Published in final edited form as:

Ultrasound Med Biol. 2006 May ; 32(5): 673–681.

ULTRASOUND ASSESSMENT OF ANGIOGENESIS IN A MATRIGEL MODEL IN RATS

Susanne M. Stieger^{*}, Susannah H. Bloch[†], Oded Foreman[‡], Erik R. Wisner^{*}, Katherine W. Ferrara[†], and Paul A. Dayton[†]

^{*} Department of Surgical and Radiological Sciences, School of Veterinary Medicine, University of California, Davis, CA, USA;

[†] Department of Biomedical Engineering, University of California, Davis, CA, USA; and

[‡] Comparative Pathology Laboratory, University of California, Davis, CA, USA

Abstract

Matrigel, a basement membrane extract, has been extensively used in *in vivo* angiogenesis. Contrast ultrasound imaging (CUI) of implanted Matrigel plugs with (+bFGF) and without basic fibroblast growth factor (–bFGF) was performed 7 and 14 d after implantation, followed by histologic analysis. Statistically significant differences between +bFGF and –bFGF plugs were apparent at d 7 in both plug size and contrast enhancement (both $p < 0.05$). Histopathology revealed differences in microvessel density (MVD) between +bFGF and –bFGF at d 7 and d 14. A significant correlation between MVD and both power Doppler contrast-enhanced area ($r = 0.65$, $p < 0.05$) and fraction of plug enhanced ($r = 0.59$, $p < 0.05$) was present. CUI of Matrigel plugs was shown to be a robust method for distinguishing between two different angiogenic states. Ultrasound measurements of blood flow in the plugs correlated with MVD, a histologic technique used to quantify tumor angiogenesis.

Keywords

Contrast ultrasound imaging; Angiogenesis; Matrigel; Tumor; Power Doppler; Rat

INTRODUCTION

Angiogenesis is a fundamental process by which new blood capillaries develop from a preexisting microvascular network stimulated by different growth factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and platelet derived growth factor. Solid tumors must continuously stimulate the growth of new blood vessels to enlarge, and furthermore, these blood vessels provide a gateway for tumor cell metastasis (Folkman and Shing 1992; Nehls et al. 1994; Wiggins et al. 1995; Carmeliet and Jain 2000; Liekens et al. 2001; Bergers and Benjamin 2003). Researchers have shown that angiogenesis is involved in many disease processes and is, in particular, an important prognostic factor in cancer development, progression and recurrence (Horak et al. 1992; Weidner et al. 1992; Wiggins et al. 1995).

Because of the fundamental role of angiogenesis in tumor development, several angiogenesis assays have been developed over the past few decades to investigate angiogenesis and antiangiogenic therapies. Some examples of commonly used assays include the chicken

embryo chorioallantoic membrane (CAM) assay (Auerbach et al. 1974), the dorsal skin fold chamber (Algire 1943), the corneal micropocket assay (Gimbrone et al. 1974; Ausprunk and Folkman 1977) and Matrigel plug or sponge assays (Malinda 2001; Akhtar et al. 2002; Kragh et al. 2003). Although *in vitro* angiogenesis assays have the advantages of simplicity and repeatability, they lack the biologic complexity of *in vivo* angiogenesis assays. Therefore, *in vivo* angiogenesis assays are necessary to more thoroughly understand this complex process. The Matrigel angiogenesis assay is easy to perform and reproducible, and the progression of angiogenesis can be observed within a few days. Matrigel is a soluble basement membrane extract of the Engelbreth-Holm-Swarm tumor. It has been demonstrated that when Matrigel is injected subcutaneously, endothelial cells migrate into the Matrigel plug and form vessels. This angiogenic process can be accelerated by adding known angiogenic growth factors, such as bFGF, to the Matrigel. The newly formed vessels can contain erythrocytes, indicating that they are functional capillaries (Passaniti et al. 1992; Malinda 2001; Auerbach et al. 2003).

Angiogenic activity is an important feature of solid neoplasms that may be predictive of biologic behavior and response to therapy but few techniques exist for noninvasively quantifying angiogenesis in a clinical setting. The only existing validated method for angiogenesis measurement *in vivo* is the histologic measurement of microvessel density (MVD). The MVD measurement in isolated regions of high vessel density (hotspots) from biopsy specimens or excised tumors appears to be useful in predicting metastasis risk, tumor growth and recurrence. However, the degree of MVD has not been proven to be a prognostic factor for angiogenic activity in a tumor or useful in planning anti-angiogenesis treatment (Hlatky et al. 2002; McDonald and Choyke 2003). In addition, MVD measurement is an invasive technique and representative tumor sampling is difficult due to tumor heterogeneity. Therefore, there is an acute need for developing imaging techniques that can quantitatively estimate angiogenesis in whole tumors, and in using these techniques to direct cancer therapy (McDonald and Choyke 2003; Miller et al. 2005).

