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Overview

Angiogenesis Assays: A Critical Overview

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Background: **Angiogenesis, the formation of new blood vessels, is an integral part of both normal developmental processes and numerous pathologies, ranging from tumor growth and metastasis to inflammation and ocular disease. Angiogenesis assays are used to test efficacy of both pro- and antiangiogenic agents.**

Methods: **Most studies of angiogenesis inducers and inhibitors rely on various models, both in vitro and in vivo, as indicators of efficacy. In this report we describe the principal methods now in use: the in vivo Matrigel plug and corneal neovascularization assays, the in vivo/in vitro chick chorioallantoic membrane (CAM) assay, and the in vitro cellular (proliferation, migration, tube formation) and organotypic (aortic ring) assays. We include description of two new methods, the chick aortic arch and the Matrigel sponge assays.**

Conclusions: **In vitro tests are valuable, can be carried out expeditiously, and lend themselves to quantification, but must be interpreted with extreme caution. In vitro tests are best viewed as providing initial information, subject to confirmation by in vivo assays. Multiple tests should be used to obtain maximum benefit from in vitro tests. In vivo tests are more difficult and time-consuming to perform, thereby limiting the number of tests that can run at any one time. Quantification is generally more difficult as well. However, in vivo assays are essential because of the complex nature of vascular responses to test reagents, responses that no in vitro model can fully achieve.**

One of the most critical technical problems in the field of angiogenesis is the accurate interpretation of the highly varied results obtained from the many assays currently in use *(1–5)*. Over the last few years there has been a logarithmic increase in the number of reports dealing with angiogenesis. Thus, of 14 851 references indexed for angiogenesis, more than one-sixth were added last year and another 1749 have been added in the first 6 months of this year. Our methods for assessing angiogenesis, however, vary dramatically in what they measure, frequently lack quantification, and are limited in their clinical relevance *(6, 7)*.

It is against this background that the following overview is presented. It will not be exhaustive; rather it will focus heavily on those assays that are in most frequent use.

Angiogenesis: Features That Lend Themselves to Assessment

Angiogenesis, or neovascularization, is the process of generating new blood vessels derived as extensions from the existing vasculature [see, for example, Ref. *(4)*]. The principal cells involved are endothelial cells, which line all blood vessels and constitute virtually the entirety of capillaries. To achieve new blood vessel formation, endothelial cells must first escape from their stable location by breaking through the basement membrane. Once this is achieved, endothelial cells migrate toward an angiogenic stimulus such as might be released from tumor cells, activated lymphocytes, or wound-associated macrophages. Behind this migrating front, endothelial cells proliferate to provide the necessary number of cells for making a new vessel. Subsequent to this proliferation, the new outgrowth of endothelial cells needs to reorganize into a patent three-dimensionally tubular structure. Each of these elements, basement membrane disruption, cell migration, cell proliferation, and tube formation, can be a target for intervention, and each can be tested in vitro. However, the critical tests for angiogenesis require a more holistic assessment, and several in vivo assays have been developed that permit a more realistic appraisal of the angiogenic response than can be obtained in vitro.

In Vitro Assays

CELL PROLIFERATION

There are numerous well-established assays for measuring cell proliferation. The most frequently used measure, the thymidine incorporation assay, will serve to introduce

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several of the key problems of validating in vitro angiogenesis assays.

Shown in Fig. 1 are three tests carried out with two test substances *(2)*. Fig. 1A represents one of the standard tests for angiogenic inhibition, the inhibition of proliferation of bovine aortic endothelial (BAE)[1] cells, and clearly shows that one of the products is a potent inhibitor of the reaction, whereas the other is ineffective. However, Fig. 1B shows a similar result obtained when the product was tested on nonendothelial, bone marrow-derived (S17) stromal cells, suggesting that the product lacked specificity inasmuch as it was no more effective in inhibiting BAE cells than it was in inhibiting S17 cells. Moreover, when the cultures were examined after only 1 h, it was apparent that the active product was simply toxic. The same result would have been obtained if one were merely to add distilled water or excess sodium chloride to the culture medium. Clearly the test did not provide a valid assessment of antiangiogenic activity.

Interesting as well was the fact that both test substances were toxic to myocardium-derived microvascular endothelial cells (Fig. 1C). These cells are more delicate and difficult to maintain, but once again the observed "inhibition" was clearly not relevant to antiangiogenic therapy.

