Video Article

Rat Mesentery Angiogenesis Assay

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Abstract

The adult rat mesentery window angiogenesis assay is biologically appropriate and is exceptionally well suited to the study of sprouting angiogenesis *in vivo* [see review papers], which is the dominating form of angiogenesis in human tumors and non-tumor tissues, as discussed in invited review papers[1,2]. Angiogenesis induced in the membranous mesenteric parts by intraperitoneal (i.p.) injection of a pro-angiogenic factor can be modulated by subcutaneous (s.c.), intravenous (i.v.) or oral (p.o.) treatment with modifying agents of choice. Each membranous part of the mesentery is translucent and framed by fatty tissue, giving it a window-like appearance.

The assay has the following advantageous features: (i) the test tissue is natively vascularized, albeit sparsely, and since it is extremely thin, the microvessel network is virtually two-dimensional, which allows the entire network to be assessed microscopically *in situ;* (ii) in adult rats the test tissue lacks significant physiologic angiogenesis, which characterizes most normal adult mammalian tissues; the degree of native vascularization is, however, correlated with age, as discussed in[1]; (iii) the negligible level of trauma-induced angiogenesis ensures high sensitivity; (iv) the assay replicates the clinical situation, as the angiogenesis-modulating test drugs are administered systemically and the responses observed reflect the net effect of all the metabolic, cellular, and molecular alterations induced by the treatment; (v) the assay allows assessments of objective, quantitative, unbiased variables of microvascular spatial extension, density, and network pattern formation, as well as of capillary sprouting, thereby enabling robust statistical analyses of the *dose-effect* and *molecular structure-activity* relationships; and (vi) the assay reveals with high sensitivity the toxic or harmful effects of treatments in terms of decreased rate of physiologic body-weight gain, as adult rats grow robustly.

Mast-cell-mediated angiogenesis was first demonstrated using this assay[3,4]. The model demonstrates a high level of discrimination regarding dosage-effect relationships and the measured effects of systemically administered chemically or functionally closely related drugs and proteins, including: (i) low-dosage, metronomically administered standard chemotherapeutics that yield diverse, drug-specific effects (*i.e.*, angiogenesis-suppressive, neutral or angiogenesis-stimulating activities[5]); (ii) natural iron-unsaturated human lactoferrin, which stimulates VEGF-A-mediated angiogenesis[6], and natural iron-unsaturated bovine lactoferrin, which inhibits VEGF-A-mediated angiogenesis[7]; and (iii) low-molecular-weight heparin fractions produced by various means[8,9]. Moreover, the assay is highly suited to studies of the combined effects on angiogenesis of agents that are administered systemically in a concurrent or sequential fashion.

The idea of making this video originated from the late Dr. Judah Folkman when he visited our laboratory and witnessed the methodology being demonstrated.

Review papers (invited) discussing and appraising the assay

Norrby, K. *In vivo* models of angiogenesis. J. Cell. Mol. Med. 10, 588-612 (2006).

Norrby, K. Drug testing with angiogenesis models. Expert Opin. Drug. Discov. 3, 533-549 (2008).

Video Link

The video component of this article can be found at http://www.jove.com/video/3078/

Protocol

1. Animals

1. Small rodents, such as rats, mice, and Guinea pigs, have been used as the animal models. Unfortunately, adult mice (many mouse strains have been assessed) tend to lack or have only a very low number of potential-parent microvessels (from which angiogenesis can originate) within their mesenteric windows, which makes studies using mice unreliable. Therefore, the assay has been developed primarily for rats.
2. The rats, which are acclimatized to a standard environment for at least 7 days following delivery from the breeder, are usually housed in pairs in a standard cage under a 12-hr light/dark cycle with free access to water and pellets. Each animal is marked. We use mostly young adult rats (typically 7-10 weeks of age of various strains but usually male Sprague-Dawley rats; in rats younger than 5-6 weeks of age the number of native microvessels tend to be low) obtained from diverse breeders. In the period prior to the experiment and throughout the experiment, the animals are weighed every second day, and always on the day of sacrifice. When animals are treated concurrently i.p. (for induction of

angiogenesis, see below) and s.c. or i.v. or p.o., they are weighed each day. This enables the establishment of experimental and control groups of equal body weight at the start of the experiment, and allows monitoring of the influence of the treatment on body-weight-gain during the course of the experiment. Since adult male rats grow robustly physiologically (gaining approximately 40-60 g per week depending on type of strain), weight-gain retardation is a sensitive substitute marker of toxicity, systemic well-being, anorexia, and failure to thrive.

