GFP-expressing vascularization of Gelfoam® as a rapid in vivo assay of angiogenesis stimulators and inhibitors

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BioTechniques 42:294-298 (March 2007)
doi 10.2144/000112382

Angiogenesis, blood flow, intravascular tumor cell trafficking, and extravasation are critical steps in tumor growth, progression, and metastasis and, therefore, are targets in worldwide drug discovery programs. The discovery and evaluation of anti-angiogenic substances have previously relied on in vivo methods such as the chorioallantoic membrane assay (1,2), the monkey iris neovascularization model (3), disc angiogenesis assay (4), and various models using the cornea to assess blood vessel growth (5–10). These models have played an important role to understand the mechanisms of blood vessel growth and its inhibition.

We recently developed a novel transgenic nude mouse for the imaging of human tumor angiogenesis. In this mouse model, the stem cell marker nestin is expressed in nascent blood vessels. A regulatory element of nestin drives green fluorescent protein (GFP) in this transgenic mouse [nestin-driven GFP (ND-GFP)] enabling nascent blood vessels to be visualized by their GFP expression. Many human and rodent cancer cell lines expressing red fluorescent protein (RFP) were implanted in the ND-GFP nude mice and grew extensively. ND-GFP was highly expressed in proliferating endothelial cells and nascent blood vessels in the growing tumors, visualized by dual-color fluorescence imaging (11). Doxorubicin inhibited the nascent tumor angiogenesis as well as tumor growth in ND-GFP mice transplanted with the B16F10-RFP murine melanoma (12).

Primary-tumor angiogenesis in the ND-GFP transgenic nude mice with orthotopically transplanted MIA PaCa-2 human pancreatic cancer expressing RFP was also visualized by dual-color imaging. Gemcitabine significantly decreased the mean nascent blood vessel density in the tumor as well as decreased tumor volume. These results demonstrated for the first time that gemcitabine is an inhibitor of angiogenesis as well as tumor growth in pancreatic cancer (13).

Angiogenesis of liver metastasis of the XPA-1-RFP human pancreatic cancer in the ND-GFP transgenic nude mice was visualized by dual-color fluorescence imaging. ND-GFP was highly expressed in proliferating endothelial cells and nascent blood vessels in the growing liver metastasis. The density of nascent blood vessels in the liver metastasis was readily quantitated by ND-GFP expression. Gemcitabine significantly decreased the mean nascent blood vessel density in the liver metastases (14).

In the present study, we developed a very convenient imageable in vivo angiogenesis assay after transplantation of Gelfoam® (Pharmacia & Upjohn Company, Kalamazoo, MI, USA) in the ND-GFP mice. We demonstrate that the Gelfoam is rapidly vascularized with GFP-expressing vessels in the presence of an angiogenesis stimulator. Anti-angiogenesis agents inhibit this process. Thus, this rapid and simple new in vivo assay can rapidly identify angiogenesis stimulators and inhibitors.

ND-GFP transgenic C57/B6 mice carry GFP under the control of the nestin second-intron enhancer. The standard image system was used for whole body image and metastatic analysis. A Model LZ12 fluorescence stereo microscope (Leica Microsystems GmbH, Wetzlar, Germany) equipped with a mercury lamp and a 50-W power supply was used. Selective excitation of GFP was produced through a D425/60 band-pass filter and a 470 DCXR dichroic mirror. Emitted fluorescence was collected through a long-pass filter (GG475; Chroma Technology, Brattleboro, VT, USA) on a Hamamatsu C5810 3-chip cooled color charge-coupled device (CCD) camera (Hamamatsu Photonics, Bridgewater, NJ, USA). Images were processed for contrast and brightness and analyzed with the use of IMAGE PRO PLUS 3.1 software (Media Cybernetics, Silver Spring, MD, USA). High-resolution images of 1024 × 724

