

## In vivo models of angiogenesis

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### Abstract

The process of building new blood vessels (angiogenesis) and controlling the propagation of blood vessels (anti-angiogenesis) are fundamental to human health, as they play key roles in wound healing and tissue growth. More than 500 million people may stand to benefit from anti- or pro-angiogenic treatments in the coming decades [National Cancer Institute (USA), Cancer Bulletin, volume 3, no. 9, 2006]. The use of animal models to assay angiogenesis is crucial to the search for therapeutic agents that inhibit

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it angiogenesis in the clinical setting. Examples of persons that would benefit from these therapies are cancer patients, as cancer growth and spread is angiogenesis-dependent, and patients with aberrant angiogenesis in the eye, which may lead to blindness or defective sight. Recently, anti-angiogenesis therapies have been introduced successfully in the clinic, representing a turning point in tumor therapy and the treatment of macular degeneration and heralding a new era for the treatment of several commonly occurring angiogenesis-related diseases. On the other hand, pro-angiogenic therapies that promote compensatory angiogenesis in hypoxic tissues, such as those subjected to ischemia in myocardial or cerebral hypoxia due to occluding lesions in the coronary or cerebral arteries, respectively, and in cases of poor wound healing, are also being developed. In this review, the current major and newly introduced preclinical angiogenesis assays are described and discussed in terms of their specific advantages and disadvantages from the biological, technical, economical and ethical perspectives. These assays include the corneal micropocket, chick chorioallantoic membrane, rodent mesentery, subcutaneous (s.c.) sponge/matrix/alginate microbead, s.c. Matrigel plug, s.c. disc, and s.c. directed *in vivo* angiogenesis assays, as well as, the zebrafish system and several additional assays. A note on quantitative techniques for assessing angiogenesis in patients is also included. The currently utilized preclinical assays are not equivalent in terms of efficacy or relevance to human disease. Some of these assays have significance for screening, while others are used primarily in studies of dosage-effects, molecular structure activities, and the combined effects of two or more agents on angiogenesis. When invited to write this review, I was asked to describe in some detail the rodent mesenteric-window angiogenesis assay, which has not received extensive coverage in previous reviews.

**Keywords:** angiogenesis assays • *in vivo* • methods • quantification • chick CAM assay • cornea assay • mesentery assay • sponge assay • matrix assay • Matrigel assay • disc assay • zebrafish assay • *Xenopus* tadpole assay • mouse • rat • rabbit

## Background

### Angiogenesis in adult organisms

In growing organisms angiogenesis, *i.e.*, the growth of microvessels from parent microvessels, is an essential part of new tissue growth. In adulthood, with the exception of tightly regulated cyclical events in the female reproductive organs, almost every normal tissue lacks substantial physiological angiogenesis, because of a balance between effects of pro- and anti-angiogenic endogenous factors. When the balance is upset in the pro-angiogenic direction, microvascular endothelial cells (ECs) switch to an angiogenic phenotype, which starts an angiogenic reaction that can either be abrogated or progress. There is considerable heterogeneity among ECs in different tissues and organs. There are also species differences that should not be ignored. Angiogenesis occurs regularly in connection with wound healing, inflammation, rheumatoid arthritis, endometriosis, diabetic retinopathy, macular degeneration, and tumor growth.

### Tumor angiogenesis

The growth of tumors beyond a minute volume is angiogenesis-dependent. Tumor cells are genomically unstable and prone to produce oncogenes, pro-

liferate, and mutate. Thus, tumors often progressively acquire a variety of phenotypes, including diverse angiogenic phenotypes. Tumors induce the production of angiogenic factors in the following ways: (i) *via* the switching of neoplastic cells to an angiogenic phenotype; (ii) by activating tumor stroma cells, such as fibroblasts, macrophages, mast cells, and leukocytes, some of which are recruited from adjacent or more distant non-tumor tissues; (iii) by release of angiogenic factors from the extracellular matrix (ECM) to which they bind; and (iv) the emergence of new epitopes in the ECM that promote angiogenesis. The pro-angiogenic contribution of non-tumor cells results from their interactions with neoplastic cells and the altered ECM.

Vascular endothelial growth factor, VEGF (VEGF-A, particularly VEGF<sub>165/164</sub>), is a major pro-angiogenic factor in most human and experimental tumors. Moreover, hypoxic cells, which results from robust cell proliferation and increased cell metabolism, produce VEGF and up-regulate VEGF-receptors on pre-existing ECs. The main functions of VEGF are to promote EC survival, induce EC proliferation and enhance the migration and invasion of ECs, which contribute to angiogenesis. VEGF regulates these functions by interacting with its tyrosine kinase receptors and transmitting signals to various downstream proteins [1]. Alternatively or as a complement to the increased expression of pro-angiogenic molecules, a local decrease in the expression of endogenous anti-

angiogenic factors may occur, with consequential stimulation of angiogenesis.

Tumor cell products that influence angiogenesis are active not only in the tumor but also in distal tissues that respond differently to these stimuli. An example of this type of distal effects is the recruitment of circulating endothelial precursors (CEPs) from the bone marrow by VEGF that is secreted from a remote tumor. CEPs are able to home to angiogenically activated microvessels in the tumor and enhance the angiogenic reaction *via* paracrine signaling [2] or by functioning as supporting cells [3].

Although VEGF is produced by both neoplastic and non-neoplastic cells, mutating tumor cells tend to acquire the ability to produce additional pro-angiogenic substances. For this reason, several pro-angiogenic stimuli are expected to occur simultaneously during the long-term progression of a tumor. Notably, ECs within tumors and in tumor-free tissues demonstrate similar signaling pathways [4]. As a result, angiogenesis studies in tumor-free tissues may reflect not only specific features of the tissue studied and the angiogenesis-modulating stimuli used, but also reflect indirectly to some degree the tumor-related features of the actual tissue and tumor angiogenesis in general. As tumor angiogenesis is primarily of the sprouting type, assays that quantify sprouting angiogenesis are well suited to studies of certain aspects of tumor angiogenesis.

The anti-tumor effects of directly or indirectly acting exogenous anti-angiogenic agents, including chemotherapeutics, on spontaneously occurring tumors in humans are probably specific to individual tumors. This is due to genomic heterogeneity among neoplastic cells and the cross-talk that takes place between these cells and the stromal host immune cells and fibroblasts, the ECs, and the ECM within the tumor tissue [5–7]. Moreover, the influence of exogenous anti-angiogenic agents on angiogenesis may be site-dependent [5, 8, 9].

### **Basic requirements for good angiogenesis assays**

Due to the complex cellular and molecular activities of angiogenic reactions, *in vivo* studies are more informative than *in vitro* studies providing that the biology of the assay and the experimental design are

relevant; *in vitro* studies of ECs are, however, in many instances a necessary complement to *in vivo* experiments. Trauma, either physical or chemical (abnormal osmolarity, altered pO<sub>2</sub> tension, changes in pH or toxicity) that leads to cell damage induces an inflammatory reaction. Since several pro-angiogenic cytokines are released during inflammation from tissue-bound and circulating cells, which include platelets, this reduces the sensitivity and specificity of any trauma-based assay. Assays in which the new blood vessels are close to tissue-air interfaces may allow exposure to artificially high concentrations of oxygen, as in the corneal micropocket and the chick CAM assays (see below).

It has been proposed that a test material that induces angiogenesis should be designated as being angiogenic only when it is in a non-inflammatory state. Clearly, care should be taken to avoid or reduce to a minimum any inflammatory reaction in the test tissue. The test substance for inducing an angiogenic response should ideally be used at a dose that approximates the physiological dose, whereas the doses used for modulating an angiogenic reaction should ideally be comparable to the range used (or that could be used) in the clinic. In many assays, the best situation is to compare test animals/samples with vehicle-exposed counterparts. However, to allow safe interpretation of the acquired data, one needs to have a good understanding of how the vehicle controls differ from the untreated controls. In this respect, the inability to control the spatial and temporal distributions of test substances *in vivo* has hindered the generation of rigorous and reliable dose-response curves (see below).

Since newly formed microvessels are delicate, histological microscopy provides the most detailed information on *in vivo* angiogenesis. Mammalian systems are considered to be more representative of human pathophysiology than, for example, the embryonic avian chorioallantoic membrane (CAM) or embryonic zebrafish assay [10].

### **Is there an ideal angiogenesis assay?**

Considering the heterogeneity of tissues and the molecular and cellular complexities of angiogenic reactions, it is not surprising that a single assay that is optimal for all situations has not yet been described, although ingenious ways have been

developed for measuring angiogenic processes. Indeed, many workers with expertise in this area have expressed a certain amount of disillusionment with the available assays. Having used the chick CAM and the rabbit cornea micropocket assays, Vallee *et al.* [11] conclude that "The design and verification of [new] specific, reliable, repeatable and precise methodology to measure angiogenesis is considered an imperative of high priority in the field of angiogenesis research". Auerbach *et al.* [12] state, "Perhaps the most consistent limitation in all these studies and approaches has been the availability of simple, reliable, reproducible, quantitative assays of the angiogenic response".