This brings up a more subtle, but very important problem: The source of endothelial cells used to validate antiangiogenic action in this example was the bovine aorta. This type of cell has been one of two principal sources of test endothelial cells, the other being human umbilical vein endothelial cells. However, all endothelial cells are not alike. As shown in the literature, not only are there differences between large-vessel-derived endothelial cells and endothelial cells of microvascular origin, but there are unique properties of endothelial cells obtained from different organ sites and even within single organs *(8–13)*. There are also species differences that should not be ignored. For example, most human endothelial cells bind *Ulex europeus* agglutinin I (UEA-I), whereas endothelial cells from mice and pigs do not. On the other hand, pig and murine endothelial cells bind *Bandeira simplifolica* lectin I (BSL-I) and BSL-4, making them targets for hyperimmune destruction were they to be implanted in patients. And perhaps most importantly, endothelial cells used in the laboratory are, by the very nature of their being in a proliferative state, different from endothelial cells that lie quiescent in the existing established vasculature. It is a well-known fact that cells in vitro both gain and lose attributes found in vivo, and it is generally not feasible to use truly primary (not passaged) endothelial cells in angiogenesis assays.

CELL MIGRATION ASSAYS

There are several tests that can be used to determine the migratory response of endothelial cells to angiogenesis-inducing or -inhibiting factors *(14)*. The most frequently used one is a blind-well chemotaxis chamber [modified Boyden chambers such as those used for classic neutrophil migration (chemotaxis) in which endothelial cells are placed on the upper layer of a cell-permeable filter and permitted to migrate in response to a test factor placed in the medium below the filter]. The most accurate measurements require cell enumeration after separation of the retained cells from the cells that have migrated across the filter. The system lends itself to testing concentration gradients and thus may well reflect the conditions that are operative in vivo. However, microvascular endothelial cells are more delicate than standard large-vessel endothelial cells or neutrophils, enumeration of traversed cells is tedious, and efforts to adapt the migration chamber for 96-well assays have met with mixed success.

In our laboratory we have developed a 96-well two-dimensional cell migration assay that measures cell motility and can be readily quantified *(15, 16)*. As shown in Fig. 2, a monolayer of 1-μm beads is deposited on the bottom of 96-well plates. Endothelial cells (100 cells/well) are then placed in the well, along with test medium. Cell movement is scored after 24 h (phagokinetic track assay), and the assay lends itself to computer-assisted quantifi-

[1] Nonstandard abbreviations: BAE, bovine aortic endothelial; BSL, *Bandeira simplifolica* lectin; FGF, fibroblast growth factor; VEGF, vascular endothelial growth factor; CAM, chick chorioallantoic membrane; FITC, fluorescein isothiocyanate; and PBS, phosphate-buffered saline.

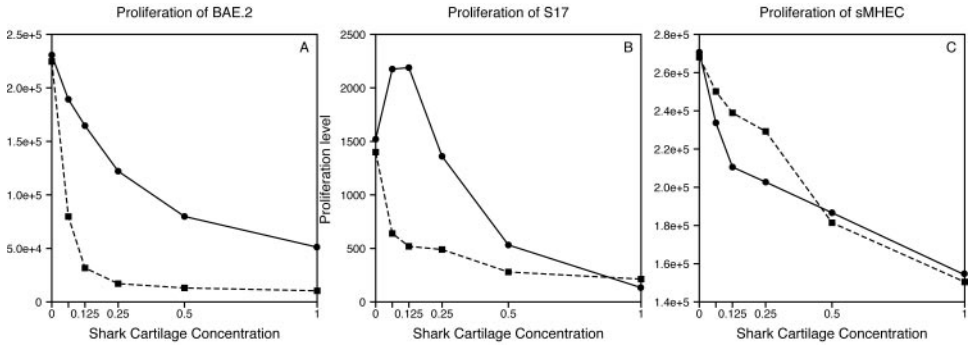

Fig. 1. Proliferation assay using [^{3}H]thymidine incorporation.

(*A*), BAE cells; (*B*), murine S17 bone marrow-derived stromal cells; (*C*), myocardium-derived microvascular endothelial cells (*smHEC*) *(2)*.

