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Brain Tumor–Induced Angiogenesis: Approaches and Bioassays

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<http://dx.doi.org/10.5772/53182>

1. Introduction

Investigation on tumor angiogenesis has taken a discontinuous path through history. Studies on blood vessels of the human body are documented for the first time in the 17th century BC by the description of a heartbeat. The Ebers Papyrus (16th century BC) reports how the heart is connected to arteries and primarily describes the high vascularization of tumors. Later, Hippocrates (460-370 BC) made a description that should have a great impact on cancer research: He interpreted the vessels around a malignant tumor as claws of a crab and therefore named the disease ‘*karkinos*’. From there on tumor vessels had been described with no conceptual impact on therapeutic approaches. In 1971, Judah Folkman kicked a stone with his landmark papers on tumor-induced angiogenesis, thereby promoting a completely new view on cancer biology and therapy. He showed evidence that the tumor is not able to grow beyond a certain barrier without a network of newly recruited microvessels, stating that the tumor stimulates the proliferation of host endothelial cells via secreted factors. Folkman concluded that tumor growth is an angiogenesis-dependent process and thus anti-angiogenic approaches could be a promising new therapy. [1, 2].

Since then the field of angiogenesis developed dynamically, becoming an actual research avalanche in the past decades. Key molecules and their receptors have been identified, certain hierarchies in signaling have been unveiled and angiogenesis is named as one of the ten hallmarks of cancer development [3]. However, the therapeutic approach to brain tumors is more complicated and has been hampered for tumor heterogeneity, delivery of drugs to the central nervous system and neurotoxic side-effects still represent the main challenges. Furthermore, the role of participating cells remains to be unraveled, i.e. whether interactions between host and tumor cells are required for vessel formation [4, 5, 6].

Blood vessels deliver oxygen and nutrients and are crucially needed for cell survival, cell function and evacuating carbon dioxide and metabolic waste. All cells of the human body reside in a 100 μm -radius to a capillary blood vessel. The close connection between brain and vessels is seen during brain development and axon growth, insofar as the same guidance molecules are recruited for axon targeting and vessel growth [7]. In particular proliferating cells in a tissue depend strongly on continuous blood supply and have an immanent aptitude to foster angiogenesis. They create a microenvironment of contact-dependent (short-range) and secreted (long-range) factors which promote the generation and growth of blood vessels. Thus, it is tempting to speculate that neoplasms have to evolve angiogenic abilities to induce neovasculature in order to develop and progress in size [8, 9, 10]. Beside normal development, physiological tissue remodeling, tumor growth, metastasis and inflammation, angiogenesis is associated with a wide range of other pathologies such as cardio-vascular and ocular diseases [11].

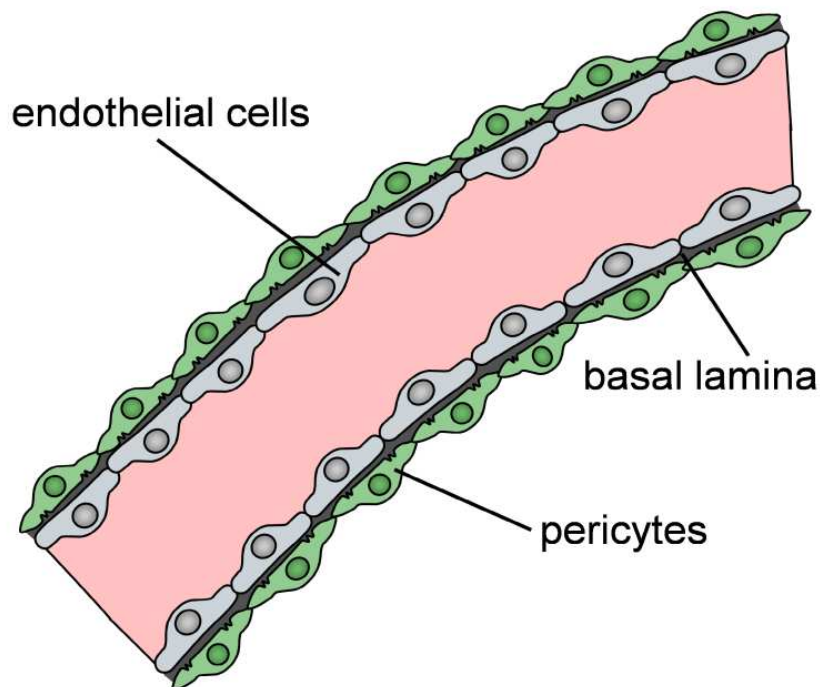


Figure 1. Vessel scheme showing the three main cellular components. The inner layer consists of endothelial cells, lining the inside (lumen) of the vessel. The tube is covered on the outer side by pericytes and smooth muscle cells. The relation of endothelial cells and pericytes is specifically in the brain with a ratio of 1:1. In between is a layer of extracellular matrix tissue known as basal lamina.