Moreover, as summarized by Jain *et al.* [13], "An ideal assay for quantitative angiogenesis studies must satisfy the following requirements: (1) the release rate [R] and the spatial and temporal concentration distribution [C] of angiogenic factor(s)/inhibitor(s) should be known for generating the dose response curves; (2) if neoplastic cells are used as a source of angiogenic factors, they should be genetically well defined in terms of oncogene expression and production of growth factors (stimulators and inhibitors); (3) the assay should provide a quantitative measure of the structure of the new vasculature (*e.g.*, vascular length [L], surface area [A], volume [V], number of vessels in the network [N], fractal dimensions of the network [Df], and extent of basement membrane [BM]); (4) it should provide a quantitative measure of the functional characteristics of the new vasculature (*e.g.*, EC migration [MR], proliferation rate [PR], canalization rate [CR], blood flow rate [F], and vascular permeability [P]); (5) there should be a clear distribution between newly formed and pre-existing host vessels; (6) tissue damage should be avoided, since it may lead to formation of new vessels; (7) any response seen *in vitro* should be confirmed *in vivo*; (8) such an assay should permit long-term and, if possible, noninvasive monitoring; and (9) it should be cost-effective, rapid, easy to use (routine), reproducible and reliable". Unfortunately, no single assay fulfills anything like all of these criteria.

The major assays are presented below in approximate chronological order of their first publication, and additional important assays are then presented in brief. The assays most frequently used in current practice appear to be the CAM, Matrigel plug, and corneal micropocket assays [14]. Some assays, such

as the CAM, Matrigel plug, and zebrafish assays, are considered suitable for the large scale screening of new compounds. The strong and weak characteristics of the major assays are summarized in Table.

## The corneal micropocket assay

Folkman and associates introduced the corneal micropocket assay [15] and the chick CAM assay in 1974 [16–19] thereby laying a firm foundation for systematic angiogenesis research.

## The test tissue

The cornea is covered on the ventral and dorsal surfaces by epithelium. In the rat, the cornea is 250–255  $\mu\text{m}$  in thickness, while it is somewhat less thick in mice. The ventral surface is covered by non-keratinizing stratified squamous epithelium abutting onto Bowman's membrane, which is composed of fine collagen fibers embedded in the ECM. The cornea is richly innervated. The long ciliary nerves course through the sclera and divide to form 70–80 myelinated nerves (in man) that branch at the corneal periphery. The numerous free nerve endings that are present ensure extreme sensitivity to touch. Therefore, the cornea is a sensory organ.

Beneath Bowman's membrane, the corneal stroma is composed of broad sheets of tightly bound, parallel collagen fibers (corneal lamellae), which are embedded in an ECM composed mainly of sulfated glycosaminoglycans covalently bound to protein. The stroma constitutes ~80% of the corneal thickness in rats. To provide maximum mechanical strength the direction of the collagen fibers alternates in each layer. Between the lamellae, there are sparse, inactive, spindle-shaped fibroblasts (keratocytes). The regular parallel arrangement of the collagen and the paucity of cells render the cornea translucent. The dorsal surface is covered by a single layer of polygonal cells (corneal endothelium) that pump fluid from the stroma, thereby preventing the excessive hydration of the ECM, which would result in the opacification of the cornea. It has been suggested that the cornea, prior to vascularization is an immunologically privileged site [15].

After trauma, the regeneration of the stroma, and the migration of adjacent keratocytes into

**Table 1** Strengths and weaknesses of the major *in vivo* angiogenesis assays discussed here

Assay	Advantages	Disadvantages
<b>Corneal micropocket</b> <sup>1, 2</sup>	New vessels are easily identified Used in rabbits, rats, and mice Permits non-invasive observation and long-term monitoring Quantitative Mammalian Immunologically privileged site before vascularization Angiogenesis by sprouting	Atypical angiogenesis, as the normal cornea is avascular Technically demanding, especially in mouse eye Traumatic technique Ethically questionable The cornea is not a highly relevant site for tumor growth Exposure to oxygen via surface can affect angiogenesis Non-specific inflammatory response with some compounds Expensive
<b>CAM</b>	Technically simple Inexpensive Suitable for large-scale screening Permits non-invasive observation Suitable for mammalian xenografts	Very sensitive to increase in oxygen tension Visualization of new vessels can be difficult Non-mammalian Embryonic Angiogenesis by sprouting and intussusceptive growth Accelerates or suppresses organogenic angiogenesis (up to Days 10–11) Non-specific inflammatory reactions common Drugs that require metabolic activation cannot be assessed
<b>Mesentery</b> <sup>1, 2</sup>	The adult tissue is vascularized and lacks significant physiologic angiogenesis Truly quantitative, allowing dose-response studies <sup>3</sup> in terms of microvessel spatial extension, density and number and length of microvessel segments and sprouts (in rat) Minimal trauma, if any, is inflicted in the test tissue The test tissue is visceral; visceral organs are frequent sites of tumor metastasis Suitable for measurement of growth factor-induced signaling in intact microvessels Suitable for intravital microscopy Suitable for molecular-activity studies Angiogenesis by sprouting	Time consuming Technically somewhat demanding Mice are less suitable for quantitative angiogenesis analysis than rats Rats demand approximately 10 times greater quantity of test agents than mice Does not allow real-time observations
<b>Sponge/matrix implant</b> <sup>1, 2</sup>	Technically simple Inexpensive Well tolerated Time course of response can be recorded Suitable for study of tumor angiogenesis	Time consuming Encapsulated by granulation tissue Sponge composition varies, making inter-experimental comparisons difficult Variable retention of test compound within implant The subcutaneous tissue is not a highly relevant site for tumor growth Animals have to be kept singly
<b>The disc assay (DAS)</b> <sup>1, 2</sup>	Technically simple Assesses wound healing and angiogenesis Quantitative analysis	Encapsulated by granulation tissue The subcutaneous tissue is not highly relevant for tumor growth
<b>Matrigel plug</b> <sup>1, 2</sup>	Technically simple Suitable for large-scale screening Rapid quantitative analysis in chambers	The Matrigel is not chemically defined Difficult to make plugs uniform in 3-dimensional shape (except in chambers) Analysis in plugs time consuming The subcutaneous tissue is not highly relevant for tumor growth Expensive Not responsive to VEGF (in chambers)
<b>Zebrafish</b>	Intact whole animal Technically simple Allows gene analysis of vessel development Truly quantitative Large numbers of animals available for statistical analysis Relatively fast assay Suitable for large-scale screening	Non-mammalian Embryonic Expensive to maintain in breeding condition

Modified from [10, 29].

1) In-bred and out-bred mouse and rat strains are available. 2) Knock-out and SCID mice are available. 3) As with most, if not all, current angiogenesis assays, the release rate and the spatial and temporal distribution of angiogenic factors/inhibitors are not fully known, however.

the wound, begins within the first 24 hrs and increases rapidly thereafter [20]. During an inflammatory response in the cornea, very high numbers of neutrophilic leukocytes and lymphocytes are frequently seen to migrate between the lamellae of the stroma [20].

### **Assay set-up**

The earliest use of intracorneal grafts to assess angiogenesis was in rabbits [15]. Using a special spatula, a pocket is made in the stroma from the dome to the limbus so that the base of the pocket toward the limbus is wider than the insertion point at the dome. The pocket is designed to reach 1–2 mm from the limbus, while the central pocket ends 5–7 mm from the limbus (in rabbits). Empty pockets that extend  $\geq 1$  mm from the limbus do not usually stimulate corneal angiogenesis. The modulation of the angiogenic response by different stimuli can be assessed by the implantation of multiple pellets into parallel micropockets in the same cornea [10, 21]. Angiogenesis mainly occurs through sprouting from the adjacent limbal area [10, 15].

Test substances are usually delivered in a slow-release polymer that is placed in the micropocket. Many of these formulations cause irritation, which leads to inflammatory reactions that can compromise angiogenesis quantification [10, 12, 14]. The introduction of cells and test substances in polyvinyl sponges avoids or reduces the use of sustained release polymers. The release kinetics for slow-releasing carriers and polymer sponges within the cornea differ over time, with the tendency to produce an initial burst before the release becomes linear [10, 22]. A single injection of a test substance will usually diffuse quickly and will not provide a sufficiently long stimulus for new capillary formation.

### **Analysis**

It is recommended that an operator independent of the surgeon should record daily the levels of angiogenesis, edema, and inflammatory cellular infiltration in the cornea [23]. The evolution of the angiogenic response in transparent corneas can be studied non-invasively using a slit lamp or a stereomicroscope. Intravenous (i.v.) administration of fluorescein-labeled high-molecular-weight dextran is

frequently employed for definitive visualization of corneal angiogenesis [10, 24]. The angiogenic response is often quantified on a 0 to +4 scale by photography and measurement of the capillary growth into the cornea. A non-invasive method for recording the entire pattern of corneal neovascularization over time in individual living animals has been developed [25]. The technique couples video data acquisition methods with computerized analysis of the video images. Background electronic signals are reduced and contrast is enhanced in each montage with the aid of image processing. Finally, vessel area is calculated by pixel counting after establishing the density range of vessel identification. Alternatively, histological methods can be applied at predetermined time-points.