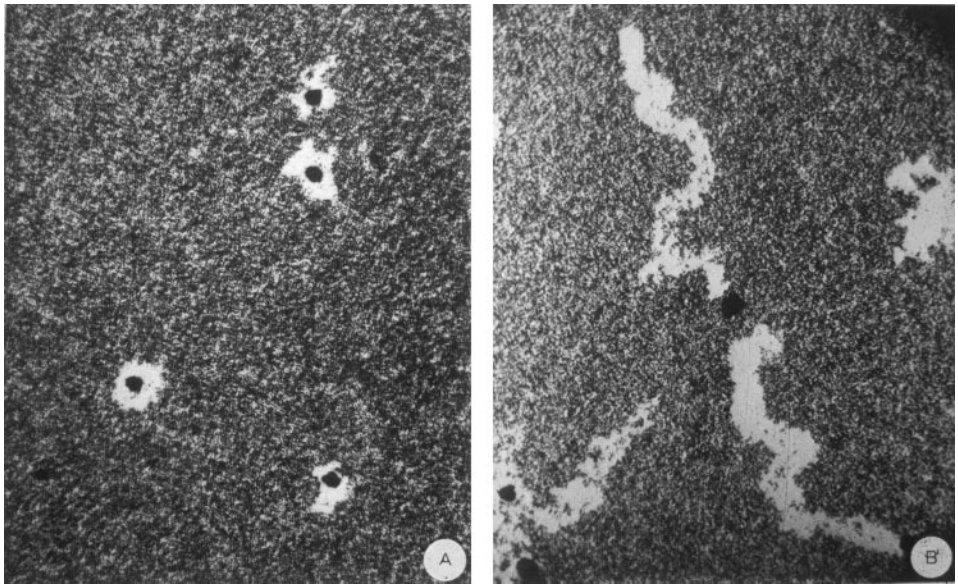

Fig. 2. Cell movement (phagokinetic track) assay *(16, 17)*.

(*A*), Eoma cells in low concentration of fetal bovine serum (10 mL/L) after 24 h; (*B*), Eoma cells in 50 mL/L fetal bovine serum after 24 h *(16)*.

cation. A typical result is shown in Fig. 3, in which a cytokine mixture, in this case a supernatant collected from mixed lymphocyte culture *(17)*, was tested for concentration-dependent efficacy in inducing an increase in cell movement. The assay is equally useful for testing inhibitory factors, such as endostatin or TNP 470, and motility-enhancing factors, such as fibroblast growth factor-2 (FGF-2) or vascular endothelial growth factor (VEGF), and because of its ease, permits utilization of several different endothelial and nonendothelial cell types to control for specificity of the observed response.

TUBE FORMATION

One of the most specific tests for angiogenesis is the measurement of the ability of endothelial cells to form three-dimensional structures (tube formation) *(18)*. Endothelial cells of all origins appear able to form tubules spontaneously, given time in vitro to lay down appropriate extracellular matrix components. Tube formation can be enhanced by use of collagen or fibrin clots to coat plastic culture dishes. Tube formation on these clots is reasonably faithful to the in vivo situation, and the formation of tight junctions can be confirmed by electron

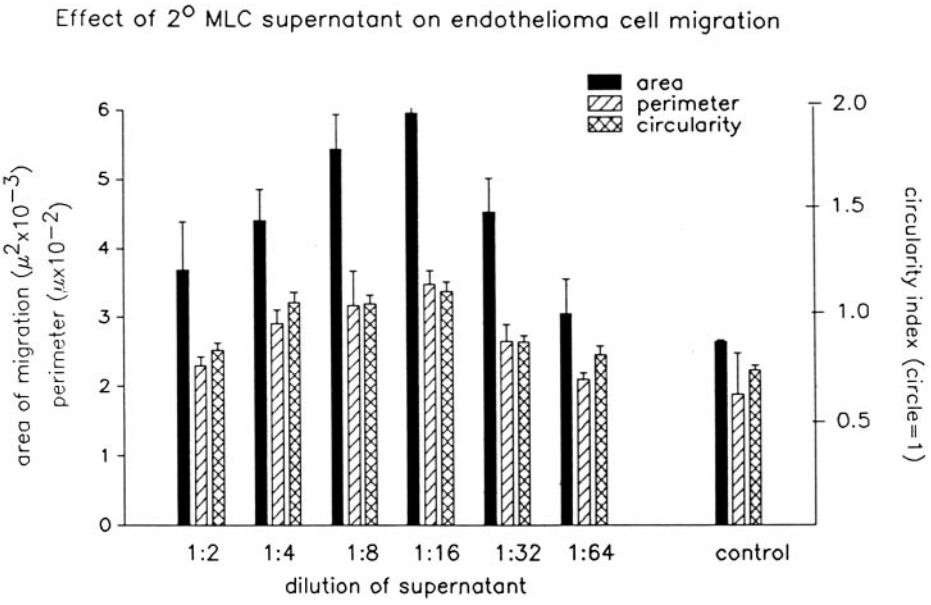

Fig. 3. Image analysis of migration tracks made in 1 mL/L serum with added supernatants from mixed lymphocyte culture *(17)*.