During development, two physiological processes of vessel growth can be distinguished: First angiogenesis as the sprouting of vessels and capillaries from pre-existing ones, and second vasculogenesis which describes the process of *de novo* formation of vessels from bone marrow-derived progenitor cells. In the mature state in adulthood new vessels are believed to be formed solely through angiogenesis. In contrast, tumor cell proliferation appears in an uncontrolled

manner with replicative immortality and sustained angiogenesis is reinforced by vasculogenesis. While in physiological processes e.g. wound healing, angiogenesis is merely active transiently, an “angiogenic switch” is almost always turned on in tumor development. It appears plausible that the angiogenic switch is redox dependent and controlled by counterbalancing positive and negative factors, either inducing or blocking angiogenesis [12, 13]. The angiogenesis-inducing factors, such as vascular endothelial growth factor-A (VEGF-A), acidic and basic fibroblast growth factors (FGF1/2), bind to their corresponding receptors on endothelial cells. VEGF-A is one of the major players in embryonic vasculo- and angiogenesis as well as in endothelial cell survival. The expression of VEGF-A and FGF-1/-2 can be chronically upregulated by hypoxia and oncogenes. However, the blood vessels produced by chronically dominant proangiogenic factors show characteristic morphological aberrations: The tumor-induced neovasculature is marked by a high-density capillary microvessel network with extreme vessel branching, auto-looping, tortuous, disorganized and enlarged vessels.

2. Identification of key angiogenic factors

Several hypotheses have been raised regarding the importance of tumor-induced angiogenesis in development and metastasis of tumors. Dr. Folkman summarized the work of insightful investigators at that time, such as Algire and Chalkley [14], Warren [15], and Greenblatt and Shubik [16], who first termed the process of ‘tumor angiogenesis’. Thus, Folkman coined the concept of treating solid tumors by inhibiting angiogenesis.

Folkman’s visionary hypotheses consisted of the ideas that primary tumors remain dormant in terms of angiogenesis up to a maximum size of approximately 1-2 mm in diameter. Tumor growth beyond that avascular state, switches angiogenesis on by perverting the circumvent mature host blood vessels to start sprouting towards and probably infiltrate the tumors. This process creates a network of new capillaries within and around the tumor bulk. This idea of an angiogenic switch was supported by an orphan vascular growth factor [17] produced by tumors, which has been initially termed ‘tumor angiogenesis factor’ (TAF) [18]. Therefore it would seem plausible to affect the tumor growth by diminishing or even preventing angiogenesis via blocking TAF or its receptors. This ‘dormancy-inducing’ therapeutic approach was thought not to be curative in the common sense but suggested the prevention of further tumor expansion. At best it would result in sustained regression of established tumor bulks to a size of 1-2 mm in diameter so that survival is possible through diffusion and a blood vessel supply is not necessary.

During the following decade Folkman’s ideas though very logical and envisioned but still hypothetical attracted little scientific interest. The situation challenged with the identification and cloning of the first pro-angiogenic factor in the mid 1980s when Sing *et al.* isolated the **basic fibroblast growth factor** (bFGF/FGF-2) from chondrosarcoma [19]. The authors showed that bFGF is a tumor-derived growth factor, which can induce angiogenesis in different models [20]. bFGF belongs to a group of structurally related signaling molecules, all binding fibroblast growth factor receptors (aFGF/FGF-1, FGFRs). Up to now, the FGFR-family consists of four

members: FGFR1, FGFR2, FGFR3 and FGFR4. Alternate splicing of FGFR1-3 gives rise to 'b' and 'c' variants resulting in seven FGFR-subtypes that can be present on the cell surface. Beside monitoring critical functions during normal development of vertebrates and invertebrates [21, 22], FGF-1 and FGF-2 take part in the promotion of endothelial cell and fibroblast proliferation and organizes endothelial cells into tube-like structures. As FGF is quite ubiquitous in the human body it is thought to have a higher angiogenic potential than other angiogenic factors (VEGF, PDGF) [23].