Developing imaging strategies to evaluate angiogenic and anti-angiogenic responses and comparing the accuracy, specificity and sensitivity of different imaging techniques requires a standardized, reproducible model. Many angiogenesis imaging studies have been performed in tumor models (Fleming and Brekken 2003; Kiessling et al. 2003; Krix et al. 2003), but comparison across different studies is difficult because of differences in tumor cell lines, animal subjects, techniques and equipment. The Matrigel implant model allows the evaluation of pro- and antiangiogenic compounds. In addition, this model mimics the hypoxic microenvironment of a tumor, providing a good model to study tumor angiogenesis (Hasan et al. 2004). However, published studies using imaging methods for the evaluation of angiogenesis in Matrigel models are sparse. Phongkitkarun et al. (2004) used contrast-enhanced functional computed tomography (fCT) in a rat Matrigel model, but reported that fCT-derived measurements did not correlate well with MVD or other histologic measures of angiogenesis. Leong-Poi et al. (2003) successfully used contrast-specific ultrasound imaging of a mouse Matrigel model to study both blood flow and targeted contrast agent retention, but did not compare ultrasound-derived measurements to MVD. Lucidarme et al. (2004) used contrast ultrasound to study Matrigel plugs in both mice and rats. This study reported a near-significant correlation between ultrasound degree of enhancement and macroscopic angiogenic response in mice. However, these researchers found that the thicker rat skin interfered with ultrasound assessment of Matrigel plugs injected on either side of the spine.

Here, we report the results of a preliminary study using Matrigel plugs implanted in the caudo-ventral region of a rat. We were interested both in evaluating this model for angiogenesis imaging, and in comparing our contrast ultrasound-derived measurements to MVD, a standard measurement of angiogenesis.

MATERIALS AND METHODS

Animals

Eleven adult male, outbred albino Sprague-Dawley[®] rats (*Rattus norvegicus*), 400 to 450 g, were obtained from HSD (Harlan Sprague Dawley Inc., India-napolis, IN, USA). The animals were housed in a standard animal facility where light was controlled in a 12-h light-dark cycle. The animals were fed a standard diet and given free access to food and water. The animals were observed daily for abnormal clinical signs. All animal experiments were approved by the Institutional Animal Use and Care Administrative Advisory Committee.

Matrigel preparation

Growth factor reduced Matrigel (BD Biosciences, Bedford, MA, USA) was prepared just before use under sterile conditions. Matrigel was thawed overnight on ice at 4°C to become liquid. Care was taken that the Matrigel was at no time during handling warmed to room temperature. After the Matrigel was thawed, it was mixed on a vortex shaker and placed on ice until any bubbles dissipated. Two separate test conditions of Matrigel were prepared: a growth-factor enriched preparation, and a control. The enriched preparation (+bFGF) included 2500 ng of bFGF (BD Biosciences), and the control (−bFGF) included cold phosphate-buffered saline with the Matrigel to match the volume of the enriched sample. Syringes were loaded with the Matrigel solutions (1.8 mL each) and placed on ice.

Matrigel implantation

The rats were anesthetized by placing each rat into an induction chamber and introducing aerosolized isoflurane. Once anesthetized, the rat was removed from the chamber and anesthesia was maintained using isoflurane/O₂ administered by mask delivery. The preloaded syringes were used to slowly inject the Matrigel solution subcutaneously into the caudal ventral area (inguinal area) of the right and left side of the rats, the growth-factor enriched Matrigel (+bFGF) on the right side and the growth-factor reduced Matrigel (−bFGF) on the left side. The needle remained under the skin for about 10 s to allow the Matrigel to solidify before removing the needle at the end of the injection. A small, ovoid bump formed under the skin at each injection site. Care was taken that all rats were injected at approximately the same sites. Placement of the Matrigel plugs in the inguinal area allowed easy ultrasound access without significant artifacts attributable to respiratory motion.

