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Vascular-specific quantification in an in vivo Matrigel chamber angiogenesis assay

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Abstract

The study of angiogenesis as a therapeutic target requires reliable in vivo assays that can provide physiologically relevant data. A murine in vivo Matrigel-based angiogenesis assay is presented here which includes the quantitative assessment of vascular-specific indicators of neovascularization. Matrigel containing 175 ng/ml bFGF is encapsulated in synthetic chambers which are implanted subcutaneously in C57/B16J mice. Ex vivo implants can be imaged to qualitatively view perfused vasculature within the chambers, or histologically processed to confirm the presence of vascular-specific tissue within the Matrigel. Viable cells are recovered from the excised chambers and quantified cytometrically using endothelial cell-specific markers CD34 and CD144, and for a marker of nucleated cells, Hoechst 33342.

Thalidomide, 200 mg/kg/day, was tested using the assay and was found to inhibit angiogenesis by 46%. Angiogenesis inhibitors secreted by LL/M27 tumors were also characterized, where tumor-bearing mice showed a 73% inhibition of angiogenesis compared to tumor-free controls. Analysis of the number of nucleated cells in these samples failed to show a strong correlation with the number of endothelial cells, indicating that quantification of nonvascular-specific tissue in in vivo angiogenesis assays may not be sufficient.

This new assay provides an objective, comprehensive determination of the vasculature-specific response of both endogenous and exogenous angiogenesis inhibitors in vivo, and also creates new opportunities for obtaining primary murine endothelial cells.

Keywords: bFGF; Carbocyanine; CD34; CD144; Cytospin; Hoechst 33342; Lewis lung; Matrisperse™; Primary endothelial cells; Quantitative microscopy

Introduction

Angiogenesis is a crucial step in cancer progression (Folkman, 1971), and a higher proliferation rate of tumor endothelial cells, compared with 'normal' tissues, makes tumor vasculature a promising target for anti-cancer therapies that should have low systemic toxicity (Hobson and Denekamp, 1984). A significant challenge in developing anti-angiogenic agents involves the in vivo assays, which may have limitations related to quantification, variability, relative invasiveness, cost or physiological relevance (Jain et al., 1997; Hasan et al., 2004).

Subcutaneous injection of a gel comprised of extracellular matrix components is a popular noninvasive approach, as vasculature entering the matrix is unambiguously new and distinct from host tissues. The Matrigel plug assay (Passaniti et al., 1992) is widely used and has undergone a variety of modifications. A recent improvement to the assay involves encapsulation of the Matrigel in a plexiglass chamber prior to subcutaneous implantation in the legs of rodents (Kragh et al., 2003; Ley et al., 2004). Nylon mesh windows in the chamber permit growth of vasculature into the lumen. In addition to establishing a clear boundary between matrix and host tissues, this method promotes consistent surface area exposures, a parameter which is typically more difficult to control in the Matrigel plug assay.

Here we describe a new in vivo angiogenesis assay method based on chambers similar to those described but created from sections of flexible plastic tubing that are positioned at the ventral midline in the pelvic region of mice. This placement draws a considerable angiogenic response that is subsequently quantified using an innovative method. Immediately following

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excision of implants, viable cells are isolated and labeled with a nuclear stain and an endothelial cell marker for cytometric analysis. The result is a vasculature-specific, comprehensive and objective determination of the response of angiogenic stimulants and inhibitors in vivo that permits a distinction between vascular-related cells and accompanying stromal cells.

We have explored the utility of this Matrigel chamber angiogenesis assay by investigating the anti-angiogenic activity of both exogenous and endogenous agents. The ability of the assay to effectively illustrate the activity of a drug administered systemically was tested using thalidomide. Thalidomide has been shown to have anti-angiogenic properties, and is in clinical use for the treatment of multiple myeloma (D'Amato et al., 1994; Hattori and Iguchi, 2004). While several mechanisms of action for thalidomide have been proposed, the molecular targets remain only partially characterized. Assays capable of providing more specific information about the activity of thalidomide and its many analogues would be desirable.

The potent anti-angiogenic activity of endogenous molecules secreted by a variety of primary tumors has been documented (for a recent review, see Folkman, 2004). The intrinsic anti-angiogenic activity of a LL/M27 tumor, a metastatic variant of Lewis Lung carcinoma, was also used to validate this assay as a considerable, quantifiable amount of angiogenesis inhibition occurs with no associated toxicity. We characterized this anti-angiogenic activity by assessing Matrigel chambers from tumor-bearing mice, tumor-free mice and mice that had their tumors surgically removed on the day of chamber implantation.

Materials and methods

Animals

Male C57/B16J mice, 8–13 weeks old weighing 27–33 g, were bred and maintained in our institutional animal facility in accordance with the Canadian Council on Animal Care guidelines. The experiments described in this report were approved by the Animal Care Committee of the University of British Columbia. A pre-emptive analgesic, 2 mg/ml of acetylsalicylic acid (Sigma Chemical, St. Louis, MO), was provided via drinking water for 7 days prior to chamber implantation, after which the concentration was increased to 4 mg/ml. During all surgical procedures, animals were maintained under general anesthetic using isoflurane (Baxter Corporation, Mississauga, ON).