The interest in the research field of angiogenesis actually started to grow in the mid and late 1970s when the group of Dvorak et al. [24, 25] published their paper on one pro-angiogenic factor originally discovered in the late 1970s as a protein secreted by tumors that could increase the permeability of the microvasculature to plasma proteins [26], thus termed **vascular permeability factor (VPF)**. It is the founding member of the later termed **vascular endothelial growth factor-** (VEGF-) family, a group of closely related cytokines (VEGF, VEGF-B, VEGF-C, VEGF-D, PDGF, VEGF-F) identified by Ferrera and colleagues [27, 28] with functions in the physiological and pathological processes of vasculogenesis, angiogenesis and lymphangiogenesis. VPF/VEGF (or VEGF-A) is the most important: It is essential for developmental vasculogenesis and angiogenesis, it increases the permeability of microvasculature to plasma proteins, a characteristic especially known for tumor vasculature in the beginning of tumor stroma generation [29]. VEGF is clinically relevant due to its widespread overexpression in different human cancers and is a candidate for evaluating the prognosis of individual patients. Unlike FGF, which is nonselective for endothelium and stimulates division in many different cell types, VEGF-A is a mitogen specific for the vascular endothelium. VEGF-A is a highly conserved dimeric glycoprotein (34-45kd), constituted of two chains arranged in an antiparallel fashion and bonded via disulfides. The coding region of VEGF-A on chromosome 6 consists of eight exons. Depending on the alternate splicing three different isoforms are prominently encoded in human cells with polypeptides of 189, 165 and 121 amino acids. Despite physical differences, the three VEGF-A isoforms apparently have identical biological activity when in solution.

VEGF-A signals through two major transmembrane tyrosine kinase receptors binding the factor with high affinity. The first is termed flt-1 or VEGF receptor 1 (VEGFR1) and the second is flk-1/KDR (VEGF-receptor-2, VEGFR2). Both tyrosin kinase receptors are mainly though not exclusively expressed on vascular endothelium. Especially endothelial cells of newly formed blood vessels and the vasculature of tumors expressing VEGF-A have a highly elevated expression of both receptors [30]. VEGF-A is overexpressed by the vast majority of cancers [31] and premalignant lesions (e.g. precursor lesions of breast, cervix and colon cancer) and, furthermore, correlates positively with malignant progression [32, 33].

Previously, neuropilin-1 (NRP-1, NP-1) a semaphorine receptor involved in axon guidance during brain development [34, 35] has been shown to play a role in angiogenesis-independent malignant progression [36] by increasing the affinity of various VEGF ligands to the primary VEGF-receptors. Cariboni et al. were also able to illustrate a KDR and blood vessel independent way via VEGF-NRP-1 interactions [37]. Its isoform, neuropi-

lin-2 receptor probably modulates the affinity of VEGF-C and -D to VEGFR-3 and is thought to be important in lymphangiogenesis [38, 39].

Although the specific biological functions mediated by the different receptors are not established precisely, it seems likely that VEGFR-2 is one of the major players in tumor-induced angiogenesis and carcinogenesis. Inasmuch as VEGFR-2 is responsible for the microvascular permeability and the subsequent proliferation and migration of endothelial cells, it is holding a unique position in tumor angiogenesis:

As tumor-derived VEGF-A binds to receptors on the tumor cells themselves, VEGF-A creates a self-perpetuating loop and induces angiogenesis and carcinogenesis in a paracrine or autocrine fashion. The possibility of a therapeutic intervention stimulated academic centers as well as the biotech and pharmaceutical industries to develop VEGF-R-blockers. *Inter alia*, Sunitinib, Imatinib or Avastin/Bevacizumab have so far been created and are used as therapeutics in various cancers.