Imaging groups

Seven rats were studied using a SONOLINE Elegra[™] ultrasound system (Siemens Medical Solutions USA, Inc., Ultrasound Division, Issaquah, WA, USA) with a VF13-5 linear array transducer in B-mode at 10.3 MHz and in power Doppler mode at 7.2 MHz, 100% transmit power and a soft tissue thermal index (TIS) of 0.5. Two of these seven rats were sacrificed for histologic examination after ultrasound examination on d 7; the remaining five were imaged on d 7 and d 14 and sacrificed on d 14. Four additional rats were studied using a SONOLINE Antares[™] ultrasound system (Siemens Medical Solutions USA, Inc., Ultrasound Division) with a VF10-5 linear array transducer in B-mode at 8.9 MHz, and in a greyscale multi-pulse imaging sequence (Cadence[™] contrast pulse sequencing, CPS) at 4.4 MHz, 0.2% power and an MI of 0.24 (Fig. 1). All four rats imaged using the Antares system were sacrificed on d 14.

Ultrasound imaging

Each rat was anesthetized using the same protocol as described above for the Matrigel implantation. The animal was placed in dorsal recumbency on a heating pad in a custom-made plastic holder used to maintain stable positioning during the ultrasound exam. A 24-gauge catheter was inserted into the tail vein for the injection of ultrasound contrast agent. The groin

area was shaved in all animals to yield a proper contact surface, and was covered with acoustic coupling gel. A gel standoff pad, independently suspended by a ring stand to reduce pressure on the underlying Matrigel plugs, was placed over the area to be imaged. The Matrigel plugs in all animals were first individually imaged in B-mode. The width, depth and height dimensions were recorded using the electronic calipers on the ultrasound system. Then, the transducer was positioned such that both Matrigel plugs were imaged at their largest transverse cross sections. The transducer was mechanically fixed in this position with an articulated arm for the remainder of the study.

Next, the ultrasound system was set to power Doppler or a greyscale multi-pulse imaging mode, and a baseline precontrast image was recorded. The greyscale multi-pulse imaging mode sends multipulse sequences per image line with varied phase and amplitude. Selective processing of the received echoes and subsequent addition of all echoes rejects linear fundamental signals from tissue and retains nonlinear echoes from the contrast agent (Phillips 2001). We evaluated varying frequencies with the greyscale multi-pulse imaging sequence and found that a transmission center frequency of 4.4 MHz provided the best contrast-to-tissue signal in this mode.

A lipid-shelled, perfluoropropane-filled ultrasound contrast agent, (MRX-815, ImaRx Therapeutics, Tuscon, AZ, USA), which is a research agent, was prepared according to the manufacturer's instructions. This agent contains microbubbles of $\sim 1.8 \pm 1.5$ microns in diameter, and is observed to persist in the rat circulation on the order of 5 min (as measured by contrast video intensity decrease to 20% of maximum; data not shown). Zero point four (0.4) mL of contrast agent was diluted with 2.6 mL of sterile saline and a 0.1 mL bolus of this diluted ultrasound contrast agent was injected IV, followed by a 0.2 mL saline flush to ensure that no residual agent remained in the catheter. Contrast-enhanced images were recorded 1 to 3 min after injection in all animals.

Histopathology and immunohistochemistry

After ultrasound imaging 7 or 14 d post implantation, the rats were sacrificed and the Matrigel plugs were removed along with underlying abdominal muscle and overlying skin for better histologic orientation. Routine paraffin-embedded histologic sections were cut at 5 μ m and stained with hematoxylin and eosin (HE). Immunohistochemistry was performed on 4 μ m thick paraffin-embedded sections following a routine protocol previously established (Norris et al. 2005) with the following modifications. The deparaffized sections were immersed in steam heated Citrate Target Retrieval (Dako Corporation, Carpinteria, CA, USA) for 20 min at 98° C. Affinity purified, goat-polyclonal CD31 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as a primary antibody, followed by a biotinylated rabbit-anti-goat secondary antibody (Vector Laboratories, Burlingame, CA, USA), which was diluted in 1:500 PBS/TritonX (Sigma-Aldrich Corp., St. Louis, MO, USA) with 1% heat inactivated normal rat serum.

Three hotspot areas containing the maximum number of discrete microvessels were identified by visually scanning the entire section of each tissue sample. A graticule with 16 squares of 1 mm $\times$ 1 mm was oriented over each hotspot so that the maximum number of graticule lines was on or within areas of highlighted vessels. In each hotspot, only vessels in which the endothelial cells formed a complete lumen within the field were counted using $\times 20$ magnification. The mean of the counts of the vessels from the three hotspot areas was calculated for each Matrigel plug (Fox 2001).