Note concentration-dependent effect on cell movement.

microscopy, With the discovery that Matrigel [a matrix-rich product prepared from Engelbreth–Holm–Swarm (EHS) tumor cells whose primary component is laminin *(18, 19)*] can evoke endothelial cell tube formation within 24 h, tube formation assays have achieved a prominent place in the array of angiogenesis measures (Fig. 4) *(18–21)*. One word of caution, however, is that cultured cells of nonendothelial origin, such as fibroblasts, may also exhibit a response to Matrigel [Ref. *(16)* and unpublished observations]. It is also critical to control the protein concentration of the Matrigel used, because not all commercial preparations of Matrigel promote tube formation in vitro (H. Kleinman, NIH, Bethesda, MD, personal communication, and our own unpublished observations).

Organ Culture Assays

THE AORTIC RING ASSAY

The recognition that angiogenesis in vivo involves not only endothelial cells but also their surrounding cells has more recently led to a move to assess angiogenesis by organ culture methods. Of these, the rat aortic ring assay has become the most widely used *(22, 23)*. In it, the isolated rat aorta is cut into segments that are placed in culture, generally in a matrix-containing environment such as Matrigel. Over the next 7–14 days, the explants are monitored for the outgrowth of endothelial (and other) cells as this is affected by the addition of test substances. Quantification is achieved by measurement of the length and abundance of vessel-like extensions from the explant. Use of endothelium-selective reagents such as fluorescein-labeled BSL-I *(24, 25)* allows quantification by pixel counts. Although in the past the culture conditions required complex media and outgrowth had to be monitored over at least 1 week, recent modifications have permitted the use of defined media and a shorter period of culture time.

This in vitro assay system is considered by many to come closest to simulating the in vivo situation, not only because it includes the surrounding nonendothelial cells but also because the endothelial cells have not been preselected by passaging and thus are not in a proliferative state at the time of explantation and thus more representative of the real-life situation. On the other hand, angiogenesis is primarily a microvascular event, making the aorta a less than ideal choice.

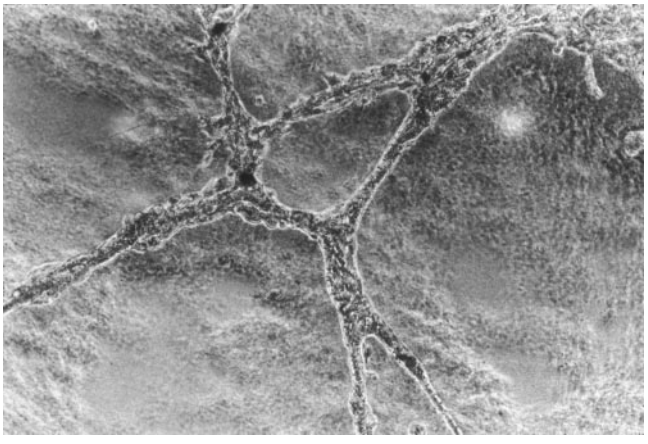

Fig. 4. Tube formation on Matrigel.

Murine myocardium-derived endothelial cells *(24)* were seeded on Matrigel. Tube formation was observed within 24 h.

THE CHICK AORTIC ARCH ASSAY

The chick aortic arch assay represents a major modification of the rat aortic ring assay. Originally developed for the specific purpose of testing thalidomide (which had previously been shown to have limited effects in rodents but strong effects in chick embryos), the assay avoids the use of laboratory animals, is rapid with an assay time of 1–3 days, and can be carried out in serum-free medium *(26)*. Aortic arches are dissected from day 12–14 chick embryos and cut into rings similar to those of the rat aorta. When the rings are placed on Matrigel, substantial outgrowth of cells occurs within 48 h, with the formation of vessel-like structures readily apparent (Fig. 5). If the aortic arch is everted before explanting, the time can be reduced to 24 h. Both growth-stimulating factors, such as FGF-2, or inhibitors, such as endostatin, can be added to the medium, where their effect becomes easily measured.