We work in a high-standard animal care facility and considerable efforts are made to minimize the levels of stress experienced by the animals. The current ethical guidelines are those established by Workman P. *et al.* (Guidelines for the welfare and use of animals in cancer research. *Br. J. Cancer 102, 1555-77, 2010*). The Animal Ethics Committee of the University of Gothenburg approved these studies.

2. Pro-angiogenic Intraperitoneal Treatment

1. The candidate pro-angiogenic factor to be tested is dissolved and diluted in the endotoxin-free saline used for infusion of patients (even at extremely low levels, endotoxin is pro-angiogenic). All solutions are made up freshly on the day of injection, sterile-filtered (Millipore 0.22-µm filtration), checked for neutral pH (7.1-7.3), and used at room temperature.
   The test agent is injected at low or very low concentrations. For example, rat $rVEGF_{164}$ (564-RV/CF; R&D Systems Europe, Ltd., Oxon, UK), which is the predominant VEGF-A isoform in rats, is diluted to 96 pmole/ml in endotoxin-free saline, frozen and thawed and a volume of 5 ml is injected i.p. into the rat. We use a 5-ml syringe with 0.8-mm needle (green needle, 21G) for i.p. injection. This treatment, given twice daily for 4.5 days, *i.e.*, from Monday morning (Day 0) to Friday morning (Day 4), induces a robust sprouting angiogenesis response in the mesenteric test tissue, peaking at around Day 21.
   Administering the solution i.p. ensures that the entire volume is delivered into the peritoneal cavity (and not into the intestines, bladder or any other organ). The rapid injection of 5 ml allows tactile confirmation by the operator that the fluid jet has passed through the abdominal wall into the abdominal cavity.
   Following, prior to or concurrently with the i.p. pro-angiogenic treatment, any test agent of choice can be administered systemically.

3. Systemic Administation of Angiogenesis-modulating Agents

1. Any route other than the i.p. route can be used, since i.p. treatment may affect the ongoing angiogenic reaction through the induction of inflammation or possibly by activating mast cells in the test tissue, which can lead to a strong pro-angiogenic response. Besides oral administration or single injection s.c. or i.v., we often use s.c. administration using osmotic minipumps (one or more, with varying volumes and pumping times) to deliver the solution at a constant rate for up to two or several weeks.
2. **Continuous subcutaneous infusion of test agents**
   Filling and implantation of osmotic minipumps
   Usually on Day 5, *i.e.,* one day after the end of the i.p. VEGF treatment, osmotic minipumps (for example, Model 2ML2, with constant pumping rate of 5.0 µl/h for 14-15 days; Alzet Osmotic Pumps, Mountain View, CA, USA) are filled under sterile conditions with the test agent or the appropriate vehicle. After storage in sterile 0.9% (w/v) NaCl overnight at 37°C, the pump(s) are surgically implanted s.c. on the backs (at the side of the spinal column in the region of the scapulae) of rats that have been anesthetized using isoflurane gas (Isoba vet; Schering-Plough Animal Health, Merck & Co., Inc., Whitehouse Station, NJ, USA), initially in an airtight transparent chamber and subsequently via a mask (to minimize the stress caused to the following animal, the chamber is flushed with air before it is reused). The skin incision made for pump implantation is sutured immediately post-implantation using silk thread.
   Since the animals gain weight physiologically during the experimental period and the test agents are administered at a constant rate, the actual dosage selected for a test drug per kg of body weight is higher than the average dosage at the beginning of the infusion period and is lower than the average dosage at the end of the infusion period.
   Continuous infusion, as described here, can be viewed as an extended form of metronomic treatment.