Image analysis

The ultrasound images were analyzed off-line using custom software written in Matlab (Mathworks, Inc., Natick, MA, USA) that allowed the user to specify regions of interest (ROIs)

defining each Matrigel plug. Image areas were scaled appropriately by calibration with a scale bar included in the ultrasound image. In the contrast-enhanced images, areas of contrast enhancement were defined as follows: 24-bit power Doppler images were separated into individual 8-bit red, green and blue images. Colored pixels were identified by their deviation from greyscale (where red, green and blue pixel values are equal). A threshold of 50 was used, so if for a given pixel in the image the absolute difference between the red value and the green value, or the green value and the blue value, exceeded 50, the pixel was considered colored, and therefore contrast-enhanced. This thresholding method was found to be sensitive and specific for the segmentation of the color pixels.

In the greyscale multi-pulse imaging sequence, the 8-bit greyscale image taken before contrast injection was subtracted from the image taken after contrast injection. If a postcontrast pixel value exceeded the corresponding precontrast pixel value by 10, the pixel was considered contrast-enhanced. Again, this threshold was determined by a heuristic method.

The cross-sectional area of each ROI (cm²) was extracted from a precontrast B-mode image in each plug. From postcontrast images at a frame rate of 0.2 Hz, we determined ROI cross-sectional area (cm²), fraction of pixels enhanced (number of enhanced pixels/number of pixels in ROI) and enhanced area (cm²). In cases where more than one postcontrast image was saved from one animal, we averaged the results over the saved images ($n = 1$ to 5) to reduce any bias in image selection.

In four rats imaged with the Elegra system, post-contrast images were obtained at frame rates of 13, 7, 5, 2, 1, 0.5, 0.2 and 0.03 Hz. These images displayed increasing enhanced area with decreasing frame rate due to microbubble destruction by high-intensity power Doppler pulses. Matlab was used to create one summary image showing first time of enhancement at each pixel in the image, and to calculate the overall fraction of enhanced pixels and the average time of enhancement in each plug ROI. The time of first enhancement was used to provide information about the difference in dynamic behavior of the newly formed vessels in each Matrigel pellet.

Statistics

Two-tailed paired Student's *t*-tests were used to compare results from the right and left plugs in each rat. Correlation between variables was assessed with Pearson's correlation coefficient, *r*. Results were considered statistically significant at $p \leq 0.05$.

RESULTS

The injection of the growth-factor enriched (+bFGF) and growth-factor reduced (−bFGF) Matrigel was easy to perform and was accomplished without any complications in all rats. None of the rats developed reactions at the site of injection and all rats tolerated the Matrigel plugs well. We were able to catheterize the tail vein of all the rats for the ultrasound contrast imaging studies.

Ultrasound observations of the Matrigel plugs

In B-mode, Matrigel plugs displayed a moderate echogenicity, between the anechoic appearance of the gel standoff pad and hyperechoic appearance of the skin. In all the Matrigel plugs, subtle hyperechoic and hypo-echoic spots and bands were observed. All Matrigel plugs could be differentiated from the surrounding tissue (Fig. 2, top images). At d 7, the +bFGF plug was obviously larger than the −bFGF plug, but this difference was less apparent by d 14. Precontrast power Doppler or contrast imaging mode images showed very little or no blood flow inside the plugs at d 7 and 14. After contrast administration, blood flow was observed in all plugs and was generally more prominent peripherally. At d 7 the +bFGF plug showed

significantly greater enhancement than the -bFGF plug; at d 14 this difference was less evident (Fig. 2, bottom images). These qualitative observations were confirmed by quantitative analysis (Table 1), as described in detail below.

Matrigel plug size

Seven d postimplantation, the +bFGF Matrigel plugs were significantly larger than the -bFGF plugs. The average plug volumes, calculated from the ultrasound caliper measurements using the formula for the volume of a prolate ellipsoid, $\text{length} \times \text{width} \times \text{height} \times \pi/6$, were 1.52 cm^3 and 0.76 cm^3 for +bFGF and -bFGF plugs, respectively ($p < 0.01$) (Fig. 3a). The average cross-sectional areas calculated from precontrast B-mode ROIs in these same cases were 0.85 cm^2 and 0.54 cm^2 ($p < 0.01$) (Fig. 3b).

Fourteen d post implantation, the average +bFGF and -bFGF plug volumes were 1.15 cm^3 and 0.91 cm^3 , and cross-sectional areas were 0.68 cm^2 and 0.56 cm^2 . There was no statistically significant difference between plug sizes at this time point.