### **Advantages and disadvantages of the assay**

With respect to angiogenesis assays, the normal cornea has the unique advantage of having no pre-existing blood vessels. Many regard the elicitation of an angiogenic reaction in the cornea as the most convincing demonstration of true neovascularization [15, 26].

However, the fact that the normal cornea is an avascular structure makes this assay somewhat atypical, since normal tissues, with few exceptions, are vascularized. Moreover, the corneal pocket itself is inaccessible to the many blood-borne factors that can influence angiogenesis [12]. The rabbit corneal assay has been modified and adapted to mice [27, 28] and rats. The assay is regarded as technically difficult and surgery becomes more difficult as eye size decreases in animals smaller than the rabbit [29]. One major pitfall is the induction of non-specific inflammation. Therefore, it is important (at the termination of the experiment) to obtain histological sections to determine whether the presence or extent of any an inflammatory response [30].

The rabbit cornea assay has been studied using transmission and scanning electron microscopy [31]. In sham-operated controls, strongly vacuolated keratocytes are present at the borders and especially at the bottom of the pocket. A large number of these cells are also found in the region between the pocket and the epithelium. These cells undergo necrosis. Changes are also observed in the surface epithelium. In basic fibroblast growth factor (bFGF)-mediated angiogenesis, neutrophils, monocytes and a few

mast cells migrate in front of the sprouting capillaries that emanate from the limbus. Based on the observations of inflicted lesions and alterations, it is suggested that an angiogenic substance placed in the corneal pocket is not the only angiogenic stimulus in the test, but acts together with many other stimuli caused by the surgical manipulations [31].

Importantly, ethical problems can be encountered when using an assay that involves a major sensory organ. Additional disadvantages of the assay include (i) the short duration of the assay (< 7 days) [32]; (ii) the time required; (iii) the cost associated with the use of rabbits; and (iv) the low number of substances that can be tested [26]. Since the surgical technique is demanding and time-consuming, relatively few animals (~20 mice) can be grafted in a single setting [14, 32]. One additional disadvantage is that tumors other than those that originate from an animal of the same genetic background as the test animal may induce an immune response once they are vascularized. - For instructive technical details, see [33].

## The chick chorioallantoic membrane (CAM) assay

As noted, Folkman and associates introduced this assay in 1974 [16–19]. Originally used by embryologists to study the developmental potential of embryonic tissue grafts, the CAM assay has been adopted to the study of tumor angiogenesis as well as to testing pro- and anti-angiogenic factors.

### Test tissue

The CAM of the chick is formed by the fusion of the somatic mesoderm of the chorion with the splanchnic mesoderm of the allantois during the fourth and fifth days of embryonic development [34]. This highly vascularized membrane serves as the initial respiratory system of the avian embryo and is responsible for gas exchange until Day 19 of incubation with a total duration of 21 days [35].

Until Day 10 of incubation, the CAM vascular ECs have the morphological characteristics of immaturity and relatively undifferentiated cells and exhibit an intrinsically high mitotic rate [17]. CAM angio-

genesis undergoes three phases of development. In the early phase (Day 5 to Day 7), the major mechanism of capillary network growth is sprouting. In the intermediate phase (Day 8 to Day 12) intussusceptive microvascular growth (IMG) prevails. IMG is a capillary system that grows by the insertion of transcapillary tissue posts that form new intercapillary tissue profiles, which subsequently grow to full-size intercapillary meshes [36, 37]. From Day 8 to Day 12, sprouts are no longer present, since they have been replaced by IMG [10]. By Day 12 or 13, the chorioallantois lines the entire shell membrane and its expansion is complete [see 17].

### Assay set-up

Fertilized hen's eggs are incubated at 37°C for 72 h and prepared for grafting by removing enough albumen to minimize adhesion of the shell membrane. Tissue grafts or carriers that contain test substance are then placed directly onto the CAM through a rectangular opening made in the eggshell. The test substance is prepared either in carriers such as slow-release polymer pellets, absorbed by gelatin sponges, or is air-dried onto plastic discs. Elvax 40 and Hydron, from which sponges and membranes can be made, have been reported to be inert when applied to the CAM [26]. However, it is often difficult to evaluate fully the potential influences of the carrier on the outcome, as discussed below. In this model, angiogenesis is quantified three or four days after grafting.

The CAM assay carried out *in ovo*, as just described, is technically relatively simple. A complementary method, which permits the growth of chicken embryos in Petri dishes *in vitro* from the third day of incubation, has been described [16]. Technically, this is an *in vitro* assay, although strictly speaking it is a whole-animal assay. After 3–6 additional days of incubation, during which time the CAM develops, the grafts can be monitored throughout subsequent development. This allows for the quantification of blood vessels over a wider area of the CAM than is possible *in ovo*. Additional advantages of the *in vitro* CAM assay are the large number of samples that can be tested at any one time and the short period of time required for a response to occur (2–3 days) [32]. For CAM *in vitro*, the test substance can be placed on the under-

side of coverslips [14]. The CAM assay has reportedly been carried out more generally *in ovo* rather than *in vitro* [22].

The timing of the CAM angiogenesis response is clearly essential. Between Days 5 and 12 of incubation, the experimentally induced acceleration or suppression of the constant embryonic organogenic angiogenesis can be assessed. The endogenous embryonic angiogenesis is governed by a host of undefined growth factors in addition to VEGF, which is essential in all embryonic angiogenesis. *De novo* angiogenesis, *i.e.*, initiation of an angiogenic reaction by a pro-angiogenic factor that overcomes the effects of inherent angiogenesis inhibitors in a tissue that lacks physiological angiogenesis, can thus only be accomplished from Day 12 onwards. A non-specific vasoproliferative response is far less likely when the test material is grafted as soon as the CAM begins to develop, at which point the host immune system is still immature [10, 38].

By applying an anti-angiogenic substance to some sort of carrier directly onto the approximately 5- to 10-day-old CAM vasculature, *in ovo* or in a Petri dish, it is, in principle, rather easy and straightforward to observe the suppression of ongoing organogenic angiogenesis.

The grafting of tissue onto the chick CAM is a procedure that has been used by embryologists for many decades and the first evidence of tumor-induced angiogenesis *in vivo* using the chick CAM was obtained in 1913 [39, 40]. The CAM appears to be ideal for investigating tumor-induced angiogenesis, as the host immune system is not fully developed. Tumors grafted onto the CAM remain non-vascularized for a couple of days, after which they can be penetrated by new blood vessels and begin a phase of rapid growth. The rate of growth during this vascular phase is reportedly highest for implants of rat Walker 256 carcinosarcoma on 5- and 6-day-old embryos [41] and for human malignant multiple myeloma cells on 8- and 10-day-old embryos [42]; the growth rate decreases the later the day of implantation. The time of onset of tumor angiogenesis appears to be independent of the immunological state of the chick embryo, although the rate of tumor growth after vascularization may be modified by the onset of immunity [41]. Adult normal tissues do not revascularize, while most embryonic tissues revascularize in 1 to 2 days by reperfusion of the existing graft vasculature [18].

## Analysis

Care must be taken not to misinterpret normal age-dependent alterations in the CAM vascular architecture as specific responses to tested agents. When an angiogenic compound is tested, it is advisable to use two independent observers. Since the test material is placed on pre-existing vessels, it is difficult to distinguish angiogenesis from artifactually increased vascular density, due to the rearrangement of pre-existing vessels following contraction of the membrane [26, 32]. Occasionally, one observes a "spoke and wheel" vascular pattern of the CAM, which previously has been interpreted as a sign of strong angiogenesis. However, this particular vascular pattern may represent either neovascularization or local buckling and contraction of the CAM or the distortion of the supply vessels due to fibrosis and may not result from a local increase in capillary numbers [43]. In spite of observer training, large inter-individual differences in the numbers of vessels counted are often observed [44]. A report that claims a reliable assessment by using statistical analysis has been published [45]. Imaging techniques such as the measurement of bifurcation points in a designated area around the test material, have also improved the reliability of the assay [46].

Monitoring the test area *in ovo* is difficult and reliance must be placed on the examination of the experimental site at the end of a prescribed period of incubation. However, in carrier sponges, or in the CAM itself, electron microscopic analysis, the determinations of content and synthesis of DNA, protein, collagen and basement membranes [47], as well as analyses of gene expression in infiltrating cells, including ECs, can be performed. The experimental stimulation of angiogenesis appears to involve all cells of the CAM [48, 49], *i.e.*, all of the cell types in the CAM are stimulated to proliferate, as evidenced by autoradiography.

## **De novo VEGF-mediated angiogenesis**

As VEGF is the most critical regulator for new vessel formation and a key pro-angiogenic factor in embryos, hypoxia, and most tumors, the response that VEGF evokes [50, 51] in the CAM assay is of interest. In the CAM of 13-day-old chick embryos, in which endogenous angiogenesis is completed, VEGF applied as a droplet that



dries on a part of the thermanox tissue culture coverslip and is then applied to the CAM is the only one of several growth factors tested in this way, including bFGF, angiogenin, and PDGF-BB, which is an EC mitogen and therefore a potent *de novo* angiogenic factor [50]. In the CAM of embryos of this age, VEGF induces vascular growth in the region of the capillaries and also in the pre- and post-capillary vessels by both sprouting angiogenesis and IMG. It is notable that when the minute thermanox disc is applied alone at Day 11, it induces a strong vascular response. Following application on Day 12, a positive or negative response to the carrier is observed, whereas on Day 13 no such carrier effect is seen. However, the small disc continues to compress the intra-ectodermal capillary plexus of the CAM on Day 13 [51]. Therefore, the possibility cannot be ruled out that the small disc itself contributes in some way to the angiogenic responsiveness seen after the application of VEGF on Day 13. - For instructive technical details, see [46, 52].