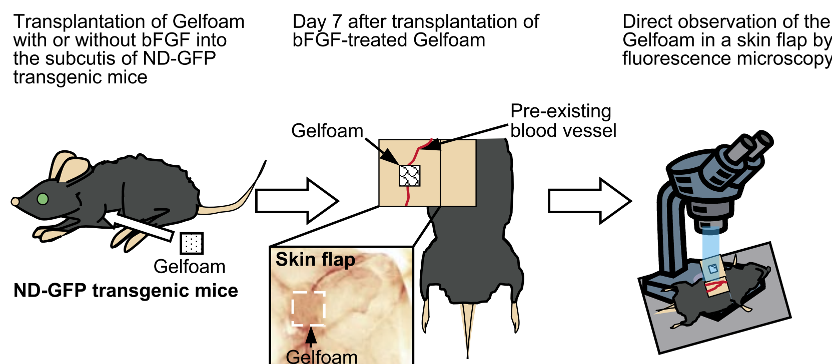

Figure 1. Experimental scheme for Gelfoam-implantation angiogenesis assay. Gelfoam (5 × 5 mm), with or without 300 ng β fibroblast growth factor (bFGF) in 75 µL RPMI 1640 medium, was transplanted into the subcutis on both flanks of nestin-driven green fluorescent protein (ND-GFP) transgenic mice. Skin flaps were made at day 7 after transplantation of Gelfoam under anesthesia. Angiogenesis was quantified in the Gelfoam by measuring the length of ND-GFP-expressing nascent blood vessels using fluorescence microscopy imaging to visualize the Gelfoam in the skin flap.

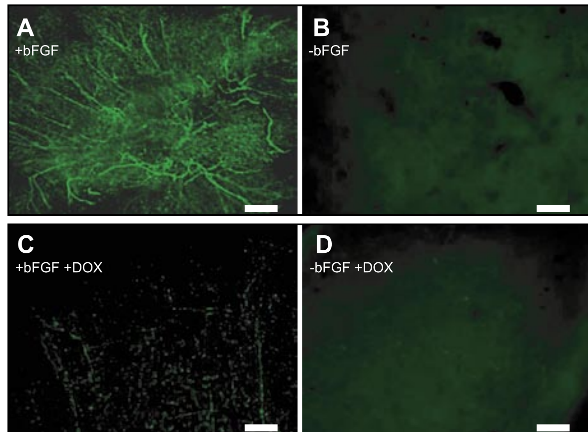

Figure 2. Angiogenesis of implanted Gelfoam with green fluorescent protein (GFP)-expressing vessels. Nestin-driven GFP (ND-GFP) mice were given daily intraperitoneal (ip) injections of 0.9% NaCl solution at day 0, 1, and 2 after transplantation of Gelfoam with or without β fibroblast growth factor (bFGF). (A) At day 7 after transplantation of Gelfoam with bFGF, the ND-GFP-expressing nascent blood vessels formed a network on the surface of Gelfoam in the skin flap. The ND-GFP-expressing nascent blood vessels had many branches that were connected to each other. (B) At day 7 after transplantation of Gelfoam, without bFGF, into the subcutis of ND-GFP transgenic mice, the ND-GFP-expressing nascent blood vessels were very sparse. (C and D) The Gelfoam-transplanted ND-GFP mice were treated with 5 µg/g doxorubicin (DOX) at day 0, 1, and 2 after transplantation. Doxorubicin significantly decreased the blood-vessel density in the (C) presence or (D) absence of bFGF. Scale bar, 500 µm.

pixels were captured directly on an IBM PC or continuously through video output on a high-resolution Sony VCR (Model SLVR1000; Sony, Tokyo, Japan).

ND-GFP transgenic mice, 6–8 weeks old, were anesthetized with tribromoethanol. Gelfoam (5 × 5 mm) was treated with and without 300 ng β fibroblast growth factor (bFGF; Chemicon, Temecula, CA, USA) in 75 µL RPMI 1640 medium (Cellgro, Herndon, VA, USA). The treated Gelfoam was then transplanted into the subcutis on both flanks of the ND-GFP transgenic mice. The mice were given daily intraperitoneal (ip) injections of 5 µg/g doxorubicin or 0.9% NaCl solution (vehicle control) at day 0, 1, and 2 after transplantation of Gelfoam. Skin flaps were made at day 7 after transplantation of Gelfoam under anesthesia. Angiogenesis was quantified by measuring the length of ND-GFP-expressing nascent blood vessels in the Gelfoam in the skin flap by in vivo fluorescence microscopy imaging (Figure 1). The vessels on the surface were counted under fluorescence microscopy. Each experimental group consisted of five mice.

Co-localization of ND-GFP fluorescence and CD31 in frozen sections of the vascularized Gelfoam was detected with the anti-rat immunoglobulin horseradish peroxidase detection kit (BD Pharmingen, San Diego, CA, USA) following the manufacturer's instructions. The primary antibody was anti-CD31 MAb (1:50; Chemicon). Substrate-chromogen 3,3′-diaminobenzidine staining was used for antigen staining. The experimental data are expressed as the mean ± SD. Statistical analysis was performed using the two-tailed Student's *t*-test.