Contrast-enhanced area

Seven d post implantation, the average enhanced area within each plug was 0.60 cm^2 (59% enhanced) for the stimulated plugs and 0.18 cm^2 (28% enhanced) for the nonstimulated plugs ($p < 0.01$) (Fig. 3c and d). Fourteen d post implantation, the average enhanced area within each +bFGF plug was 0.36 cm^2 (47% enhanced) and 0.29 cm^2 (40% enhanced) for the -bFGF plugs.

Average time to enhancement

An example composite image of power Doppler contrast enhancement 7 d post implantation is shown in Fig. 4. In this case, the mean time to first enhancement was 1.4 s in the +bFGF plug and 7.9 s in the -bFGF plug. The average enhancement times over the four cases, where varying frame rates were acquired, were 2.5 s at 7 d post implantation and 1.1 s at 14 d post implantation for +bFGF plugs, and 8.0 s at d 7 and 2.6 s at d 14 for -bFGF plugs. The difference between +bFGF and -bFGF plugs was statistically significant at d 7 ($p = 0.01$) but not at d 14.

The average fraction of plug enhanced at the lowest frame rate, 0.2 Hz (30 s between frames) was 74% and 77% for +bFGF plugs at d 7 and d 14, respectively; and 58% and 65% for -bFGF plugs at d 7 and d 14, respectively. The differences between +bFGF and -bFGF plugs approached statistical significance ($0.05 < p < 0.1$) at both time points.

Histologic observations

The formation of new blood vessels was observed in all the plugs. In many vessels, intravascular erythrocytes were seen, indicating the presence of blood flow in these vessels. Most vessels ranged from 6 to $30 \mu\text{m}$ in diameter, with rare dilated vessels reaching $60 \mu\text{m}$ in diameter. Vessels 6 to $15 \mu\text{m}$ in diameter were most common throughout all examined sections. In all plugs, there was a higher density of vessels along the periphery of the plugs, and vessels of larger diameter were seen primarily on the margins of the plugs with decreased diameter and density toward the center. In the two rats examined 7 d post implantation, a higher density of vessels was observed in the +bFGF plug compared to the -bFGF plug. On d 14 post implantation, there were larger diameter vessels within the plugs compared to d 7; in addition, vessels could be seen throughout the entire plug, although the periphery still showed higher vascular density as well as increased vascular diameter compared to the center of the plug (Fig. 5a and b). At this time point, the +bFGF and -bFGF plugs were more similar in appearance. A diffuse immune response was observed in all plugs, characterized by multifocal infiltrates of macrophages and smaller numbers of lymphocytes and plasma cells and rare neutrophils. The immune response increased over time and was more prominent on d 14 than on d 7.

Microvessel density

The average microvessel densities in the +bFGF plugs and -bFGF plugs removed at d 7 were 21 and 16, respectively. The two rats analyzed at this time point both displayed a lower hotspot density in the -bFGF plug, however, this difference was not statistically significant because of the small number of samples.

At d 14, the average hotspot densities in the +bFGF plugs and -bFGF plugs were 31 and 22, respectively. This difference was statistically significant with $p = 0.03$.

Correlation between microvessel density and contrast enhancement

Overall, microvessel density measurements displayed a significant correlation with power Doppler enhanced area ($r = 0.65$, $p < 0.05$), and enhanced fraction ($r = 0.59$, $p < 0.05$) (Fig. 6). The correlation between the microvessel density and the enhanced area in the greyscale multi-pulse imaging sequence was not statistically significant; however, only four rats were imaged.

DISCUSSION

The Matrigel model is a straightforward and reproducible model of angiogenesis in the rat. Tail vein catheterization was fairly straightforward; this procedure is much easier in rats than in mice because of the large size of the tail vein. Angiogenesis was observed in the Matrigel plugs in all animals. Compared to tumor models, Matrigel plugs display a less variable development pattern, and therefore a smaller number of animals is required in a study to detect statistically significant differences in angiogenesis. Studies of tumor models report a loss of up to 40% of animals due to death or unpredictable tumor growth rates (Schroeder et al. 2001).