More recently a second family of ligands and receptors specific for vascular endothelial cells has emerged: the **angiopoietin-tie-system** as non-redundant regulator of endothelial cell activation [40, 41]. Curiously, in this case was that the receptors were discovered first (tie-2/tek and tie-1) and remained orphan receptors until angiopoietin-1 (ang-1) and angiopoietin-2 (ang-2) have been found to be ligands of tie-2 by Yancopoulos *et al.* Ang-1 is essentially expressed by pericytes and smooth muscle cells, whereas ang-2 is stored in the granules of endothelial cells (Weibel-Palade bodies) and secreted after stimulation. Most interestingly ang-1 and ang-2 are antagonistic ligands with a high binding affinity to tie-2. While ang-1 functions as agonist to tie-2 promoting angiogenesis, inhibiting vessel leakage and suppressing inflammatory gene expression [42, 43], ang-2 behaves oppositely, causes endothelial cell apoptosis and as a result regression of newly formed vessels [44]. The situation changes completely when ang-2 acts in combination with VEGF-A, for the two work in and promote angiogenesis [45].

After focusing the first two decades of research on pro-angiogenic growth factor stimulators and how to intervene in their pathways to exogenously block the process of angiogenesis, **endogenous inhibitors** entered the scene and a large, growing family of antiangiogenic basement membrane proteins, hormone metabolites and apoptosis modulators has been discovered. Among others thrombospondin-1, interferon α/β , arresten, canstatin, angiostatin, tissue inhibitors of matrix metalloproteinases (TIMP) and vascular endothelial growth inhibitor (VEGI) have been proven to be endogenous inhibitors (Table 1).

Today, it is accepted that endogenous inhibitors activate a cellular 'brake' mechanism. This mechanism leads to altered cell-cell-interactions, malignancy associated with induced angiogenesis and other diseases like ocular disorders or rheumatoid arthritis when turned off. In case of dominant endogenous inhibitors and deactivated angiogenic switch, launching angiogenesis is (almost) impossible.

Name	Description
Angiopoietin-2 (Ang-2)	Non-matrix derived; stored in Weibel-Palade bodies; inhibits EC proliferation and migration; antagonist of ang-1; in clinical trial [46, 47]
Angiostatin	Non-matrix derived; 38-45kDa, involves either kringle domains 1-3, or smaller kringle 5 fragments [48, 49]
Arresten	Matrix derived; 26-kDa; from type IV collagen; selectively inhibits EC tube formation, interferes with FGF-2 [50, 51]
Canstatin	Matrix derived; 24-kDa; from type IV collagen; inhibits EC migration and tube formation dose-dependently [52]
Endorepellin	Matrix derived; from perlecan; inhibits several aspects of angiogenesis [53, 54]
Endostatin	Matrix derived; 20-kDa; zinc-binding fragment of type XVIII collagen; blocks angiogenesis, primary tumor growth and metastasis; interferes with FGF-2; Phase II [55]
Interferon α/β	Non-matrix derived; anti-viral proteins; Phase III (Interferon- β) [56]
Interleukins	Leukocyte-derived; heterogeneous superfamily [57]
2-Methoxyestradiol	Estradiol metabolite; Phase II [58]
Meth-1/-2	Non-matrix derived; proteins containing metalloprotease and thrombospondin domains [59]
Platelet factor-4	Inhibits FGF-2-induced EC proliferation [60]
Prolactin fragment	Derived from prolactin; 16-kDa; blocks angiogenesis; inhibits VEGF-induced Ras-activation [61]
Thrombospondin-1/-2	Matrix derived; large extracellular matrix protein; Phase II (Thrombospondin-1) [62]
TIMP	Non-matrix derived; suppress MMP activity; pluripotent effect on EC growth, apoptosis and cell differentiation [63]
Troponin I	Cartilage-derived; inhibits EC proliferation and angiogenesis [64]
Tumstatin	Matrix derived; 28-kDa from type IV collagen; apoptosis of EC [65, 66]
VEGI	174 amino acid cytokine; TNF-superfamily; autocrine apoptosis in EC [67]
Vasostatin	Non-matrix derived; fragment of calreticulin; selectively inhibits EC proliferation and angiogenesis in response to stimulus; suppresses tumor growth [68]