Quantification of endothelial cell outgrowth in both the aortic ring and the aortic arch cultures can be achieved by the use of fluorescein-labeled lectins such as BSL-I and BSL-B4 or by staining of the cultures with labeled antibodies to CD31. Standard imaging techniques are useful both for the enumeration of endothelial cells and for delineating the total outgrowth area.

Unlike the adult aorta, embryonic arch endothelial cells share many properties with microvascular endothelial cells. However, they are obtained from growing embryos and are therefore undergoing rapid cell division before explantation and exposure to angiogenic mediators.

In Vivo Assays

There is little doubt that the evaluation of angiogenesis-influencing factors is ultimately best made in vivo. The earliest of these in vivo assays involved the preparation of diffusion chambers made with Millipore filters *(27)*, as well as various other chamber techniques designed to monitor visually the progress of neovascularization of implanted tumors. Histologic observations even now provide the most detailed information concerning in vivo angiogenesis *(28)*. Improved techniques for monitoring blood flow by Doppler or radiologic approaches augment the information available from standard histologic observations *(29)*.

Many in vivo assay systems, however, have been developed that are easier to perform and permit better quantification. These include the chick chorioallantoic membrane (CAM) assay, an in vivo Matrigel plug assay, and a group of assays that use implants of sponges containing test cells or substances.

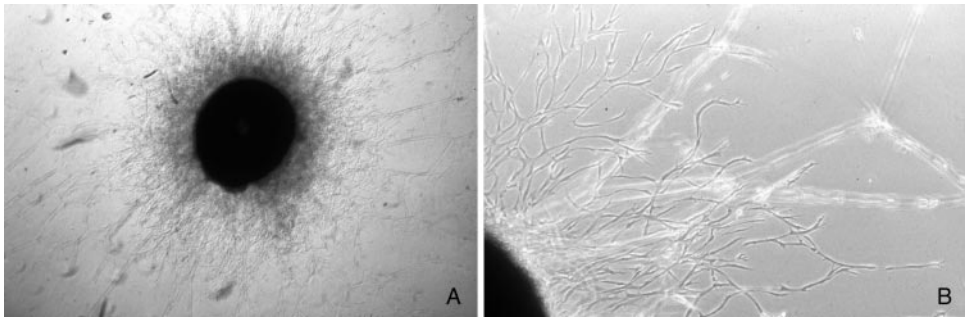

Fig. 5. Outgrowth of cells from the explanted aortic arch.

Panel A shows the total explant area. *Panel B* shows more details of the outgrowth area, in which endothelial cells are seen to form tube-like structures.

THE CAM ASSAY

The original CAM assay was described by experimental embryologists more than 50 years ago and has long been a mainstay for the study of embryonic organ development. In its original form, the CAM of day 7–9 chick embryos was exposed by making a window in the egg shell, and tissue or organ grafts were then placed directly on the CAM. The window was sealed, eggs were reincubated, and the grafts were recovered after an appropriate length of incubation time. The grafts were then scored for growth and vascularization. Initially, assessment of the angiogenic reaction was limited to ranking the vascularization on a 0 to 4 basis *(30, 31)*, but more recently, imaging techniques such as the measurement of bifurcation points in a designated area around the test material *(32)* have improved the quantification of the assay.

In a modification of this in ovo method, the entire egg contents were transferred to a plastic culture dish (whole embryo culture) after 72 h of incubation (Fig. 6A) *(33)*. Although technically this may be considered an in vitro assay, it is a whole-animal assay. After 3–6 additional days of incubation (Fig. 6B), during which time the CAM develops, grafts can be made more readily than within the egg shell and can be monitored throughout the time of subsequent development. Angiogenic stimulation by tumors or immunocompetent allografts or xenografts (graft-vs-host reaction) yields extensive angiogenesis over the next several days *(34)*. Test substances can be adminis-