Ultrasound at 10 MHz was well-suited to imaging 1 cm³ plugs, and the administration of ultrasound micro-bubble contrast agent through the tail vein allowed assessment of the degree of vascular development within each plug. Power Doppler imaging as well as greyscale multi-pulse imaging have shown promise to be very sensitive to small quantities of ultrasound contrast agent with a still reasonable spatial resolution. Ultrasound has been shown to be of such high sensitivity to contrast agents that even a single bubble echo can be detected. Additionally, contrast ultrasound imaging has the advantage over other contrast imaging techniques that the microbubbles are strictly intravascular and do not diffuse out into the extravascular space (Ferrara et al. 2000). In our study, the most common vessels were of capillary size, and in rare cases vessels up to 60 µm in diameter were present. Both power Doppler imaging and greyscale multi-pulse imaging are sensitive to small quantities of ultrasound contrast agent. This high sensitivity, combined with the intravascular nature of the agent, resulted in successful imaging of the microvasculature even though the spatial resolution (200 to 350 µm) was larger than any single microvessel.

We were able to identify differences in both size and blood flow within Matrigel plugs at two different stimulation levels: one with Matrigel alone, and one where basic fibroblast growth factor was added. The differences between these two angiogenic states were clearer 7 d post implantation than 14 d post implantation. We hypothesize that the significant differences at d 7 can be explained by the fact that the +bFGF plug shows a more rapid angiogenesis compared to the -bFGF plug, in accordance with previous studies (Passaniti et al. 1992). The increased angiogenesis of the stimulated Matrigel plug results in better perfusion and also increases the volume of the plug, because of vessel ingrowth and neovessel formation. The differences in the volume, enhanced fraction and enhanced area are less prominent 14 d postimplantation

because of the increased angiogenesis in the nonstimulated plug: even growth factor-reduced Matrigel will induce angiogenesis over time.

We did observe 15% to 30% variability in quantitative variables derived from the Matrigel assay due to physiological differences among the rats, however, the internal control provided by placing two plugs in the same rat was useful in accounting for this variability, and the variance we observed was much smaller, in our experience, than that observed in tumor xenografts. Both power Doppler imaging and the greyscale multi-pulse imaging sequence allowed good visualization of blood flow in both +bFGF and -bFGF Matrigel plugs. We found a significant correlation between power Doppler enhancement and histologic microvessel density count. A more sophisticated analysis such as destruction-reper-fusion imaging might be used to differentiate between blood flow and vascular volume.

CONCLUSION

Contrast-assisted ultrasound assessment of a Matrigel plug model in rats was shown to be a robust method for distinguishing between plugs in two different angiogenic states. Ultrasound measurements of blood flow in the plugs 7 and 14 d post implantation correlated with microvessel density, a widely used histologic measure of angiogenesis. The lower variability of Matrigel models compared to tumor xenograft models, combined with the relative size and robustness of rats compared to mice, make this model suitable for evaluating a wide range of imaging techniques. This technique might also prove to be a useful model for comparing different imaging methods performed at different research facilities.

Acknowledgements

The authors acknowledge the support of NIH 1R21CA098692, NIH CA 72062 and NIH BRP 103828, as well as the use of ultrasound equipment provided by Siemens Medical Solutions USA, Inc., Ultrasound Division, Issaquah, WA, USA. We thank ImaRx Therapeutics, Tuscon, AZ, USA for providing ultrasound contrast agents for this study.

References

- Akhtar N, Dickerson EB, Auerbach R. The sponge/Matrigel angiogenesis assay. *Angiogenesis* 2002;5:75–80. [PubMed: 12549862]
- Algire GH. An adaption of the transparent chamber technique to the mouse. *J Natl Cancer Inst* 1943;4:1–11.
- Auerbach R, Kubai L, Knighton D, Folkman J. A simple procedure for the long-term cultivation of chicken embryos. *Dev Biol* 1974;41:391–394. [PubMed: 4452416]
- Auerbach R, Lewis R, Shinnars B, Kubai L, Akhtar N. Angiogenesis assays: a critical overview. *Clin Chem* 2003;49:32–40. [PubMed: 12507958]
- Ausprunk DH, Folkman J. Migration and proliferation of endothelial cells in preformed and newly formed blood vessels during tumor angiogenesis. *Microvasc Res* 1977;14:53–65. [PubMed: 895546]
- Bergers G, Benjamin LE. Tumorigenesis and the angiogenic switch. *Nat Rev Cancer* 2003;3:401–410. [PubMed: 12778130]
- Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. *Nature* 2000;407:249–257. [PubMed: 11001068]
- Ferrara KW, Merritt CR, Burns PN, et al. Evaluation of tumor angiogenesis with US: imaging, Doppler, and contrast agents. *Acad Radiol* 2000;7:824–839. [PubMed: 11048880]
- Fleming JB, Brekken RA. Functional imaging of angiogenesis in an orthotopic model of pancreatic cancer. *J Cell Biochem* 2003;90:492–501. [PubMed: 14523983]
- Folkman J, Shing Y. Angiogenesis. *J Biol Chem* 1992;267:10931–10934. [PubMed: 1375931]
- Fox, SB. Microscopic Assessment of Angiogenesis. In: Clifford, Murray J., editor. *Angiogenesis protocols*. New Jersey: Human Press; 2001. p. 29-43.

