



Evaluating Compounds Affecting Angiogenesis

Angiogenesis is a highly regulated process essential to reproduction and wound healing. In the normal response to injury, several factors act to initiate the formation of vascular sprouts from existing vessels. The newly formed vasculature is highly branched and contains unproductive dead-end and loop structures. Aberrant vessels are resorbed as vascular remodeling occurs. Uncontrolled angiogenesis is a major contributor to a number of disease states, including arthritis, diabetes-related blindness, psoriasis, and tumor growth and metastasis. While anti-angiogenic compounds would be useful in treating diseases having uncontrolled angiogenesis, pro-angiogenic compounds are needed in wound healing and may be useful in minimizing tissue damage following ischemia damage from heart attacks or stroke.

One focus of our cancer research program is the relationship between hypoxia and angiogenesis induction in tumor growth and metastasis. During the course of our studies we have developed assays to investigate both the pro- and anti-angiogenic effects of potential new drugs. In addition to performing basic research, we offer our clients several assays to evaluate angiogenesis. These include:

- In vitro endothelial cell proliferation, migration, differentiation, and tube formation assays
- Ex ovo chick chorioallantoic membrane (CAM) assay
- In vivo Z-chamber model in mice and rats
- Human xenograft and syngeneic tumor models in mice and rats

Endothelial Cell Proliferation, Migration, Differentiation, and Tube Formation Assays

Human umbilical vein endothelial culture (HUVEC) populations are very responsive to compounds that modulate angiogenic response. Pro-angiogenic compounds promote increased growth and differentiation rates in these cells. Activated endothelial cells become highly chemotactic. We can evaluate the effect of compounds on the growth rate of HUVEC cells and, by using Boyden chambers, a compound's ability to induce migration through a defined matrix.



Bridging the drug development gap

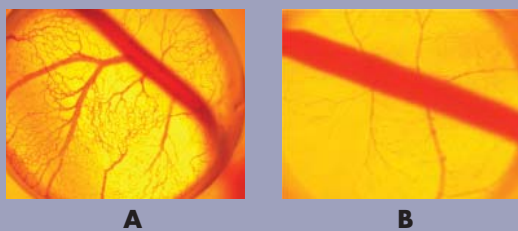
When cultured in the presence of angiogenic agents, HUVEC cells become stimulated and form tube-like structures 1 to 2 days following introduction of the angiogenic agent. In addition to time-dependent formation of tube-like structures, there is a time-dependent decrease in cell-covered surfaces. Tube formation is quantified by using phase contrast microscopy. While there are several commercially available kits for evaluating tube formation, custom matrices allow the evaluation of specific factors involved in angiogenesis (e.g., VEGF). We use a variant of the methods described by Chalupowicz et al. (*J. Cell Biology*, 1995, 130, 207-215) for performing the endothelial tube formation assay.

While these assays are an inexpensive way to evaluate a large number of compounds, the results are variable and many times reflect the effects of the environment more than the test compound. We highly recommend the use of companion studies to confirm the results of HUVEC proliferation, migration, and tube formation assays.

CHICK CHORIOALLANTOIC MEMBRANE (CAM) ASSAY

In a typical angiogenesis evaluation using the CAM assay, fertilized chick eggs are incubated at 37°C and specific humidity (60%) for 3 to 4 days. The shell is cut open and the embryo with intact CAM is carefully transferred to a Petri dish and incubated in a sterile incubator 3 to 4 more days. Blood vessel formation becomes apparent by this time. O-rings, used to reduce compound diffusion, are placed on regions of the CAM where blood vessel proliferation is pronounced. The test article is then introduced inside the O-ring and incubation continued for several days in the sterile environment. Angiogenesis is evaluated by using a dissecting microscope and a digital imaging system.

The CAM-tumor assay is similar to standard the CAM assay, except that tumor cells are placed inside the O-rings along with the test article. Tumor cell survival and angiogenesis are visualized and recorded.



Normal angiogenesis observed in CAM development (a) versus decreased vascularization in the development of the CAM (b) when treated with an anti-angiogenic compound.

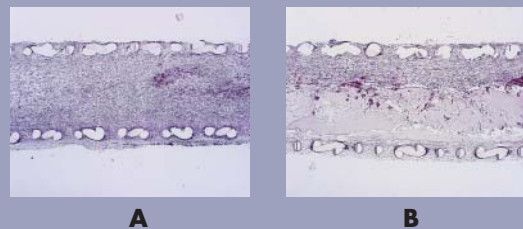
While the CAM assay is a step closer to an in vivo model, the processes that control embryonic angiogenesis may be significantly different than those responsible for injury- or cancer-induced angiogenesis.

Z-Chamber Model

The Z-chamber is an innovative way to study angiogenesis. Fibrinogen and thrombin are added through a port in the side of the chamber, and a fibrin clot rapidly forms.

The chamber is implanted in the subcutaneous space of the animals and subsequently harvested for evaluation. The two sides of the chamber are nylon mesh (180 µm pore size), which allows test agents, nutrients, proteins, and even cells to enter and leave the chamber. Our standard protocol involves inserting four chambers per animal, which greatly increases the statistical test number, n , while minimizing the number of animals needed for an experiment.

Chambers are available for both mouse (5 mm ID) and rat (10 mm ID). Although Z-chambers were designed to study wound healing and tumor growth, the chamber design allows us to design custom animal models for other inflammatory diseases and tissue generation.