Table 1. Summary chart of various endogenous inhibitors of angiogenesis

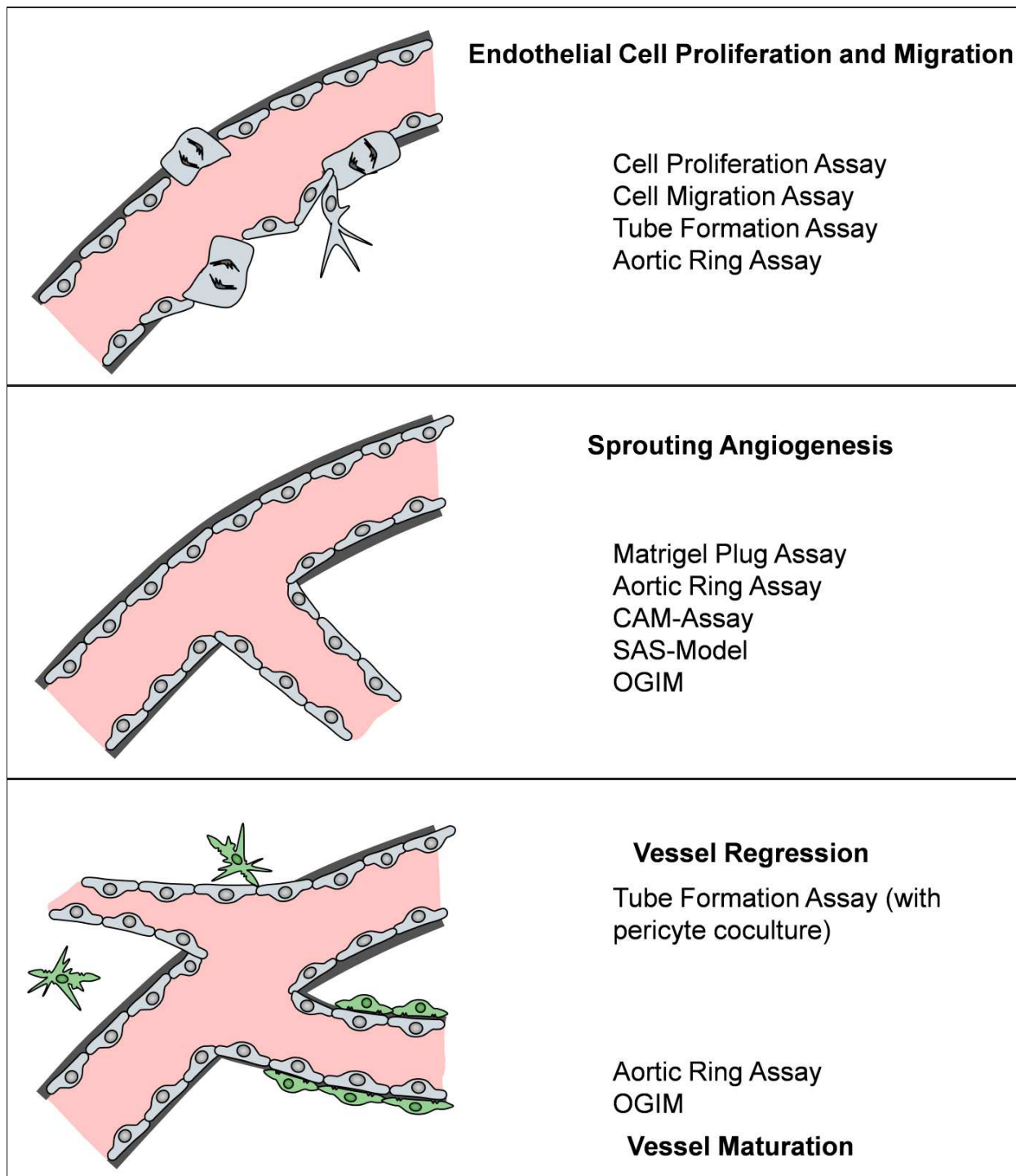


Figure 2. The key steps of angiogenesis. After binding of angiogenic factors to their receptor, endothelial cells start to proliferate and migrate towards the source of angiogenic activity (upper panel). This induces the sprouting and formation of a new branch (middle). Newly sprouted vasculature can either undergo maturation or regression (lower panel). Depending on the biological events of interest different bioassays are available with particular advantages for gauging.

3. *In vitro* assays to study brain tumor angiogenesis

The human brain is made up of various cell types of different lineages (neuroepithelial, epithelial and mesenchymal traits). Brain cells include astrocytes, oligodendrocytes, neurons, microglia, ependyma and vessels which interact with each other and form a particular environment via signaling molecules. Therefore the most important issues for *in vitro* bioassays are to reduce such complexity and to make single biological events measurable in a reasonable way. *In vitro* assays allow a high level of redundancy and permit the researcher to focus on single biological events.