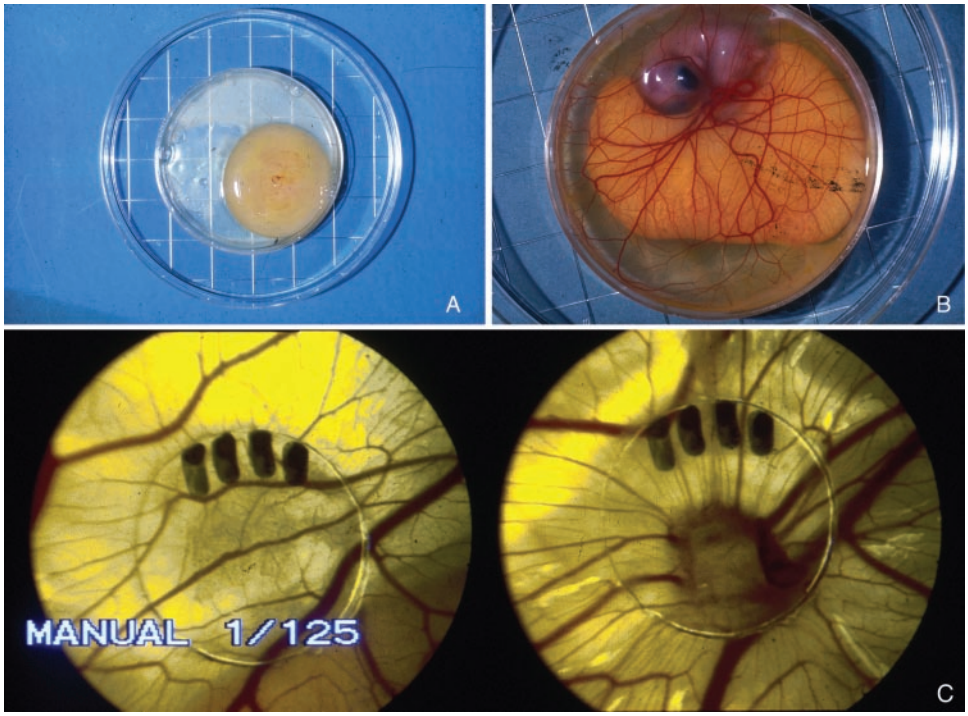

Fig. 6. CAM assay.

(*A*), chick embryo at 72 h of incubation immediately after its placement in a culture dish. (*B*), chick embryo after 1 week of additional growth *(33)*. (*C*), effect of FGF-2 (bFGF) placed on the underside of plastic coverslips. (*Left*), 0 h; (*right*), 72 h.

tered by placing them on membranes or on the underside of coverslips (Fig. 6C). Inhibitors can be assessed by their effect either on the normal development of the CAM vasculature itself or on induced angiogenesis such as the FGF-2-evoked angiogenesis shown in Fig. 6C.

Among the most valuable features of CAM assays are the relative ease of carrying out the assays, the ready availability of experimental material, and for the explant method, the feasibility of carrying out multiple tests on individual CAMs as well as of monitoring the reaction throughout the course of the assay. However, the tests are run on chicken cells, which may limit the utility of the assay, and there is always the underlying concern that the CAM itself is undergoing rapid changes both morphologically and in terms of the gradual change in the rate of endothelial cell proliferation during the course of embryonic development.

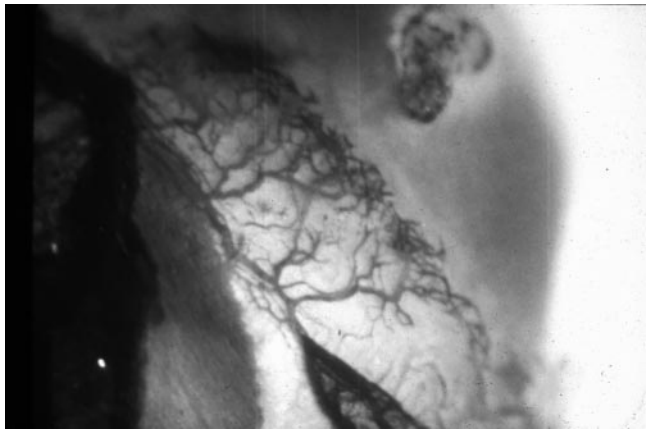

Fig. 7. Cytokine-induced angiogenesis in the mouse cornea.

Note ELVAX pellet. Sea Star Factor (interleukin-1) incorporated into the pellet (*upper right*) has induced vessels from the limbal vasculature. India ink injection.