Gelfoam implanted in ND-GFP mice was rapidly vascularized with ND-GFP-expressing blood vessels. Angiogenesis was quantified in the Gelfoam by measuring the total length of ND-GFP-expressing nascent blood vessels in a skin flap by in vivo fluorescence microscopy imaging (Figure 1). At day 7 after transplantation, the ND-GFP-expressing nascent blood vessels were observed forming a network on the surface of the bFGF-treated Gelfoam in the skin flap (Figure 2). Implanted Gelfoam that was not treated with bFGF was not vascularized. The ND-GFP vessels in the Gelfoam stained positively for CD31, demonstrating the presence of endothelial cells (Figure 3). Day 7 was chosen as an arbitrary time point to measure the GFP vessels in the implanted Gelfoam. The Gelfoam can be analyzed at any time point, and an optimal time for measurement would depend on the angiogenesis drug being tested.

ND-GFP mice that received ip injections of doxorubicin (5 µg/g) at day 0, 1, and 2 after transplantation of Gelfoam, with or without bFGF, had fewer ND-GFP-expressing nascent blood vessels than NaCl-treated mice (see Figures 2 and 4). Future experiments will address the destruction of preformed vessels in Gelfoam.

Angiogenesis plays an important role in physiologic and pathologic processes (15). Angiogenesis occurs normally during embryonic development, tissue regeneration, the menstrual cycle, and pathologically in cancer, proliferative retinopathy, and rheumatoid arthritis (15,16). Because the growth of solid tumors requires an adequate blood supply, agents that block angiogenesis inhibit tumor growth. The process of blood vessel formation is crucial to tumor development providing a source of oxygen and nutrients. The blood vessels that form to nourish a small tumor can help the tumor grow and eventually metastasize. Angiogenesis is usually determined by immunohistochemical staining of tumor tissue using various antibodies specific for endothelial cells.

In a previous study, we demonstrated that nascent blood vessels expressed nestin (12). We took advantage of this fact to transplant Gelfoam in mice in which ND-GFP is expressed in nascent blood vessels. The ND-GFP transgenic mouse model with transplanted Gelfoam readily enables the visualization and quantification of nascent angiogenesis. Vessel function

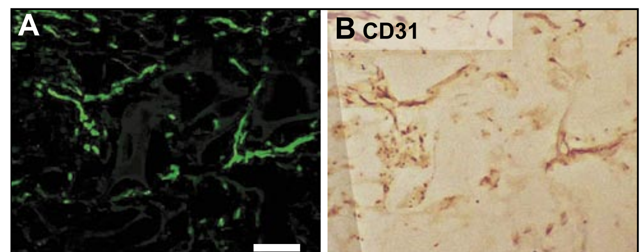

Figure 3. Co-localization of green fluorescent protein (GFP) and CD31 in Gelfoam blood vessels. Frozen sections were made at day 7 after transplantation of Gelfoam treated with β fibroblast growth factor (bFGF), into the subcutis of nestin-driven GFP (ND-GFP) transgenic mice. (A) Nascent ND-GFP-expressing blood vessels were growing into the Gelfoam. (B) Immunohistochemical (IHC) staining showed that CD31 (B), and ND-GFP fluorescence (A) co-localized in the newly formed ND-GFP-expressing blood vessels. Scale bar, 100 µm.

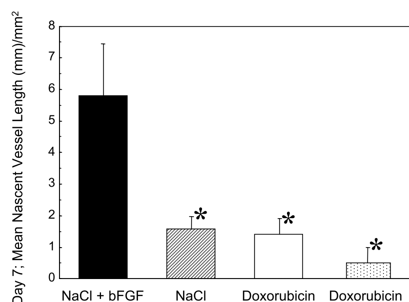

Figure 4. Efficacy of doxorubicin on Gelfoam angiogenesis. At day 7 after transplantation of Gelfoam with β fibroblast growth factor (bFGF), treatment with doxorubicin significantly decreased the mean nascent blood vessel length per mm^2. *$P < 0.001$ versus NaCl controls.

in the Gelfoam will be assessed in future experiments. Distinction of nascent and mature blood vessels will also be carried out in future experiments. In addition, measurement of total fluorescence and integration by confocal microscopy will be carried out in future experiments. This model can rapidly identify angiogenesis stimulators and inhibitors. The assay should be important to identify more effective agents for targeting angiogenesis.

ACKNOWLEDGMENTS

This work was supported by National Cancer Institute grant nos. CA099258 and CA103563 (to AntiCancer, Inc.), National Institutes of Health (NIH) grant no. R21 CA109949-01, and American Cancer Society RSG-05-037-01-CCE (to M.B.).

COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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Received 10 August 2006; accepted 13 November 2006.

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