Besides pericytes and the vessel-surrounding tissue, endothelial cells lining all blood vessels of the body are the prominent cells involved in neovascularization. New blood vessel formation follows in principle four cardinal steps: First step is that endothelial cells need to adhere, proliferate and permeabilize the environmental boundaries. Therefore endothelial cells undergo cell division and start to secrete proteases to degrade the matrix and break through the basal lamina, which is the second inner layer of the vessel. Second, migration and invasion towards angiogenic stimuli needs to be facilitated (VEGF, FGF, PDGF, etc.). Endothelial cells use for instance integrins to travel in tandem. This event is connected with the sprouting of newly formed vessel barbs. As a source, angiogenic stimuli can be secreted by activated lymphocytes, tumor cells, microglia and macrophages. Thirdly, endothelial vessel sprouts form new vessels and recruit cellular components to line up a fully functional vessel. The fourth key step is the modeling and reorganization process. Newly formed vessels can mature or undergo regression. For each of the four key steps several *in vitro* assays have been developed.

3.1. Tube Formation Assay (TFA)

In the past we considered the vascular endothelium as a passive structure which acts like a filter between the blood in the vessel lumen and the vessel wall itself. In fact endothelial cells are active members of the vascular homeostasis playing a vital role in the coagulation and the fibrinolysis system as well as in adhesion and aggregation of blood platelets via secreted activators and inhibitors. These processes (among others) can be studied *in vitro* using human vascular endothelial cells isolated from umbilical veins.

Human umbilical vein endothelial cells (HUVECs) were isolated from freshly obtained human umbilical cords the first time by Eric A. Jaffe in 1973 [69]. They were able to identify the cell morphologically (Weible-Palade bodies) and immunologically (ABH antigens fitting to the donor's blood type) as human endothelial cells and demonstrated that it is possible to culture them for a period of time. Endothelial cells of all origin seem to have the ability to form three-dimensional structures (tubes, cobblestone pattern), which can be fostered by coating the plastic culture dishes with either collagen or fibrin clots. The formation of tight junctions between endothelial cells can be confirmed by electron microscopy.

The tube formation assay is a viable assay, which can be accomplished within a day. Image analysis is used generally to measure the endothelial cells ability to form tube-like structures. Further endothelial cell adhesion, migration and invasion have been reported [70]. Though

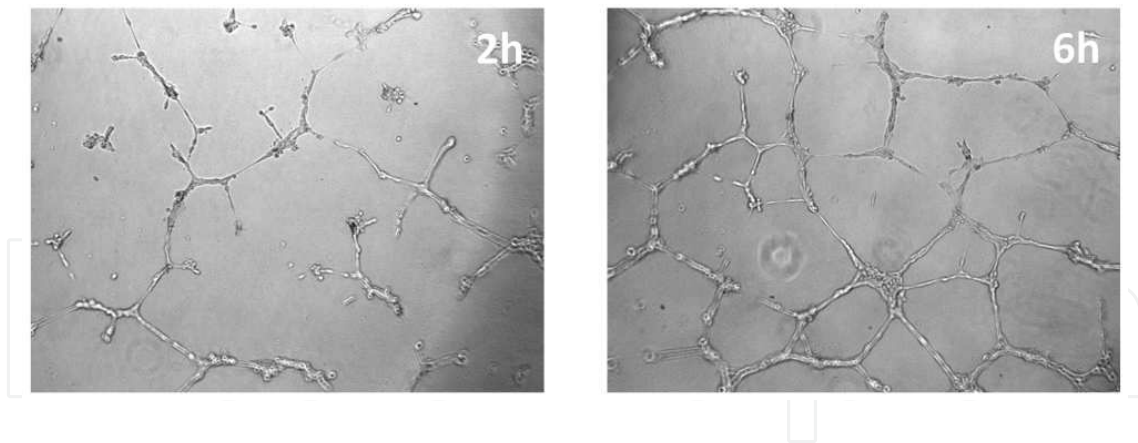


Figure 3. Tube formation assay in time lapse modus. Representative photographs of tubes formed by human umbilical vein endothelial cells (HUVECs). The left picture is taken 2 h after plating and shows beginning tube formation. The right picture is taken 6 hours after plating and shows the typical 'cobblestone' forming. Images are given as x10 magnification.

very reliable in studying the reorganization, the TFA represents only a small part of angiogenesis and allows specific predictions of the nature of different endothelial cell strains rather than testifying the angiogenic process itself.