4. Ending the Experiment

1. One animal at a time is placed in a transparent airtight chamber, which is flushed with CO_2 gas, which rapidly kills the animal. The chamber is flushed with air before being used again.

5. Harvesting Tissue Samples

1. As shown in the DVD, the abdominal wall is opened and the small-gut mesentery is identified, including the most distal part that reaches the ileo-cecal valve (the junction of the small intestine and the large intestine). Four (or more) of the most distally located "windows" are numbered and spread onto objective standard untreated Superfrost Plus-slides (DAKO Denmark A/S, Glostrup, Denmark). It is important to avoid, if possible, contaminating the samples with blood, gut contents or other bodily fluids, as these materials may be stained non-specifically, although this does not invalidate the subsequent immunohistochemical visualization of microvessels.
2. Each mesenteric window specimen with attached small gut segment is spread on a slide and dried at room temperature for 20 minutes. Then, the intestinal stump is cut off, while the intact membranous mesentery specimens are stored at -20°C for extended periods. This is shown in the DVD.

It should be mentioned that the tissue and patent vasculature can be visualized prior to harvest with transmitted light intravital microscopy.

6. Protocol for Immunohistochemical Staining of Microvessels

1. **Day 0**
   The specimens are allowed to thaw from -20°C to room temperature, dried in a cabinet at 60°C for 1 hr, and kept at room temperature overnight.

2. **Day 1**
   Place the slides in staining slide holders (in every second track) in a staining jar and wash twice with TBS (pH 7.8) for 10 minutes at room temperature. The solution is discarded and replaced with the next solution, and so on for all the following steps.
3. **Trypsin treatment**
   Fill the staining jar that will harbor the staining slide holder with trypsin solution (25° C for 20 minutes). Discard the solution and incubate twice with 2.5% sucrose solution (room temperature for 10 minutes each time). Discard the solution and rinse a couple of times in *aqua dest* (room temperature for a few minutes each).
4. **Blocking**
   Fill the staining jar that will harbor the staining slide holder with 0.5% H_2O_2 in methanol (room temperature for 30 minutes). Discard the solution and rinse with *aqua dest* (room temperature for a few minutes). Discard the solution and rinse twice with TBS (pH 7.4) (room temperature for 8 minutes each time).
5. **Incubation**
   All incubations are in a humidified chamber at room temperature. Approximately 150 µl of each of the solutions listed below are used per slide. The slides are placed in a humidified chamber and the solutions are pipetted onto the specimens (the non-membranous parts do not need to be covered by the solutions).
   For each of the following steps, carefully shake off all of the solution before the subsequent solution is added.
   1. **Incubation with Normal (blocking) serum** (horse) (ABC kit) 3 drops *yellow solution*/10 ml dilution buffer for 20 minutes.
   2. **Incubation with Primary antibody** ARU0181, which labels rat vascular endothelial cells. The antibody is diluted 1:600 with dilution buffer (Biosource) (100 µl diluted 40-fold [frozen] plus 900 µl dilution buffer) and incubation takes place overnight at 4°C.

6. **Day 2**
   The slides are removed from the humidified chamber, the primary antibody is discarded, and the slides are placed in a staining slide holder, which is submerged in the staining jar and rinsed twice with TBS (pH 7.8) for 8 minutes.
   1. **Incubation with biotinylated antibody** (ABC kit)
      The slides are placed horizontally in a humidified chamber and incubated with 1 drop of *blue solution*/10 ml dilution buffer for 30 minutes. Discard the solution and place the slides in a staining slide holder submerged in the staining jar and rinse twice with TBS (pH 7.4) for 8 minutes.
   2. **Incubation with the ABC reagent**
      The slides are again placed horizontally in a humidified chamber and the following solutions are pipetted onto the membranous parts of the specimens: 2 drops of *orange solution* plus 2 drops of *brown solution*/10 ml TBS (pH 7.4) for 30 minutes. Mix carefully 30 minutes before use! Discard the solution and place the slides in the slide holder in the staining jar and rinse twice with TBS (pH 7.4) for 8 minutes.