Cross-sections of a Z-chamber used in assessment of wound healing. As a granulation tissue grows, inflammatory, endothelial, and other wound-response cells are recruited into the chamber. The fibrin clot in the control chamber (a) has been replaced by a network of vascularized stroma (granulation tissue) by Day 10, while the chamber removed from an animal treated with an anti-angiogenic compound (b) shows greatly reduced granulation tissue thick

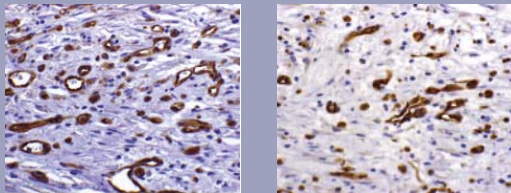
Immediately after incurring a wound, the body responds with a series of events that result in tissue remodeling and scar tissue formation. An early event in the process is the formation of a fibrin clot. Having fibrin as its provisional matrix, the Z-chamber mimics the environment observed in the very early stage of wound healing. Agents designed to affect wound healing mechanisms, such as angiogenesis, can be administered systemically or directly through the chamber port. Because wound healing is a well-ordered process, the chambers can be removed at distinct time points during the healing cascade (inflammation, proliferation

and remodeling) to evaluate the test agent's effectiveness.

Z-chambers can be analyzed in a variety of ways, including extent of granulation tissue formation or tumor growth, vascular density, vascular permeability, immunoblotting, cell sorting, and fluorescent, confocal, and MR imaging. Histology/immunohistology slices can be fixed and stained for a variety of cell markers and cytokines.

Advantages of the Z-chamber design include:

- Rapid in vivo screen for pro-angiogenic, anti-angiogenic, and anti-tumor compounds
- Minimal animal handling and surgical expertise required
- Four chambers per animal and 6- to 12-day studies lead to cost savings in time and the number of animals needed
- Weight loss and stress are not observed in animals
- Choice of chamber or systemic administration of test agent
- Test agent effects on wound healing and tumor biology can be studied simultaneously
- Ability to customize matrix components to assess tumor-matrix interactions

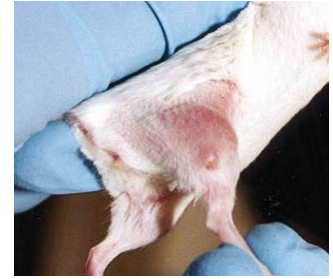


Differences between vascular density of controls (a) and animals treated with anti-angiogenic agent (b) can easily be observed in these sections of fibrin Z-chamber labeled with a blood vessel marker.

Tumor cells can be introduced along with the fibrinogen/thrombin mixture inside the chambers. Even though the nylon mesh size would allow the cells to move out of the chamber, the fibrin environment appears natural to the tumor cells and they have a tendency to remain in the chamber. The chambers containing tumor cells are surgically implanted, four chambers per animal, into the subcutaneous space of the animal. The study time of 6 to 12 days depends on the number of cancer cells introduced inside the chamber and the cells' normal growth rate.

XENOGRAFT AND SYNGENEIC TUMOR IMPLANTS

Human xenograft implants in nude athymic mice or syngeneic transplants in appropriate rodent models are the industry standard for assessing the success of an anti-cancer compound whether it works by directly affecting the growth of the tumor or by controlling processes such as angiogenesis. Tumor cell cultures or tumor fragments are implanted either ectopically or orthotopically and the tumors grown to a specified size. The animals are randomized, placed into groups, and the test groups treated with a prescribed drug regimen over the test period. Typically, tumor sizes are measured twice a week and weights once a week. The animals are sacrificed at the end of the test period and tissues are removed for pathological evaluation.



There are a number of sources for obtaining cancer cell lines. Whenever possible, we like the client to supply the cell line so that the in vivo studies are performed from the same cell line source as the initial in vitro studies.

Some human tumor lines that we commonly use in xenograft studies are:

- Breast (MDA-MB231, MCF-7)
- Lung (A549)
- Colon (HT25)
- Prostate (DU-145, PC-3)
- Ovary (Ovcar-3, SKOV-3)
- Brain (U87)
- Pancreas
- Fibrosarcoma
- Leukemia

Examples of syngeneic cell lines are:

- Mouse leukemia (L1210, P388)
- Mouse melanoma (B16)
- Rat gliosarcoma (9L)
- Rat adenocarcinoma (R230)

PATHOLOGY AND FOLLOW-ON STUDIES

Once the tissues have been collected, a variety of follow-on studies can be performed. We have exceptional expertise in human and animal pathology. Our capabilities include:

- Basic histology (H&E stain)
- Use of special stains (e.g., Masson Trichrome)
- Proliferation and apoptosis immunostains

- Immunohistochemistry with both monoclonal and polyclonal antibodies
- Fluorescent immunohistochemistry
- Cellular hypoxia measurements
- Blood vessel density measurements
- In situ hybridization
- Receptor binding and activity assays
- ELISA and Western blots on treated animal tissue and cell lysates for enzymes, cytokines, and other protein targets
- Zymogels for protease activity (e.g., matrix metalloproteases)
- RNA isolation, PCR, RT-PCR, and SuperArray®
- FACS analysis
- Biochemical assays for target effectiveness on treated animal tissues
- Custom assays

You Make the Call

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