3.2. Cell proliferation assays

There is a large variety of assays to measure cell proliferation. Well-established ones are for example the MTT-assay, in which living cells reduce 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole to purple formazan, and the thymidine incorporation assay using BrdU (5-bromo-2'-deoxyuridine), an analogue of thymidine. Both assays are performed to detect proliferating cells in living tissue, Especially the BrdU incorporation assay is most frequently used for cell proliferation studies in angiogenesis.

The cells used in proliferation assays are endothelial cells. However, it matters from which source the endothelial cells are isolated since they differ not only between large-vessel-derived endothelial cells and ones originated from microvasculature. They also seem to have distinctive characteristics when obtained from different organs and even when taken from different sites in one single organ (71, 72, 73). Another fact is the total difference between species, which cannot be ignored: One example are the pig and murine endothelial cells, which bind BSL-1 and BSL-4 (lectin from *Bandeira simplifolia*) and are therefore a target for hyper-immune destruction when implanted into patients.

The most common cell types used in this assay are either bovine aortic endothelial cells or human umbilical vein endothelial cells. During culture in the laboratory these cells are by nature in a proliferative state, while they are more in a quiescent, non-proliferative state *in vivo*. Therefore endothelial cells *in vitro* depend on the environment in which they are kept during culturing.

3.3. Cell migration assay

Cell migration is the movement of cells from one area to another induced by chemical signals. Generally cell migration plays a vital role in processes like cell differentiation, tumor metastasis and wound healing. In angiogenesis studies the focus is mainly laid on migrating endothelial cells. In terms of angiogenesis one should speak of cell invasion, for endothelial primarily degrade the basal lamina before migrating toward an angiogenic stimulus.

The assays most frequently performed are the blind-well chemotaxis chamber and the scratch-wound assay. The blind-well chemotaxis chamber is a modified Boyden chamber as used for classic neutrophil chemotaxis. Instead, endothelial cells are placed on a cell permeable filter. When an angiogenic stimulus is added into the medium below the filter, the cells start to migrate. The system is very useful in concentration-dependent effects.

In the other preferred assay, the scratch-wound assay [74, 75], HUVECs are used to be seeded into trans-wells. When the cells are 85-95% confluent a wound/scratch is set. The test is used to measure the time needed under different conditions (drug treatment, etc.) to close the wound again.

3.4. Aortic Ring Assay (ARA)

The Rat Aortic Ring Assay, originally developed by Nicosia *et al.* [76] phenocopies all the key steps in the angiogenesis process: matrix degradation, migration and proliferation of endothelial cells and structural reorganization. The basis of this assay is the excised aorta (of rats or mice) from which the connective tissue has been removed. The aortic tube will then be cut into rings or small pieces (aortic explants) and taken into culture with collagen gel. Several days after culture initiation the aortic tissue gives rise to a microvascular network of branching endothelial tubes, a process which is triggered by the dissection procedure and the growth factors produced by the rings/explants.

Since angiogenesis *in vivo* not only involves endothelial cells, but also pericytes, the basal lamina and other surrounding cells, researchers have moved to assess angiogenesis by organ culture methods. Of these, the rat aortic ring assay has become one of the most widely used ones. In particular, it has several advantages compared to "normal" *in vitro* single-cell culture: As the assay is based on explants, it does not only consist of endothelial cells which have been selected via cell passaging, but of endothelial cells and multiple other cell types which are in a non-proliferative state and therefore closer to a real-life environment. It is even possible to recapitulate all of the key steps of endothelial cell sprouting or angiogenesis: Matrix degradation, that is to say breaking through the basal lamina, which is the second layer of mature vessel enveloping the endothelial cells, migration toward a signal source, proliferation and finally reorganization or tube-forming can be analyzed. After several days in culture image analysis is used to measure length and abundance of the sprouting endothelial cells.