### Advantages and disadvantages of the assay

Largely owing to its simplicity and low cost, the CAM is currently the most widely used *in vivo* model for the study of both pro- and anti-angiogenesis [29, 52]. Indeed, the CAM assay has been used to identify almost all the known angiogenic factors.

There are several unfortunate artifacts that make validation difficult. Any irritant, such as the shell dust generated during the excision of a rectangular opening in the shell, and any sliver of shell membrane that protrudes and touches the CAM will cause an inflammation-mediated angiogenic reaction. The CAM has a tendency to be overly sensitive [32, 53]. An angiogenic reaction may be induced by the mere presence of seemingly inert foreign materials on the CAM [53]. The membrane is also extremely sensitive to changes in oxygen tension, which makes the sealing of the opening in the shell critical [22]. It is recommended to wait 3 days after making the opening in the eggshell before adding the test substance, to check for any inflammatory response [29]. *In vitro*, careful control of the pO<sub>2</sub> level is essential. Drugs that require metabolic activation cannot be assessed using this method.

### The rodent mesentery angiogenesis assay

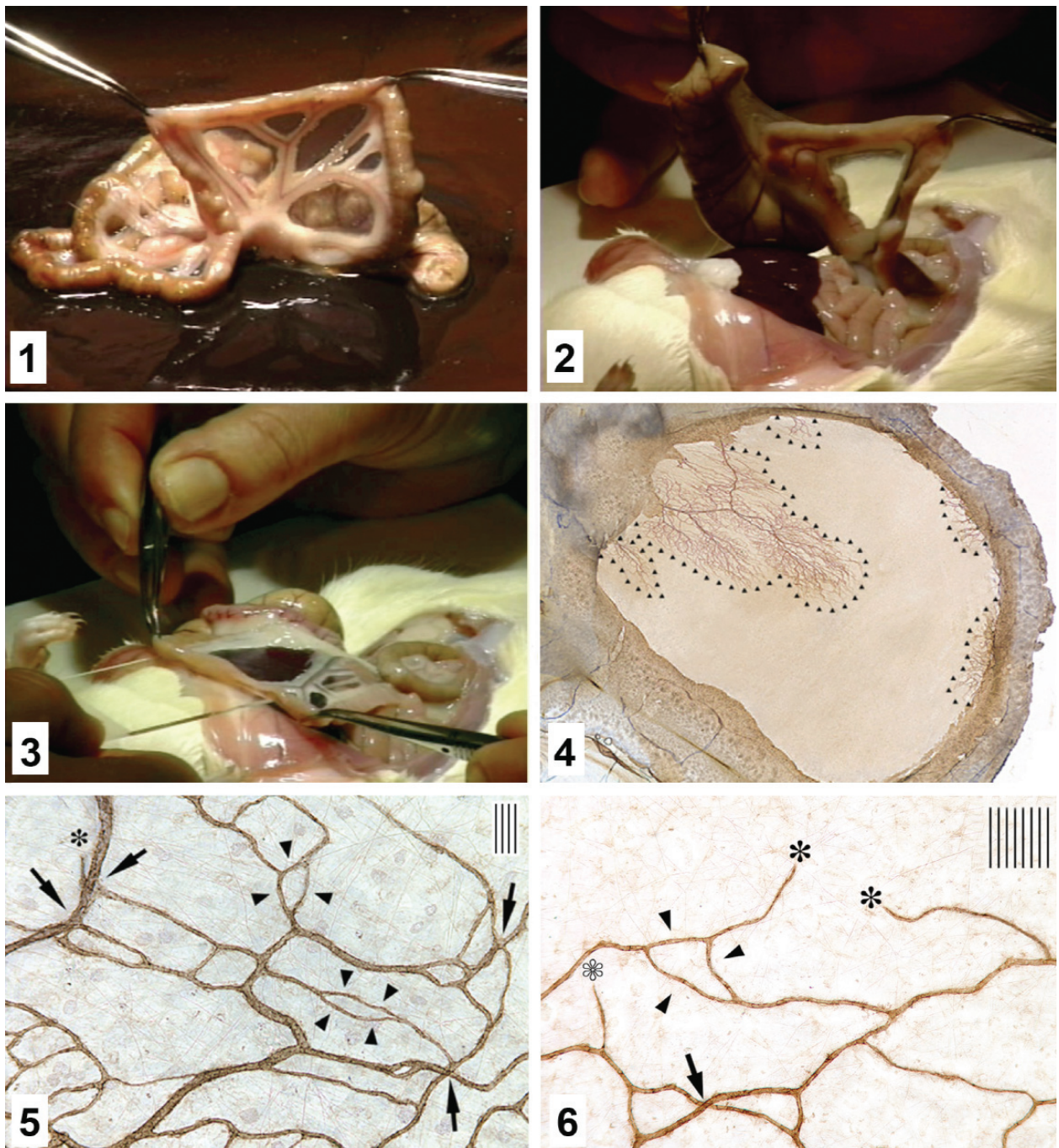
The rat mesenteric-window angiogenesis assay was introduced by our laboratory in 1986 [54] and has been developed and refined [55–60].

#### Test tissue

The ease with which the small-gut mesentery of small rodents can be exteriorized from the abdominal cavity and the transparency of its thin membranous "window"-like parts has made it a favorite test tissue for demonstrations of the microcirculation using intravital microscopy [61] especially as the exteriorized mesentery is considered to be an ideal system for physiological measurements [62]. There are no significant structural differences between the microcirculations of the intestinal mesentery of the mouse, rat, guinea pig, rabbit, cat, and dog [61, 63].

In adult male Sprague-Dawley (SD) rats, there are some 40–50 mesenteric very thin "windows", each of which is distinctly framed by fatty tissue that contains a portal artery-vein pair (Figure); similar windows occur in several small rodent species. When whole-mount specimens of single windows on objective slides are examined microscopically, a comparatively large central part of each window is normally avascular, whereas microvessels are found somewhat asymmetrically towards the border. In rats, usually more vessels are found on the intestinal side, but without significant differences in vessel density between different areas of the window [64]. The mesenteric test tissue is a 5–10 μm-thick membrane that is covered on both sides by a single layer of mesothelial cells abutting onto a delicate basal membrane, sandwiching a tissue space that contains fibroblasts, mast cells, macrophages (histiocytes), as well as occasional eosinophils and lymphocytes [65]. It is one of the thinnest tissues found in the body. In the avascular parts, mesothelial cells and fibroblasts predominate overwhelmingly; among these, fibroblast normally make up ~52% and mesothelial cells ~48% in adult male SD rats [66]. The mesentery also contains connective tissue elements such as collagen, elastin, and elastic fibers of varying caliber.

Spreads of intact mesenteric windows on objective slides enable detailed microscopic analysis of all the cellular and vascular components that are present



**Figure** Illustration of the rat small-gut mesenteric-window angiogenesis assay. Panel 1 shows the entire excised small gut with its mesentery containing some 40–50 transparent membranous "window"-like parts. As a standard, we sample four windows for analysis, i.e. the most distal ones adjacent to the ileocecal valve. When harvesting the test tissue, *i.e.*, the window-like membranous parts of the mesentery which are extremely thin (~5–10  $\mu\text{m}$  thick), the intact tissue, still connected to the small gut, can be spread on objective slides (panels 2 and 3). Following staining with an antibody specifically directed against rat endothelium, the entire virtually two-dimensional microvessel network is visualized (panels 4–6). The intact specimens are ideal for the quantification of objective microvessel variables such as spatial extension, density, number and length of individual microvessel segments, frequency of intersection, frequency of interconnecting loop formation, number and length of individual sprouts, as described in [55–60, 89]. The distance between two adjacent lines in panels 5 and 6 is 10  $\mu\text{m}$ . In panel 5 there is one sprout (asterisk), which is not located at the edge of the network. In panel 6 two sprouts at the network edge and one sprout close to the edge are shown (asterisk). Arrows indicate intersections and arrowheads indicate inter-connecting loops (not all intersections and loops are indicated).

[56, 57, 67, 68]. The number of vessels at the window periphery, in terms of the number of microvessels per mm circumference, is significantly increased in 15-week-old male SD rats compared with 5.5-week-old rats. This demonstrates a type of slowly occurring physiological angiogenesis that appears to be limited to the most peripheral parts of the windows [69]. An age-related increase in the number of peripheral microvessels occurs also in female SD rats [70, 71]. However, the data suggest that there is no significant increase in the number of peripheral vessels per mm of perimeter, vessel density or vascularized area in the mesenteric windows in untreated male SD rats over the age of ~7 weeks [72]. In mesenteric windows from the most distal part of the mesentery (that we analyze as a standard), untreated adult male SD rats show no statistically significant angiogenesis over a period of 2 to 3 weeks, which is the usual duration of angiogenesis experiments.