THE CORNEAL ANGIOGENESIS ASSAY

This assay is still considered one of the best in vivo assays, inasmuch as the cornea itself is avascular. Thus, any vessels seen in the cornea after stimulation by angiogenesis-inducing tissues or factors are new vessels. The original method was developed for rabbit eyes *(35)*, but has been adapted to mice *(36, 37)*, now the most frequently used test animal. In brief, a pocket is made in the cornea, and test tumors or tissues, when introduced into this pocket, elicit the ingrowth of new vessels from the peripheral limbal vasculature. Slow-release materials such as ELVAX (ethylene vinyl copolymer) or Hydron have been used to introduce test substances into the corneal pocket. In our laboratory, we primarily use sponge material to hold test cell suspensions or substances to induce angiogenesis because the slow-release formulations are frequently toxic.

To test inhibitors of angiogenesis, one can monitor the effect of such inhibitors on the locally induced (e.g., sponge implant) angiogenic reaction in the cornea (e.g., by FGF, VEGF, or tumor cells). The test inhibitors can be administered orally or systemically, the latter either by bolus injection or, more effectively, by use of a sustained-release method such as implantation of osmotic pumps loaded with the test inhibitor *(38, 39)*.

The vascular response can be monitored by direct observation throughout the course of the experiment. This requires a slit lamp for the rabbit but needs only a simple stereomicroscope in mice. Definitive visualization of the mouse corneal vasculature was once achieved by injecting India ink (Fig. 7). More recently, use of fluorochrome-labeled high-molecular weight dextran has become the method of choice (Fig. 8, A and B) *(40)*. Methods for quantification include measuring the area of vessel penetration, the progress of vessels toward the angiogenic stimulus over time, or in the case of fluorescence, histogram analysis or pixel counts above a specific (background) threshold.

There are considerable advantages to the corneal angiogenesis assay: the ability to monitor progress of angiogenesis, the absence of an existing background vasculature in the cornea, and the ability to use mice as experimental animals. On the other hand, the surgical procedure is demanding, so that relatively few animals (~20 mice) can be grafted at a single setting. In addition, the space available for introducing test material is limiting, inflammatory reactions are difficult to avoid, and the site, although ideal for visualization, is atypical precisely because the cornea is avascular.

THE MATRIGEL PLUG ASSAY

In contrast to the mouse corneal angiogenesis assay, which requires considerable technical skill, the Matrigel plug assay is not difficult to administer *(41)*. Matrigel containing test cells or substances is injected subcutaneously, where it solidifies to form a plug. This plug can be recovered after 7–21 days in the animal and examined histologically to determine the extent to which blood vessels have entered it. Quantification of the vessels in histologic sections is tedious but accurate *(41)*. Fluorescence measurement of plasma volume can be achieved using fluorescein isothiocyanate (FITC)-labeled dextran 150 *(42)*. Quantification can also be achieved by measuring the amount of hemoglobin contained in the plug *(41)*. However, the hemoglobin assay may be misleading because blood content is much affected by the size of vessels and by the extent of stagnant pools of blood.

In our laboratory we have recently modified the Matrigel plug assay to permit clearer delineation of neovascularization *(39)*. In this modified assay, the sponge/Matrigel assay, Matrigel alone is first introduced into the mouse. A sponge or tissue fragment is then inserted into the plug. New vessels can then be measured by injection of FITC-dextran as described for the corneal assay. Panels C and D in Fig. 8 illustrate an experiment in which mouse mammary adenocarcinoma fragments were introduced into the Matrigel plugs. Alzet pumps containing either endostatin or phosphate-buffered saline (PBS) were im-