7. **Staining with DAB (0.05%)**
   This step must be performed in a ventilated chemical hood!
   Place the slides and add the 3,3-diaminobenzidine (DAB; Sigma-Aldrich) solution using a pipette: 0.1% DAB solution in TBS (pH 7.4) [frozen] is diluted 1:1 with a mixture of 10 µl H_2O_2 and 7.5 ml TBS (pH 7.4). This solution is sterile-filtered just before use (to eliminate any particles that may obscure the microscopic picture). Stain the specimens for 8 minutes. Discard the solution and place the slides in the staining slide holder submerged in the staining jar. Rinse under running tap water and then in *aqua dest* for a few minutes.
8. **Dehydration**
   The slides are kept in the staining slide holder. Rinse by discarding and refilling the staining jar containing *aqua dest*/ethanol: *aqua dest*, 70% alcohol, 95% alcohol, absolute alcohol, with 2 minutes in each solution.

7. Imaging and Assessing the Microvascular Network in Each Mesenteric Window Specimen After Immunohistochemical Staining of Vascular Endothelial Cells

As shown in the movie, microvessels are microscopically visualized *in situ* in the intact tissue.

1. First, we use a drawing microscope with magnification range from 14 to 34 (Leica Wild M 3Z) to pencil on white paper the entire window area, as well as the entire *vascularized area* (VA) per mesenteric window. VA, as a percentage of the entire area, is easily measured using computerized or non-computerized planimetry (or simply by cutting out and weighting the image of the whole window, and the images of all its vascularized parts).
2. Using another drawing microscope, individual microvessel profiles, which are readily identified, are drawn in black ink on white paper at 80 magnification within randomly chosen fields. This is the starting point for the subsequent computerized morphometric assessment of *microvascular length* (MVL), which is basically a measurement of microvascular density.
3. Optionally, variables related to microvascular pattern formation, as well as those for the assessment of *the number of microvascular sprouts* and *length of individual microvascular sprouts* (No. SP and Le. SP, respectively) can be determined. -The optimal contrast between the depicted black vessel structures and the white background facilitates detailed analyses of the images in most commercial morphometric computerized programs.
4. In most cases, the major variables are VA and MVL, which when multiplied together generate the *total microvascular length* (TMVL).

8. Statistical Analysis

We usually use the standard non-parametric Mann-Whitney *U*-test to analyze unpaired (two-tailed) observations. A mean of four mesenteric windows per animal is used as the independent data-point for each variable of the mesenteric window. The criterion for statistical significance is $P \leq 0.05$.

Discussion

The assay was introduced in 1986[3] and has successively been further developed and refined in our laboratory[7,10-12]. As discussed in review papers (invited)[1, 2], the assay compares well with other mammalian *in vivo* angiogenesis assay in regard particularly to important features such as the adult test tissue is natively vascularized and lacks physiologic angiogenesis; minimal trauma, if any, is inflicted upon the tissue; truly quantitative variables are measured, which allows sound statistical analysis regarding dose-response and molecular-activity studies; sprouting angiogenesis occurs, which is predominating in normal tissues and tumors; and toxicity data are easily accumulated in rats that grow robustly physiologically in adulthood. Disadvantageous features may include the fact that the assay is relatively time-consuming, especially when assessing the number and length of individual microvessel segments and microvessel sprouts; that it does not allow real-time/serial observations; and that it is hardly suitable for mice since many mesenteric windows in mice lack potential parent microvessels from which new capillaries can originate.

Real-time observations are, however, enabled by intravital microscopy where the exteriorized but intact mesenteric windows are splayed out over a microscope stage while the animal is anesthetized, as discussed in[1].

Significant advances in angiogenesis research have been achieved in the past decades using models such as the corneal micropocket, chick chorioallantoic membrane, and s.c. Matrigel plug assays, which are useful but ultimately limited tools. Future clinical applications of anti-angiogenic and pro-angiogenic therapies necessitate the establishment and validation of more sensitive and biologically relevant, truly quantitative pre-clinical models, such as the one described in the current report.

Disclosures

No conflicts of interest declared.

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