Immunostaining the microvascular network of rat mesenteric windows *in situ* shows that the microvascular bed is composed of arterioles, metarterioles, precapillaries, midcapillaries, postcapillaries and venules [73]. During normal maturation, PDGF- $\beta$  and its receptor PDGFR- $\beta$  are expressed in a pattern that is consistent with a role for PDGF in mediating the microvascular development process [74]. During angiogenesis in the rat mesenteric window, capillary sprout ECs and pericytes migrate preferentially along resident elastic fibers. The prevalence of this phenomenon is influenced by both the applied angiogenic stimulus and the anatomical position of the sprout within the network [75]. Recently, it has been shown that in the untreated adult rat mesentery neuron-gial antigen 2 is expressed by all perivascular cells (mature and immature smooth muscle cells, and pericytes) along arterioles, but is absent in venous smooth muscle and pericytes [76]. However, during the microvascular remodeling of mast-cell-mediated angiogenesis (see below), the venules dynamically upregulate neuron-gial antigen 2 expression [77].

### Assay set-up

Any solution that is injected i.p. (providing its volume is not diminutive) will rapidly reach all the target microvessel cells, as well as all non-vascular cells, in the test tissue. This is because the mesothe-

lial layer of cells that covers both sides of the mesenteric window is highly permeable to compounds within a wide range of molecular-weights [78]. The test tissue is thus untouched mechanically and unaffected by wound-healing-induced angiogenesis, as no surgery is involved. In effect, i.p. injection(s) of an agent the pro-angiogenic effect of which is being tested, are usually made once or twice daily for up to five consecutive days (Monday morning to Friday morning). Results suggest that a single injection of a pro-angiogenic protein of this kind at low concentration does not provide a sufficiently long or potent stimulus to overcome the influence of inherent angiogenesis inhibitors in the test tissue.

An agent that selectively activates the mesenteric mast cells in rats have been used, by which mast-cell-mediated angiogenesis was demonstrated for the first time [6, 54, 79]. Inflammatory cytokines such as IL-1- $\alpha$  [80], IL-8 [81], TNF- $\alpha$  [82], or a growth factor such as bFGF [56] or VEGF [58] have also been tested. The test agents have been given at low or very low, in certain cases at approximately physiological levels. In studies of the systemic effects of agents that may modify a particular angiogenic response in the mesentery, the test drugs are given s.c. (preferably by continuous infusion using one or two osmotic mini (Alzet<sup>®</sup>) pumps that deliver the test substance(s) at a constant rate), i.v. [83] or orally [57, 84]. To avoid any direct interference of the test agent with the target tissue, including unintentional activation of mast cells (see below), we do not use i.p. administration. This procedure mimics a clinical treatment situation in that the net effects of the systemically administered parent molecule(s) as well as of its/their metabolites can be studied.

Angiogenesis induced by VEGF, bFGF, IL-1- $\alpha$ , IL-8, and TNF- $\alpha$  is of the sprouting type [60], in agreement with other reports in fetal and adult rat [62, 70, 85, 86] and adult mouse [87] mesenteric windows.

### VEGF signaling

Since VEGF is a key pro-angiogenic factor, the response that it evokes in this assay is of interest. Measurements of VEGF-induced signaling in intact microvessels in the mesentery of 5- to 6-week-old mice have been performed [87]. At various intervals after i.p. injection of VEGF, the mesenteries were harvested, extracted, and immunoprecipitated. Similar levels of phosphorylation are observed

when the mesentery is exposed to VEGF *in vitro* or when mesenteries are harvested from mice that bear a mouse ovarian ascites tumor, which itself secretes high levels of VEGF [87]. VEGF causes increased microvascular permeability within a matter of minutes, whereas EC division, altered gene expression and angiogenesis do not occur for some hours or even days. Moreover, when ascites tumor cells are transplanted to the mouse peritoneal cavity, the mesentery exhibits a complete repertoire of biological responses that can be attributed to VEGF, including sprouting angiogenesis.

Both types of high-affinity tyrosine receptors for VEGF (VEGFR-2 and VEGFR-1) are expressed exclusively on the mesenteric vasculature. As a result, signaling events measured in the mesentery after stimulation with VEGF can be safely attributed to events that take place in the vascular endothelium of the microvasculature [87]. This approach offers many advantages, including the ability to study the responses of ECs in intact microvessels under physiological conditions, as well as the ECs of blood vessels induced by tumors and by other angiogenic stimuli. This system can therefore be regarded as a benchmark against which studies of cultured ECs can be compared for relevance to the vascular endothelium responses that occur *in vivo* in both the physiological and pathological settings [87].

#### **VEGF-mediated microvessel expansion**

The time course and potency of the response to VEGF at low graded doses have been assessed in adult male SD rats [58]. A series of technically independent angiogenesis variables was measured. Data relating to the kinetics of the angiogenic response in terms of swiftness, potency, and duration at each dose level were thus obtained. VEGF induces a rapid angiogenic response and the relative increase in microvascular spatial expansion initially dominates over the increase in microvascular density. There is no effect on the proliferation of the predominant non-vascular (fibroblast and mesothelial) cells as measured 32 hrs after an i.p. injection of VEGF, which is suggestive of a selective initial angioproliferative effect. There is no release of histamine following i.p. treatment with either VEGF or bFGF, which indicates that the mast cells in the test tissue are unaffected by these proteins at the doses given [58]; the mast cell is the major repository of histamine in the body and when activated is able to induce cell proliferation and angiogenesis (see below).

The data suggest that the dose-effect of VEGF in terms of increases in microvessel density and vascularized area are non-linear [58], as has recently been demonstrated in terms of the effects of VEGF on bovine microvascular EC proliferation *in vitro* [88]. It is noteworthy that apparently non-linear, dose-related angiogenic effects are also found in the mesenteric window assay for other heparin-binding angiogens such as bFGF [56] and IL-8 [81], which implies that a bimodal dose-activity relationship is a prevalent feature of angiogenic heparin-binding peptides.

#### **Analysis**

As noted, the molecular and cell biological studies on VEGF-induced signaling in intact mesenteric microvessels have yielded important information [87]. Owing to the small dimensions of the newly formed microvessels, their lengths and numbers can only be assessed accurately using microscopic techniques. As the test tissue provides an ideal opportunity to view the microvessels, several different strategies can be used to quantify the microvasculature. Since the membrane is natively largely avascular, the assay has some of the advantages described for the cornea, *i.e.*, angiogenesis, when it occurs, is obvious. A pre-determined number of mesenteric windows located at the most distal part of the small-gut mesentery is harvested at intervals of choice, leaving no room for arbitrariness in the selection of windows. When properly stained in the intact mesenteric window, the vessels are easy to identify, as they form continuous, virtually two-dimensional structures (Figure).

We have developed the following procedure for microvessel quantification. The entire vasculature of each of the sampled intact mesenteric windows is visualized immunohistochemically using a primary monoclonal antibody against the rat endothelium, allowing straightforward identification of even the smallest microvessels in the intact network [57, 89]. Microscopic morphometry and computerized image analysis are then employed in a blinded fashion. Initially, the total area of each mesenteric window is measured. Subsequently, the following variables can be measured objectively [56, 58]: the percentage of vascularized area, which is a measurement of

the spatial extension of the network; microvascular length, which is a composite measurement of microvessel density calculated by pixel counting; and the total microvascular length.

Optionally, the following variables can be measured: the length of the individual microvessel segments, *i.e.*, the actual distance between two successive branching points (by pixel counting); the number of microvessel segments per unit tissue volume; the number of microvessel branching points per unit tissue volume; and the number of capillary sprouts per unit tissue volume and their individual lengths (by pixel counting) at the edge of the expanding network [59, 60, 90]. The methodology can for the first time be used to report on large populations of microvessel segments and sprouts in any tissue in terms of the accurate counts and the virtually unbiased distribution of their individual lengths. All of these variables are recorded objectively and quantitatively, which is a prerequisite for molecular-activity analysis, such as of (i) the effect of heparin molecular weight, and (ii) low-molecular-weight heparin preparations displaying slightly different chemical configuration [91], and dose-response analysis. The data enable robust statistical analysis.

### **Advantages and disadvantages of the assay**

The mesenteric window incorporates three important features from a biological point of view: (i) it is natively albeit sparsely vascularized, like almost all other normal adult tissues; (ii) it lacks significant physiological angiogenesis, like most normal adult tissues; and (iii) angiogenesis can be induced with a minimum of trauma, if any, to the test tissue. Moreover, thanks to its extreme thinness, the intact mesenteric windows in rats and mice and other small rodents are ideal for quantitative analyses of microvessel variables.

To the best of our knowledge, the techniques listed for the quantitative assessment of the objective microvessel variables, such as spatial extension, density, the number and length of individual microvessel segments as well as of individual capillary sprouts *in situ* in a vascularized tissue *in vivo*, are unrivalled. As noted, sprouting angiogenesis is a characteristic of tumor angiogenesis, making the assay suitable for the study of various aspects of tumor angiogenesis.