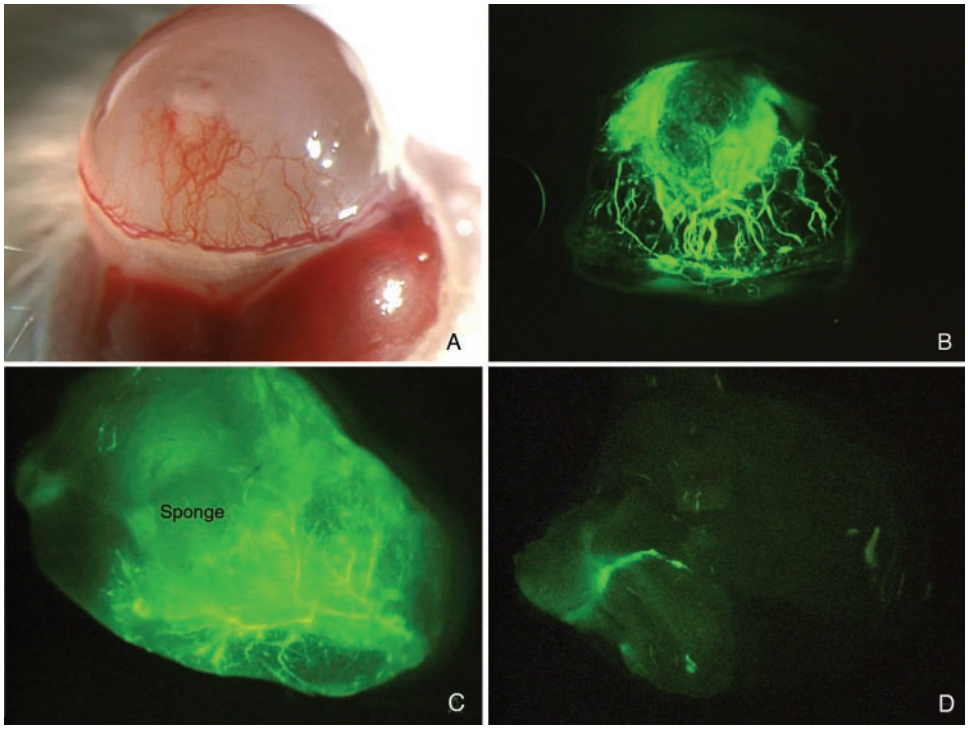

Fig. 8. FGF-2 (bFGF)-induced angiogenesis in the mouse cornea (*top*), and example of the sponge-Matrigel system (*bottom*).

(*Top*), vessel formation can be observed during the course of angiogenesis (*A*) and more readily quantified by measuring fluorescence after injection of FITC-dextran (*B*). (*Bottom*), antiangiogenic effect of endostatin on vascularization. Alzet pumps containing either PBS (*C*) or endostatin (*D*) were implanted subcutaneously and replaced after 7 days. Matrigel plugs containing 4T1 tumor fragments were removed and analyzed after 14 days *(39)*.

planted peritoneally, the pumps were replaced with new pumps after 7 days, and the plugs were recovered after 2 weeks *(39)*. Angiogenesis was readily observed in the PBS control plugs (Fig. 8C) but was virtually abolished when endostatin was administered over the 2-week period (Fig. 8D). The greatest disadvantage of the sponge/Matrigel assay is that it is more time-consuming than the standard Matrigel plug assay. When Alzet pump implants are added to the protocol, the number of animals that can be assayed (~12) becomes limiting.

Caveats and Cautions

Listed in Table 1 are caveats and cautions to consider for the in vitro and in vivo assays.

Conclusions

In vitro tests are useful, can be carried out expeditiously, and lend themselves to quantification, but must be interpreted with extreme caution. In vitro tests provide critical information and are essential first steps for validation. Multiple tests should be used to obtain maximum benefit from in vitro tests. Endothelial cell chemokinesis, chemotaxis, proliferation, and tube formation are complementary assays that need to be confirmed in vivo. Organ cultures such as the aortic ring and aortic arch assays yield important information not yielded by cell cultures because organ cultures permit interactions between endothelial cells and their surrounding heterotypic microenvironment.

Table 1. Caveats and cautions in the use of in vitro and in vivo assays.

In Vitro	In Vivo
All endothelial cells are not alike	The cornea and subcutaneous locales are not representative of the sites where angiogenesis occurs during pathologic development
Cell culture is not the same as organ culture	Species-specific differences need to be taken into account in interpreting results obtained in experimental animals
Current assays do not use primary cultures	Most test systems are artificial; established tumor lines are not the same as autochthonous tumors
Cells have been preselected for proliferative capacity	In xenografts, new vessels arise from the host rather than the graft
Endothelial cell cultures do not allow for heterospecific interactions	Because of technical difficulties, there is much variability in experimental results
Even organ cultures lack the many dynamic factors regulating angiogenesis in vivo	There is much inherent variability among animals, and accuracy in measurements, such as using imaging techniques, cannot compensate for this variability

Regardless of the amount of information that can be generated by in vitro tests, in vivo tests are an absolute necessity for accurate evaluation of angiogenesis. In vivo tests tend to be more difficult to perform and frequently require surgical skills, thereby limiting the number of tests that can readily be performed. Critical choices need to be made to decide the best animal species for a particular assay, given that results obtained in mice, for example, may not be directly applicable to patients. Currently, in vivo assays are difficult to quantify, but new methods for imaging vessels and for image analysis are emerging that may help provide quantification of in vivo experiments, quantification that is essential to studies of angiogenic and antiangiogenic reagents.

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