- Gimbrone MA Jr, Cotran RS, Leapman SB, Folkman J. Tumor growth and neovascularization: An experimental model using the rabbit cornea. *J Natl Cancer Inst* 1974;52:413–427. [PubMed: 4816003]
- Hasan J, Shnyder SD, Bibby M, et al. Quantitative angiogenesis assays *in vivo*—a review. *Angiogenesis* 2004;7:1–16. [PubMed: 15302991]
- Hlatky L, Hahnfeldt P, Folkman J. Clinical application of antiangiogenic therapy: Microvessel density, what it does and doesn't tell us. *J Natl Cancer Inst* 2002;94:883–893. [PubMed: 12072542]
- Horak ER, Leek R, Klenk N, et al. Angiogenesis, assessed by platelet/endothelial cell adhesion molecule antibodies, as indicator of node metastases and survival in breast cancer. *Lancet* 1992;340:1120–1124. [PubMed: 1279332]
- Kiessling F, Krix M, Heilmann M, et al. Comparing dynamic parameters of tumor vascularization in nude mice revealed by magnetic resonance imaging and contrast-enhanced intermittent power Doppler sonography. *Invest Radiol* 2003;38:516–524. [PubMed: 12874518]
- Kragh M, Hjarnaa PJ, Bramm E, et al. *In vivo* chamber angiogenesis assay: An optimized Matrigel plug assay for fast assessment of anti-angiogenic activity. *Int J Oncol* 2003;22:305–311. [PubMed: 12527927]
- Krix M, Kiessling F, Vosseler S, et al. Sensitive noninvasive monitoring of tumor perfusion during antiangiogenic therapy by intermittent bolus-contrast power Doppler sonography. *Cancer Res* 2003;63:8264–8270. [PubMed: 14678984]
- Leong-Poi H, Christiansen J, Klibanov AL, Kaul S, Lindner JR. Non-invasive assessment of angiogenesis by ultrasound and micro-bubbles targeted to alpha(v)-integrins. *Circulation* 2003;107:455–460. [PubMed: 12551871]
- Liekens S, De Clercq E, Neyts J. Angiogenesis: Regulators and clinical applications. *Biochem Pharmacol* 2001;61:253–270. [PubMed: 11172729]
- Lucidarme O, Nguyen T, Kono Y, et al. Angiogenesis model for ultrasound contrast research: exploratory study. *Acad Radiol* 2004;11:4–12. [PubMed: 14746396]
- Malinda, K. *In vivo* Matrigel migration and angiogenesis assays. In: Clifford Murray, J., editor. *Angiogenesis protocols*. New Jersey: Human Press; 2001. p. 47-52.
- McDonald DM, Choyke PL. Imaging of angiogenesis: from microscope to clinic. *Nat Med* 2003;9:713–725. [PubMed: 12778170]
- Miller JC, Pien HH, Sahani D, Sorensen AG, Thrall JH. Imaging angiogenesis: Applications and potential for drug development. *J Natl Cancer Inst* 2005;97:172–187. [PubMed: 15687360]
- Nehls V, Schuchardt E, Drenckhahn D. The effect of fibroblasts, vascular smooth muscle cells, and pericytes on sprout formation of endothelial cells in a fibrin gel angiogenesis system. *Microvasc Res* 1994;48:349–363. [PubMed: 7537351]
- Norris AJ, Naydan DK, Wilson DW. Interstitial lung disease in West Highland White Terriers. *Vet Pathol* 2005;42:35–41. [PubMed: 15657270]
- Passaniti A, Taylor RM, Pili R, et al. A simple, quantitative method for assessing angiogenesis and antiangiogenic agents using reconstituted basement membrane, heparin, and fibroblast growth factor. *Lab Invest* 1992;67:519–528. [PubMed: 1279270]
- Phillips PJ. Contrast pulse sequences (CPS): Imaging nonlinear micro-bubbles. *Proceedings of the IEEE Ultrasonics Symposium* New York: Institute of Electrical and Electronic Engineers 2001;2:1739–1745.
- Phongkitkarun S, Kobayashi S, Kan Z, Lee TY, Charnsangavej C. Quantification of angiogenesis by functional computed tomography in a Matrigel model in rats. *Acad Radiol* 2004;11:573–582. [PubMed: 15147622]
- Schroeder RJ, Hauff P, Bartels T, et al. Tumor vascularization in experimental melanomas: correlation between unenhanced and contrast enhanced power Doppler imaging and histological grading. *Ultrasound Med Biol* 2001;27:761–771. [PubMed: 11516536]
- Weidner N, Folkman J, Pozza F, et al. Tumor angiogenesis: a new significant and independent prognostic indicator in early-stage breast carcinoma. *J Natl Cancer Inst* 1992;84:1875–1887. [PubMed: 1281237]
- Wiggins DL, Granai CO, Steinhoff MM, Calabresi P. Tumor angiogenesis as a prognostic factor in cervical carcinoma. *Gynecol Oncol* 1995;56:353–356. [PubMed: 7535719]

