiogenic grade.

Figure 4. Fraction of plugs with a significant angiogenic response (graded II or III, see Materials and Methods section) on gross inspection as a function of bFGF dose and heparin. 1 μg/mL of bFGF induced a greater effect that was not significantly influenced by the addition of heparin.
was too small to reach statistical significance (day 3 vs day 7–9; \( P = .15 \)).

Two mice where lost because of anesthesia, therefore imaging data was available on 32 mice. The control Matrigel plugs appeared anechoic and homogeneous precontrast (Figs 6 and 7). Some of the plugs with bFGF had subtle echogenic regions (Fig 7a) precontrast. After contrast administration, the enhancement of the 32 control plugs was graded 0 (\( n = 15/32; 47\% \)) or 1 (\( n = 17/32; 53\% \)). Grade 1 reflected few hyperechoic artifacts coming from the peripheral tissues and covering partially the peripheral part of the plugs. The enhancement in the 32 bFGF added plugs was graded 0 in 12 of 32 (38%), 1 in 10 of 32 (31%), 2 in seven of 32 (22%), and 3 in three of 32 (9%). The subtle echogenic region seen within the Matrigel plugs precontrast enhanced following contrast administration (Fig 7b). Because artifacts from the surrounding tissues affected the ability to assess peripheral enhancement (graded 1 in 31% of the control plugs; Fig 7b) we only considered grades 2 and 3 as significant enhancement. No enhancement greater than 1 was seen on day 3 while eight of the 18 mice (44%) that had a positive angiogenic response as assessed macroscopically, enhanced post-contrast at or after day six (Figs 6 and 7).

Figure 5. Fraction of plugs with 1 \( \mu \)g/mL of bFGF that had an angiogenic response graded II or III on gross inspection (\( n = 23 \)) as a function of days after implantation. The angiogenic response increased at day 3 and reached maximum after day 7.

Figure 6. In vivo appearance of Matrigel plugs implanted 10 days earlier imaged at 12 MHz precontrast (a). Note that the Matrigel plugs (arrows) are anechoic, located subcutaneously on either side of the spine (arrow head). The right Matrigel plug had 1 \( \mu \)g/mL of bFGF added. Thirty seconds following the administration of 0.1 mL of AF0150 (b) nodular enhancement (graded 3) in the bFGF added Matrigel plug (arrow) is visible. No enhancement was observed in the control plug.
The percent of plugs that enhanced as a function of the macroscopic angiogenic response is shown in Figure 8. The degree of enhancement increased as the macroscopic angiogenic response grade increased \((P = .06)\); however, the number of mice was too small to reach the 0.05 level.

No rats were lost to anesthesia, despite multiple imaging sessions, making the rat a more robust model. Both control and bFGF added plugs appeared hypoechoic or isoechonic compared with the paraspinal muscles. Because the skin is thicker than in mice, the surrounding tissues were more detrimental to the ability to judge peripheral enhancement. Contrast filled the entire plug in three out of the five rats studied in both the control and the bFGF added plugs. Macroscopically, the plugs were opaque and it was more difficult to assess the angiogenic response. No histologic analysis was performed on these plugs.

The reddish tubular structures visible on gross inspection in the mouse plugs were tortuous and multi-branching vessels with a coarse and grainy texture that stained with anti-CD31. Matrigel plugs implanted 6 or more days before sacrifice had vessels in the interior of the Matrigel plug more often than those implanted 3 days before sacrifice. A typical “hot spot” of angiogenesis is shown in Figure 9. Note the sparsely organized cells in an oval fashion suggesting a cross-sectional view of a vascular wall. The periphery of the Matrigel plug had denser neovascularity compared with the deeper layers. Control plugs had a lesser quantity of the multi-branching, tortuous vasculature. Matrigel without vessels was clear and had no anti-CD31 staining. Because separate slices were examined, it was not possible to determine if the angiogenic vessels terminated in the plug or formed continuous loops.