One disadvantage is that mice are less suitable for quantitative angiogenesis analysis because many of the mesenteric windows lack microvessels from which angiogenesis can be initiated.

The "Rat Mesentery Window Angiogenesis Assay", which is a short DVD presentation providing straightforward technical information of the procedure, is available from the author on request, as long as stocks last.

### **A note on disturbance of tissue homeostasis and mast cell-secretion in the exteriorized mesentery used for intravital microscopy**

To test the effect of aseptic sham-operation on mesenteric windows, the abdominal cavity was opened in rats, and the small intestine with its mesenteric windows was gently held up with tweezers before being replaced within a few minutes into the abdominal cavity, with the peritoneum and *linea alba* being immediately sutured. Forty-eight hours later, the specific DNA activity in the mesenteric windows rose significantly and the total mast cell histamine content decreased by up to 55%, which was a sign of mast cell secretion [92, 93]. Thus, a short period of exteriorization of the mesentery causes disturbance in tissue homeostasis.

Activated mast cells are able to induce the proliferation of mesenteric fibroblasts and mesothelial cells by a paracrine mechanism, as seen in other tissues [68, 94], as well as angiogenesis in the mesenteric window [6, 54]. Indeed, activated mast cells are able to secrete several angiogenic growth factors (bFGF, VEGF, hepatocyte growth factor, TNF- $\alpha$  and several other), a number of chemokines, several enzymes and lipid-derived biologically potent agents that induce and promote angiogenesis [6]. Mast cells are activated by numerous stimuli, including trauma, which means that there is a component of mast cell-mediated angiogenesis in wound healing, tumor growth and inflammation [6]. Notably, in the adult male rat, *i.p.* injections of low doses of inflammatory cytokines such as IL-8, IL-1-alpha and TNF-alpha release ~25% of all mast cell histamine in the mesenteric window test tissue [80–82].

Therefore, it seems likely that intravital microscopy of the exteriorized mesenteries of mice and rats leads to mast cell-mediated proliferation and mast cell-mediated angiogenesis, as a symptom of markedly disturbed tissue homeostasis, which may complicate the interpretation of the results

obtained, depending on the experimental design. In fact, this was recently illustrated in a study of angiogenic effects using an adenovirus-mediated gene-transfer model in the rat mesentery, in which intravital microscopy of the exteriorized mesentery was performed [62]. Proliferating cells were observed not only in the microvessel sprouts but also among a considerable number of the non-vascular cells in the mesenteric window.

### **The sponge implant assays**

In 1987, Andrade and associates introduced an assay in which compounds of interest were injected directly into a sponge that was implanted s.c. in the rat [95].

#### **The test substance, assay set-up, analysis and advantages**

The s.c. implantation of sterile polyester sponges and the subsequent measurements of blood flow (using the  $^{133}\text{Xe}$  clearance technique) in the sponge implants as they become vascularized enable reproducible, objective, and continuous assessments of angiogenesis [95]. It is also possible to make localized injections of angiogenic substances or inhibitors and to collect exudate fluids for biochemical analysis. Variations of the sponge implant assay have been described. The sponge/matrix-implant assays have the potential advantage of replicating the hypoxic tumor microenvironment, thereby making them suitable for the study of tumor angiogenesis [10].

#### **Disadvantages**

A common problem associated with various assays of vascularization that employ matrix implants is non-specific inflammatory host responses to the matrix implants [13], whereby granulation tissue gradually encapsulates the s.c. sponge and infiltrates the substance of the sponge. In addition, sponge composition can vary, making inter-experimental comparisons difficult [29], and the use of radioactive gas is a complication. The animals have to be housed singly. - For instructive technical details, see [96].

### **Tumor growth in vascularized subcutaneous polyurethane sponges**

The growth and metastasis of human tumors in nude mice following tumor-cell inoculation into a vascularized polyurethane sponge matrix have been studied [97]. The sponge material induces a non-specific inflammatory reaction, which mediates angiogenesis and connective tissue infiltration. Two sponges are implanted in each mouse and tumor cells are inoculated into one of these vascularized polyurethane sponges, usually leading to metastases. Interestingly, most metastases are found in the second sponge graft, which does not receive inoculation of tumor cells and is implanted at the contra-lateral site of the animal.

### **The disc angiogenesis system (DAS)**

Introduced in 1988, the DAS implanted s.c. in mice provides a model of wound healing and of angiogenic responses to solid tumors and soluble substances [98].

#### **Test tissue and assay set-up**

A small disc of polyvinyl alcohol foam, which is covered on both flat sides by Millipore filters, leaving only the edge as the area of cell penetration into the disc, is used. A test agent or the suspension of tumor cells to be studied is placed at the center of the disc. The slow release of the test substance or of factors from the tumor cells is maintained by a film of ethylene-vinyl acetate co-polymer or by the use of agarose [98]. The abdomen and thorax of the mouse are convenient implantation sites.

#### **Analysis**

Paraffin sections of the entire disc can be prepared so that the areas of growth of vessels, fibroblasts, and additional connective tissue components can be measured microscopically. The measurement of the blood vessels can be performed by various methods, including point counting on histological sections, determination of intravascular volume and so on. The radial distance between the most central

portion of a vessel wall and the edge of the disc is measured and is designated as the "centripetal vessel growth". Some of the central sprouts are probably empty. Fibroblasts are always present whenever there is vascular growth in the disc. Additional cell types include lymphocytes, mast cells and histiocytes (macrophages). Collagen is observed as a fine network, whereas elastin fibers are not found in the invading vascularized connective tissue [98].

### **Advantages and disadvantages**

Quantification, as described above, is straightforward and comparatively easy to perform.

The disc is surrounded by inflammation-induced granulation tissue, since the implanted disc causes a foreign-body reaction that involves foreign body-multinucleated giant cells that are seen apposing the polyvinyl alcohol foam sponge trabecula. Moderate growth of vascularized connective tissue occurs spontaneously in the disc and is accelerated by angiogenic stimulants placed at the center of the disc [10]. The DAS does not allow continuous or kinetic observations as each disc provides information for only one point in time. - For instructive technical details, see [99].

### **Entrapment of tumor cells separated from the host immune system**

A system for monitoring tumor cell-induced blood vessel growth using a sodium alginate microbead entrapment process was developed in the early 1990's [100, 101]. The alginate polymer, which is derived from *Macrocystis pyrifera* and consists of guluronic and mannuronic acids, surrounds and sequesters cells from direct contact with their immediate environment, but permits diffusible angiogenic factors to pass through and induce neovascularization in the host. The tumor cells are protected from contact with the host immune system and can thus be evaluated for their angiogenic potential across histocompatibility or species barriers. The beads are preferably injected s.c. into the animals. Neovascularization can be monitored quantitatively by macroscopic photography, microscopic histology, by measuring the level of hemoglobin at the alginate injection site or by mea-

suring the amount of radioactive red blood cells that pool at the injection site [100, 102]. - For instructive technical details on the alginate microbead assay, see [103].

### **The matrigel plug assay**

Passaniti and co-workers introduced this assay in 1992 [104].

#### **Test substance and assay set-up**

Matrigel, which is an extract of the Engleberth-Holm-Swarm tumor, is composed of basement membrane proteins. Although it takes the form of a liquid at 4°C, Matrigel reconstitutes into a gel or plug at body temperature when injected s.c. into mice, where it is progressively surrounded by granulation tissue. The plug supports an intense vascular response when supplemented with angiogenic factors, such as bFGF [104]. This is an assay that does not require any surgical procedures and is not difficult to administer, although it is considered by some workers to be time-consuming [29].

Matrigel has not been fully defined chemically. It contains collagen IV, laminin, nidogen/entacin, heparan sulfate proteoglycan, and growth factors such as epidermal growth factor, transforming growth factor beta, platelet derived growth factor, insulin-like growth factor-1, nerve growth factor, and bFGF [105, 106]. This suggests that caution should be exercised in the interpretation of experiments on cellular activities related to Matrigel [105]. In more recent studies, ammonium sulfate-treated Matrigel, so-called growth-factor reduced Matrigel, has been used. Low-molecular-mass proteins (such as growth factors) are soluble in 20% saturated ammonium sulfate, unlike the major ECM proteins laminin, collagen IV and heparan sulfate proteoglycan [107]. With growth factor-reduced, unsupplemented Matrigel, few cells invade the plug [108]. When known angiogenic factors, such as bFGF, are mixed with Matrigel and injected s.c., ECs migrate into the plug and form vessel-like structures. ECs in Matrigel show the characteristic Weibel-Palade bodies and the cytoskeleton is re-organized. The formation of the fine networks of EC tubes elaborated by the macro- as well as the

micro-vascular ECs on Matrigel is a process that appears to be specific to these cells and mimics the formation of capillary networks *in vivo* [106].