Fig. 1.
Ultrasound imaging protocol.

Fig. 2.

B-mode and 0.2 Hz power Doppler images of basic fibroblast growth-factor enriched (+bFGF, left side of images) and growth-factor reduced (-bFGF, right side of images) plugs 7 and 14 d after implantation (arrows). In this typical case, the +bFGF plug appeared to be larger and more highly perfused than the -bFGF plug on d 7. On d 14, the plugs appeared to be approximately the same size, and noticeable contrast enhancement was visible in the nonstimulated plug.

Fig. 3.

Quantitative ultrasound measurements of Matrigel plug size and perfusion in +bFGF (closed symbols) and -bFGF (open symbols) Matrigel plugs in 11 rats. Solid bars indicate average values. (a) Plug volume, calculated using ultrasound caliper measurements of three orthogonal dimensions using the formula for a prolate ellipsoid. (b) Area of a cross sectional region of interest (ROI) defined around each plug on a saved B-mode image. (c) Fraction of each plug enhanced by ultrasound contrast agent, from frames acquired at 0.2 Hz frame rate. (d) Total enhanced area within each plug ROI. In these four measurements, significant differences were observed between the +bFGF and -bFGF Matrigel plugs at d 7 postimplantation, but not at d 14.

Fig. 4.

Sample composite image of power Doppler contrast enhancement in the rat imaged in Fig. 2, 7 d after Matrigel plug implantation. Contrast enhancement occurs in the +bFGF plug (left side of image) at higher frame rates than in the -bFGF plug (right side of image), indicating that contrast replenishment due to blood flow occurs more quickly in the stimulated plug.

Fig. 5.

Histologic image of the +bFGF Matrigel implant (core on top, margin on bottom), 7 d (a) and 14 d (b) post implantation. HE stain, Bar = 100 μ m. Higher density and larger diameter vascular structures (white arrows) are present on the margin of the implant compared to the core region. At d 14, a higher density of vascular structures as well as diffuse inflammatory infiltrate (black arrows) is present compared to d 7.

Fig. 6.

A significant correlation was observed between microvessel density and the fraction of plug enhanced in contrast-enhanced power Doppler images of Matrigel plugs. Open symbols indicate -bFGF Matrigel plugs; closed symbols indicate +bFGF Matrigel plugs.

Summary of quantitative B-mode ultrasound, contrast-enhanced ultrasound and histology results for growth-factor enriched (+bFGF) and growth-factor reduced (-bFGF) Matrigel plugs

Table 1

	Day 7		<i>p</i>	Day 14		<i>p</i>
	+bFGF	-bFGF		+bFGF	-bFGF	
Volume (cm ³)	1.52 ± 0.74	0.76 ± 0.28	0.007	1.15 ± 0.57	0.91 ± 0.41	0.28
Cross sectional area (cm ²)	0.85 ± 0.23	0.54 ± 0.15	<0.001	0.68 ± 0.22	0.56 ± 0.19	0.13
Enhanced area (cm ²)	0.60 ± 0.28	0.18 ± 0.28	0.002	0.36 ± 0.28	0.29 ± 0.24	0.35
% enhanced	59 ± 17	28 ± 12	<0.001	47 ± 27	40 ± 25	0.37
Hotspot count	21 ± 10	16 ± 9	0.06	31 ± 9	22 ± 8	0.03

Average values and standard deviations are given. Probability *p* is for a two-tailed paired Student's *t*-test between stimulated and non-stimulated plugs in each case.