**DISCUSSION**

The high sensitivity of US to microbubble contrast agents and the fact that microbubbles are limited in their distribution to the intravascular space shows great poten-
tial for noninvasive assessment of the microcirculation. In addition, because microbubbles can be destroyed by the US wave, this allows for the theoretical potential to assess tissue fractional blood volume and blood flow using functional US imaging techniques (9,10). This potential may be more advantageous than the techniques used with x-ray, computed tomography, or magnetic resonance imaging because it is affected by fewer variables. Several studies have shown the ability of functional US imaging to assess fractional blood volume and relative regional blood flow in the heart (9,11,12), kidney (13) and brain (14,15). Because of the potential to quantify relative tumor blood flow and blood volume, there is an effort to assess angiogenesis and the effects of anti-angiogenic therapy in vivo. This study aimed to optimize and assess the potential to use a stable and histologically quantifiable angiogenesis model that has been exploited by molecular biologists.

The nontumor Matrigel model proposed eliminates the variability that exists with tumor models. It allows histologic morphology indices such as microvascular density (by endothelial staining), vascular space volume (by quantification of the hemoglobin trapped in the plug), or more complex analysis of the vascular network using mathematical analysis of vascular branching. In a histologic analysis study of the Matrigel plug, Passani et al (6) showed that growth factors including a combination of 0.001 to 0.1 μl/mL of bFGF and heparin (40 units/mL) induced apparent vascular proliferation 2–3 days after injection in C57/BL mice, reaching maximal response in 3–5 days. In their study, the magnitude of the angiogenic response increased with growth factor dose but was greater in plugs supplanted with heparin, suggesting that heparin is a potent inducer of angiogenesis. In our study we tested heparin added to 0.1 and 1.0 μl/mL of bFGF. While the 1.0 μl/mL of bFGF produced a greater response than the 0.1 μl/mL, there was no significant increase with the addition of heparin.

The Matrigel plug was consistently visualized with US as a hypoechoic to anechoic space in the mouse model. Four hundred μl of Matrigel was sufficient to provide enough thickness to observe internal enhancement when angiogenesis occurred. The proper handling of Matrigel is critical for the success of the model. Care must be taken to maintain Matrigel at 4°C as well as the needle and syringe to prevent gelling. This was possible by keeping all implements on ice. Mixing bFGF and filling the syringe have to be performed with care to prevent the introduction of air bubbles. While US detected the angiogenic response precontrast in some plugs, contrast administration enhanced the detection significantly allowing the potential to assess blood flow and fractional blood volume. The enhancement was predominantly seen at the periphery of the plug and extended into the center when the angiogenic response extended centrally. When only peripheral enhancement was observed, the enhancement was

Figure 9. Anti-CD31 staining (20× magnification) of a “hot spot” of angiogenesis obtained in a bFGF treated plug with newly formed endothelial cells. Note the formation of new vessels appearing as oval-shaped (arrows) and multi-branching structures (arrowhead).
at times confused with the enhancement of the surrounding tissues, predominantly the skin and the peritoneal lining. The ability to discern true peripheral enhancement from tissue enhancement was aided by comparing the enhancement of the treated to the control plug. Because of potential bias, experiments aimed at assessing enhancement require blinding the sonographer and observer. The main limitation of this exploratory and optimization study was the absence of blinding for the qualitative assessment of US enhancement. The thickness of the rat skin made assessment more difficult. While a larger volume could have been used in rats, there were a sufficient number of plugs that only enhanced along their periphery that made assessment difficult in rats.

Our primary motivation in developing this model was for angiogenesis research using US contrast agents, particularly to quantitate the response to anti-angiogenesis therapy and to study microbubbles targeted to activated endothelial cells. The dark hypoechoic background of the Matrigel plug should provide an ideal model to assess angiogenesis imaging. The mouse model using 0.4 mL of Matrigel impregnated with 1 μg of bFGF appears well suited for these investigations. While the Matrigel model is convenient and reproducible and ideally suited for early or controlled experiments, we caution the extrapolation of results to the tumor model that maintains a constant stimulus for angiogenesis and displays both spatial and temporal heterogeneity.

REFERENCES