Matrigel chambers

Chambers were constructed using 3 mm sections of surgical grade tubing (TYGON S-50-HL, ID: 6.4 mm; OD: 7.9 mm; Wall: 0.8 mm) with 100 μm nylon filters attached at either end (Krazy Glue Original; ethyl-cyanoacrylate). Chambers were soaked in 100% ethanol and dried. Frozen Matrigel (BD Discovery Labware, Bedford, MA) was thawed overnight at 4°C, stored on ice and mixed with 175 ng/ml basic Fibroblast Growth Factor (Sigma Chemical) in 5% bovine serum albumin (BSA) (Sigma Chemical). The mixture was injected into the lumen of chambers positioned on a temperature-controlled heating plate at 37°C, allowing the Matrigel to gel rapidly. Chambers were stored at 37°C in a humidified, sterile environment, and implanted within 2 h. A 1 cm incision was made on the ventral side of anesthetized animals, along the medial plane in the pectoral region. The chamber was positioned in a subcutaneous pocket at the midline in the pelvic region 1.5–2 cm distal to the incision, which was closed using 9 mm stainless steel wound clips (Clay Adams Brand, Sparks, MD).

Tumor implantation

LL/M27 tumor cell line was generously provided by Dr. Pnina Brodt (Chung et al., 1988). Tumors were implanted by subcutaneous injection of 4.0×10^5 cells in suspension into the sacral region of mice. Tumors were grown to an average of 370 mm^3 at which time half of the mice had their tumors surgically removed and all mice had chambers surgically implanted. Remaining tumors continued to grow for the 9 day Matrigel chamber incubation period, reaching an average size of 1000 mm^3 on the day of chamber explantation.

Treatment

Thalidomide (Andrulis Pharmaceuticals Corporation, Beltsville, MD) was administered daily for 8 days via i.p. injections at a dose of 200 mg/kg as a 10 mg/ml suspension in 1% methyl cellulose (Sigma Chemical) in saline.

Cell recovery

Nine days after chamber implantation, mice were euthanized and chambers removed. Chambers were rinsed in PBS and the contents transferred to individual polypropylene tubes containing 1 ml of Matrisperse™ cell recovery solution (BD Discovery Labware). After an incubation period of 1.5 h at 4°C, suspensions were agitated using a micropipette and filtered through a 100 μm nylon mesh. The filtrate was then centrifuged and the cells resuspended in phosphate-buffered saline (PBS).

Immunostaining cell suspensions

Endothelial cells were detected using a 1:375 dilution of either purified monoclonal rat anti-mouse CD34 antibody (clone RAM34; BD Pharmingen, San Diego, CA) or purified monoclonal rat anti-mouse VE-Cadherin antibody (clone 11D4.1; BD Pharmingen). Cells were centrifuged and rinsed twice in PBS containing 2.5% BSA prior to addition of Alexa Fluor 546 goat anti-rat secondary (Molecular Probes, Eugene, OR) at a dilution of 1:350. Samples were again rinsed twice and resuspended to a final volume of 400 μl. Cells were counterstained with Hoechst 33342 (Sigma Chemical) at a concentration of 8 μg/ml for 30 min at 37°C. A 100 μl volume from each was cytospun for 8 min at 400 rpm onto Histobond slides (Marienfeld, Lauda-Koenigshofen, Germany).

Image acquisition and analysis

The imaging system consisted of a microscope (III RS; Zeiss, Oberkochen, Germany), a cooled monochrome charge-coupled device video camera (model 4922; Cohu, San Diego, CA), a frame grabber (Scion, Frederick, MD), a custom built motorized x–y stage and customized NIH-Image software (http://rsb.info.nih.gov/nih-image/Default.html) run on a G3 Macintosh computer (Apple, Cupertino, CA). The motorized stage allowed for tiling of adjacent microscope fields of view so that an image of the entire cytospun cell-containing area of interest (AOI) was obtained at 1 pixel/μm^2. Images were cropped to remove staining artifacts and debris. Using NIH-Image software user-supplied algorithms, fluorescent images were inverted and positive staining regions identified by establishing threshold values evaluated to select all pixels more than 11 standard deviations above background for the thalidomide chambers and their controls, and 5 standard deviations above background for LL/M27 tumor-bearing mice, tumor-removed mice and tumor-free controls. Hoechst 33342 staining was quantified by selecting a threshold to include all positive pixels relative to the otherwise tissue-free, zero background intensity. The number of positive pixels divided by the total number of pixels in the AOI was determined and is reported as percentages.

Statistical analysis

Statistical analysis was performed using PAST data analysis software (Hammer et al., 2001). Nonparametric equations were used in all instances; a two-tailed P value < 0.05 was considered significant, and was adjusted using the Bonferroni correction for multiple comparisons.