### Analysis

The Matrigel plugs and the surrounding granulation tissue are removed after one to three weeks and angiogenesis is quantified by immunohistochemistry (provided that an antibody directed specifically against the ECs of the host is available) or other means using histological sections (see below for Quantitative techniques for the assessment of angiogenesis in patients). The histological enumeration of blood vessel-like structures (*i.e.*, the profiles of capillary-like vessels) is considered to be difficult. On the other hand, the hemoglobin assay used to estimate the blood content of the newly formed vessels cannot distinguish between blood in the capillaries and blood in the sinuses or larger vessels [22]. Alternative methods involve quantification of the vasculature after the injection of fluorochrome-labeled, high-molecular-weight dextran [10, 14] and the quantitative assessment of vascular-specific indicators in a chamber model [109].

To achieve maximum long-term effects with test substances, the half-life of which is likely to be short in the circulation, an osmotic mini (Alzet®) pump is implanted s.c. dorsally. By introducing high-molecular-weight fluoresceinated dextran into the circulation, observations of blood flow or blood content in the Matrigel plug can be made without any further tissue processing. - For instructive technical details, see [108].

### Advantages and disadvantages of the assay

The assay also qualifies as an experimental model of tissue regeneration, in which neovascularization intimately couples with fibrosis and organogenesis and in which monocytes/macrophages play a key structural role [110]. The fact that the Matrigel plug contains no tissue other than capillary-like blood vessels can probably be regarded as a drawback, since all tissues appear to contain pro- and anti-angiogenic factors that are able to influence angiogenic reactions.

Unfortunately, this assay suffers from considerable variability, primarily because it is difficult to generate

identical three-dimensional plugs, even though the total Matrigel volume is kept constant [22]. Nevertheless, this assay is regarded as one of the best assays for the rapid screening of potential pro- and anti-angiogenic compounds [14, 108]. Recently, a modification of the assay in mice and rats using s.c. chambers that allow constant three-dimensional form and volume of the Matrigel plug, which makes the assay more reproducible, have been described [111, 112]. Notably, VEGF admixed to growth factor-reduced Matrigel does not display angiogenic activity in this particular assay [112], which is in contrast to the known role of VEGF as a very potent inducer of *in vivo* angiogenesis [112].

### The sponge/Matrigel assay

A variant of this method, which combines the Matrigel plug and sponge techniques, has recently been introduced [113]. Matrigel (500 µl) is injected s.c. into mice and allowed to solidify. Subsequently (after 20–30 min), the mice are anesthetized and the skin overlaying the Matrigel plug is gently shaved, after which a small nick is made in the skin, followed by a smaller nick in the Matrigel plug. A sterilized polyvinyl sponge that contains the test material is introduced through the nick into the Matrigel and advanced to the center of the plug with the help of tweezers. Alternatively, fragments of tumor or other tissues are introduced in a similar manner. Based on this modification, angiogenesis is directional and therefore the assay has increased sensitivity and measures angiogenesis more directly than in the standard Matrigel assay [113].

The greatest disadvantage of the sponge/Matrigel assay is that it is more time-consuming than the standard Matrigel plug assay. When osmotic mini pump implants are added to the protocol, the number of animals that can be assayed (~12) becomes limited [14].

## Whole small animal angiogenesis models: zebrafish and the *Xenopus Laevis* tadpole

### Zebrafish

In 1999, embryonic and young, growing zebrafish were demonstrated as whole animal models for screening small molecules that affect blood vessel formation [114].



### **The test animal**

Zebrafish are small tropical freshwater fish (approximately 3–4 cm long as adults) with a short generation time (about 3 months) that can be housed in large numbers and in a relatively small space. Despite the more than 400 million years that separate the last common ancestor of zebrafish and humans, many zebrafish organs are remarkably similar to their human counterparts at the anatomical, physiological, and molecular levels [115].

### **Assay set-up**

The external development of zebrafish embryos and their optical transparency during their first few days allow direct and continuous microscopic inspections of diverse developmental processes, from gastrulation to organogenesis. External fertilization permits immediate accessibility for experimentation and observation. Small molecules added directly to the water that holds the fish diffuse into the embryo and induce observable, dose-dependent effects. Anti- and pro-angiogenic molecules that are effective in mammals have been shown to exert corresponding effects in the zebrafish.

### **Analysis**

Zebrafish transparency becomes even more useful when fluorescent markers are used to label specific populations of cells, including ECs [116–119].

### **Advantages and disadvantages**

The assay allows the assessment of embryonic and organogenic angiogenesis. Overall, an impressive repertoire of genetic tools is available to modify the zebrafish genome, making it the premier small animal model for functional genomic studies. Among these tools, morpholino (MO) "knockdown" technology for reverse genetic analysis of gene function has recently emerged as a powerful approach to understanding molecular events in vasculogenesis and angiogenesis in the zebrafish [118–120]. MOs are chemically modified oligonucleotides with similar base-stacking abilities as natural genetic material but they have a morpholine moiety instead of a ribose [121]. When injected into one- to four-cell-stage zebrafish embryos, the MOs exert their effects throughout embryogenesis. An additional advantage is that zebrafish

embryos receive enough oxygen *via* passive diffusion to develop normally for several days in the absence of blood circulation, thus allowing studies of the vascular system even during perturbation of angiogenic processes [115].

Several complementary studies based on the use of *in situ* hybridization, confocal microangiography [116], lineage tracking, and transgenic strains that express enhanced green fluorescent protein (GFP) in ECs have contributed to a better understanding as to how the vasculature develops in zebrafish. Notably, the vasculature in zebrafish follows a plan that is similar but not entirely identical to that of higher vertebrates [122–124].

Compared with conventional assays, the advantages of using zebrafish to assess drug effects on angiogenesis include: (i) short assay time; (ii) easy animal maintenance; (iii) use of small quantities of drug; (iv) single dosing; (v) a quantitative assay format; and (vi) the ease of obtaining a statistically significant number of animals per test [125]. The zebrafish model is considered to offer unique opportunities to identify rapidly both novel candidate disease genes involved in angiogenesis and chemical compounds with strong therapeutic potential [115].

### ***Xenopus laevis* tadpole**

The development of *Xenopus* is rapid: as early as 4 days after fertilization, a fully developed and functional vasculature can be visualized by microscopic imaging [115]. The *Xenopus* embryo provides a potent and useful model to study the processes and factors involved in vasculogenesis and angiogenesis. Notably, *Xenopus* shares greater vascular similarity than zebrafish with higher vertebrates [115, 126]. Interestingly, *Xenopus*, in contrast to zebrafish, develops a lymphatic system [115, 127, 128], which allows molecular studies of lymphangiogenesis.

### **The directed *in vivo* angiogenesis assay (DIVAA)**

In 2003, Guedez and associates described the development and application of the DIVAA in mice [129].

## Test tissue and assay set-up

The assay involves of the s.c. implantation into mice of semi-closed silicone cylinders that are plugged at one end with a solid steel rod or sealed with silicone (so-called angioreactors). Each angioreactor is filled with only 18  $\mu$ l of Matrigel premixed with or without angiogenic factors.

## Analysis

Vascularization within the angioreactors is quantified by the i.v. injection of fluorescein isothiocyanate-dextran before recovery, followed by spectrofluorimetry. This gives a measurement of blood volume within the angioreactor. Histological examination of the angioreactor controls reveals loose granulation tissue that contains a modest infiltrate of mononuclear cells. In control angioreactors, cellular invasion of the Matrigel occurs only occasionally and there is no evidence of vascular invasion.

## Advantages and disadvantages

Only a small amount of test substance is used in each angioreactor. The DIVAA is an observer-independent, objective, quantitative assay. The angioreactors cause tissue wounding and a granulomatous tissue reaction. Notably, Matrigel is not chemically defined.

## Invertebrate angiogenesis model

Recently, *Hirudo medicinalis*, an invertebrate species that features a virtually avascular muscular body wall, was recently introduced as a model for studies of angiogenesis in avascular tissue [130]. It appears that angiogenesis and vasculogenesis in invertebrates such as *Hirudo medicinalis* share a high degree of similarity with the same processes in humans, both in terms of structural and functional properties as well as biochemical events.

## Additional pertinent angiogenesis assays

There are a number of assays, which because they are adequately described or commented upon else-

where, will only be mentioned briefly here. These include assays that use the **anterior-eye chamber** and **the rabbit ear chamber** [10], **the mouse cranial window** [13, 131], **the hamster cheek pouch**, **the dorsal skinfold** and **other skin chambers** in mice [10, 132–134], **the dorsal air-sack** or s.c. air "pouch" or "blister" method [10, 29, 135], **the hollow-fiber solid tumor model** [136–138], **intravital microscopy** [139–141], and **the Angiomouse<sup>®</sup> system** [142].

**Chronic artificial-window or chamber preparations** in rodents include: (a) the rabbit ear chamber, (b) dorsal window in the mouse/rat, (c) the cranial window in the mouse/rat and (d) collagen gel that contains angiogenic factors sandwiched within a nylon mesh [13]. These surgically implanted preparations allow non-invasive, continuous measurements of gene expression, angiogenesis and blood flow, metabolites, pH, pO<sub>2</sub>, transport of molecules and cells and cell-cell interactions. Many regard the chamber assays as the optimal systems for measuring physiological effects, such as perturbations in blood flow, but they are considered to be technically difficult [29].

In all surgically implanted chamber assays, there are inflammatory reactions to wounding and the introduction of foreign material, which lead to the formation of granulation tissues that encapsulate the chamber, which hinders the correct interpretation of outcomes. Granulation tissue is a specialized type of tissue that is characteristic of healing and its histological appearance is characterized by the proliferation of fibroblasts and capillary-like thin-walled vessels in a loose ECM. The granulation tissue progressively accumulates connective tissue matrix, which eventually produces dense fibroids that may further remodel over time into scar tissue.

A major drawback of the rabbit ear chamber is the fact that the granulation tissue takes 4–6 weeks after chamber implantation to mature before any angiogenic factor(s) can be placed in the chamber [13].

**The hollow-fiber assay** used by the National Cancer Institute (USA) involves the short-term *in vitro* culture of tumor cells inside biocompatible polyvinylidene fluoride fibers that contain gels impregnated with the test compound, followed by implantation into mice s.c. and i.p. The responses at the i.p. site are expected to reflect chemosensitivity, while the responses at the s.c. site may indicate that the test agent possesses good bioavailability and

desirable pharmacological properties. Genetically well-characterized tumor cells are used and the model is considered useful for the identification and interpretation of molecular events in tumor angiogenesis; the tumor cells can be retrieved intact at various stages after implantation without host cell infiltration [10, 137].

**Intravital microscopy** is considered [139–141] to be well suited (*i*) to studies of the systemic positive and negative feedback loops in the control and regulation of complex physiological or pathophysiological processes, including angiogenesis, and (*ii*) for critical evaluation of the significance of novel anticancer strategies in the *in vitro* in the *in vivo* situations.

**Angiomouse®** is a system in which green fluorescent protein (GFP) is used indirectly to image tumor angiogenesis [142]. The tumor cells contain GFP and new blood vessels formed by the host vasculature are not fluorescent and are consequently imaged as well-defined, dark networks against the bright green background. The footpads of mice are relatively transparent, with few resident large blood vessels, and are therefore well suited for the quantitative imaging of tumor angiogenesis in intact animals [143]. Moreover, multiphoton laser-scanning microscopy can provide high-resolution three-dimensional images of angiogenic factor expression [144]. To monitor the activity of the VEGF promoter, transgenic mice that express GFP under the control of the VEGF promoter have been constructed [145, 146]. This is a non-invasive method that allows real-time recording.

## A note on toxicity

Since it appears to be almost impossible to interpret the results for specific anti-angiogenic effects when a toxic effect is involved, it is important to have a handle on this issue regardless of the animal's age. Among adult animals, the use of rats helps considerably in this respect, as these animals grow physiologically. Adult male rats usually increase in weight by some 50–60 g per week (female rats show lower weight gain). Thus, by weighing the animals it is possible to obtain an understanding of the degree to which the treatment has slowed the increase in body weight, which can then be taken as an indication of toxic effects (including systemic

well-being, anorexia and failure to thrive). As adult rats grow considerably faster than adult mice the effect of toxic agents on body-weight gain should be a more sensitive indicator of general toxic effects in rats than in mice.

## Quantitative techniques for the assessment of angiogenesis in patients

Excised tissues are suitable for various detailed analyses related to angiogenesis using various techniques including vascular casts, histological examination of tissue sections, and quantitative autoradiography. All of these invasive methods are unable to capture real-time events. Despite these limitations and because of clinical constraints, these are currently the only practical approaches for human studies.

For some time, the measurement of intra-tumoral microvessel density (MVD) in histological sections of human tumors has been regarded as the gold standard for the quantitative evaluation of angiogenic responses [147–149]. However, the microvessel network in a tumor is the net product of microvessel formation and loss and is not a measure of ongoing angiogenesis. As discussed in [150], MVD, which is a useful prognostic indicator in patients with most types of cancer, is not a measure of the angiogenic activity or angiogenic dependence of a tumor. Actually, MVD largely reflects the metabolic burden of the supported tumor cells and a minimum MVD is determined by tumor cell metabolic demand, whereas the MVD can exceed the metabolic requirements of a tumor. MVD varies widely with tumor type. Two categories of EC-specific antibodies for staining microvessels in excised human tissue are currently available: (*i*) the pan-EC markers (such as anti-CD31, anti-CD34, and anti-Factor VIII) that can be visualized in formalin-fixed tissue, and (*ii*) the pan-EC antibodies, which bind selectively to activated or proliferating endothelium as a result of VEGF influence and they can be visualized microscopically in frozen microtome-sectioned tissues [148, 151, 152]. Examples of the latter type include anti-CD105 (anti-endoglin) [153], LM609, a monoclonal antibody against integrin  $\alpha_v\beta_3$  [154], and 3E7, GV39M and 11B5, which bind with high affinity to the VEGF:VEGFR-2 complex [151].

There are pitfalls associated with assessing MVD [147]. The results are influenced by whether the MVD is assessed at the periphery or center of the tumors. Moreover, antigen loss occurs frequently due to the use of fixatives that contain acetic acid [155]. In formalin-fixed, paraffin-embedded tissues, the MVD of the capillary-sized vessels, which are relevant with regard to angiogenesis, is dependent upon the EC marker that is used. In fact, there is a tumor-type specific selective loss of staining ability on the parts of anti-CD31 and anti-Factor VIII in malignant B-cell lymphomas, which results in a significant overall loss of visualized capillary-size vessels as compared with staining with anti-CD34 [156]. Observations of this type may have a bearing on data relating to capillary endothelium-related functional variables of proliferation, apoptosis and maturation when different double-labeling immunohistochemical techniques are used and different tumor types are analyzed.

Non-invasive methods that are currently used in patients include contrast-enhanced magnetic resonance imaging (MRI) [157], positron emission tomography (PET) and various optical molecular imaging procedures [158]. These methods allow real-time recording, but do not resolve the data at the microscopic level.

## Comments and conclusions

It is clear that no single model is able to elucidate the entire progress of angiogenesis, as there are differences between species, specific microenvironments, organ sites, whether embryonic or adult tissues are used, and the manner of administration of pro- and anti-angiogenic test substances.

In most assays it is difficult, if not impossible, to quantify angiogenesis in terms of the numbers and lengths of newly formed microvessels. To eliminate the influence of any preconceived notions, it is helpful if the analysis is performed in a blinded manner. Not surprisingly, considerable technical training is needed with almost any angiogenesis assay to ensure maximum success.

The release rate and the spatial and temporal concentration distributions of angiogenic factor(s)/inhibitor(s) should be known for the strict

generation of dose-response curves, although this appears to be very difficult to control. As a result, (i) unobjectionable dose-effect studies are very rare, and (ii) systematic, stringently controlled studies comparing two or more assays in parallel have not, to my knowledge, been published.

The interaction between two or more systemically administered agents can be complex with regard to angiogenesis-modulating effects. For example, as recently reported, a vehicle that by itself may not affect angiogenesis can play a significant role in the effect on angiogenesis of some metronomically administered chemotherapeutics, *i.e.*, those given continuously or frequently at a non-toxic dose, possibly due to redox-phenomena [89].

It can be assumed that all the assays described here yield some information of significance, but it seems unlikely that they are fully equivalent in terms of efficacy and relevance to human disease. Indeed, the relevance of the various assays to human pathology remains to be established and the results must be interpreted with care. The true predictive value of preclinical data can only be adequately assessed once the outcomes of clinical trials of the agent(s) and treatment(s) in question are known.

## Pertinent questions to be addressed

Important factors that need to be elucidated in greater depth in order to optimize angiogenesis-related therapies in the clinic include: (i) differences between species, and even between strains of experimental animals, such as mice, the most frequently used test animal, and rats; (ii) gender; and (iii) age-related differences.

## Can the relevance of preclinical studies to the clinical situation be improved?

In the future, a stronger emphasis should be placed on using pro-angiogenic test substances at approximately physiological concentrations and anti-angiogenic test agents at concentrations that are reasonable from a clinical viewpoint. Trauma of any kind that affects the test tissue should be minimized, to reduce the influence of local inflammation- and/or wound healing-mediated angiogenesis. In mammals, the use of *s.c.*

osmotic mini-pumps that deliver the test agent solution at a constant rate is an attractive mode of systemic administration, since the half-life in the circulation of the agent tested becomes unimportant. This makes comparisons of data obtained in animals and humans easier. The somewhat cumbersome course between clear-cut preclinical anti-angiogenesis data from primarily murine tumor models using in-bred animals and the significant recent results in cancer patients suggests that these models may not offer a very high degree of prognostic relevance to human cancer. Alternatively, the treatment scheduling and dosing required for successful anti-tumor therapy may differ markedly between man and mouse. This important issue has to be addressed in a systematic fashion.

Despite the complexity of angiogenesis reactions in terms of the positive and negative stimuli involved, different tissues and species, and the difficulties associated with recording relevant angiogenesis variables in objective and truly quantitative manners, the field of angiogenesis research has accelerated most remarkably and yielded data that have recently been successfully transferred to the clinic. To date, these advances pertain primarily to anti-angiogenic therapies for major cancer types, diabetic retinopathy, and macular degeneration, which are the most common causes of blindness/defective sight in the western world.

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