Although the situation in the aortic ring assay is closer to the *in vivo* environment than in dissociated single cell culture assays, one has to deal with the higher experimental variability due to animal strains and animal age. The main factors here are the incomplete removal of the surrounding connective tissue which can affect the sprouting and outgrowth of endothelial

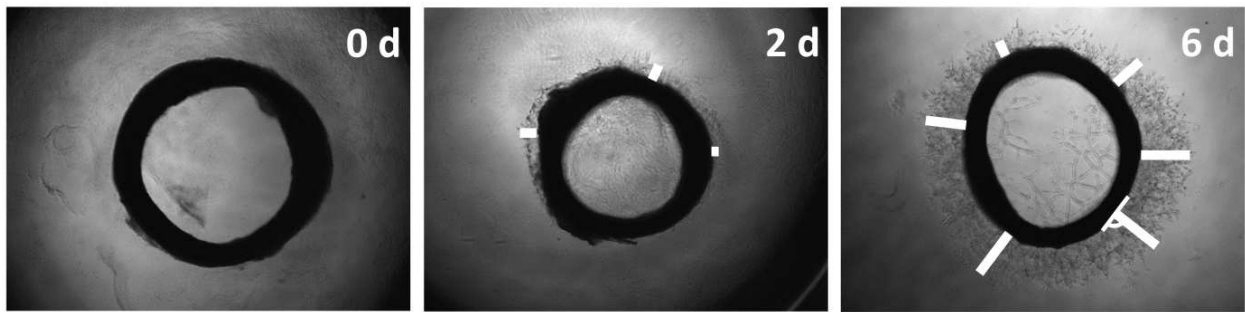


Figure 4. Aortic ring assay experiments. Phase-contrast images showing endothelial sprouting from aortic rings embedded in Matrigel and the method of quantification. To make the data comparable, we developed a tangents measurement methods. Tangents are set in respect to the ring and measurements can be conducted of the sprouting distance with 90° to the tangent (white bars).

cells, the tissue from multiple animals itself which can result in an inconstant angiogenic response and last, the evaluation of the images, for the sprouting is a three-dimensional process.

The aortic ring assay is a reliable assay to test substances for their potential to positively or negatively affect angiogenesis and is able to monitor angiogenesis activity.

4. *In vivo* assays in neuro–oncology

4.1. Chronic window preparations

4.1.1. Rabbit ear chamber

In 1924 Sandison *et al.* developed the first chronic window preparation. They implanted a transparent window chamber in the ear of a rabbit [77]. The preparation allows inspection of an *in vivo* environment from outside the body, a long-term monitoring of angiogenesis is possible. Clark *et al.* made the first steps by studying wound-healing associated angiogenesis [78, 79]. Ide *et al.* adopted the method, transplanted tumors and were able to observe strong tumour growth associated with a newly formed vascular network [80]. Their results led to the proposal of a 'vessel growth- stimulating substance' produced by the tumor.

4.1.2. Dorsal skinfold chamber

The dorsal skinfold chamber is an adaption of the rabbit ear chamber made by Algire *et al.* in the 1940s [81, 82]. A metal chamber with a transparent window is implanted in various animal species, in rodents it is the most widely used window preparation. The dorsal window can last 30-40 days. To study the effect of tumors on vessel formation, fluorescent tumor cells are implanted into the chamber and the angiogenic activity, infiltration by immune cells, tumor cell migration and tumor growth are then monitored by intravital microscopy (IVM) [83, 84].

The main limitation of this model is the non-organotypical environment of tumors other than skin cancer.

4.2. Chick embryo chorioallantoic membrane (CAM-) assay

A widely performed assay to test angiogenesis *in vivo* is the chorioallantoic membrane (CAM-) assay. Initially, Murphy *et al.* [85] implanted rat Jensen sarcoma cells into the CAM of chicken eggs and were able to show signs of tumor-induced angiogenesis. Highly promoted by Folkman *et al.*, the CAM-assay has been transposed to the developing field of angiogenesis [86].

Since the introduction of the CAM-assay almost a century ago multiple modifications of the original angiogenic assay were made which allow quantification of the angiogenic process. In addition chick embryos are immune-incompetent until embryonic day 17. Therefore grafting of cells of different species (e.g. human tumor cells) is possible so that the CAM-assay became a useful tool for analysis of the proangiogenic potential of test cells. The HET-CAM developed by Niels-Peter Lüpke in 1985 [87, 88] and the CAMVA (chorio allantoic membrane vascular assay) are the two modified CAM-assays mainly used in the field of angiogenesis.

The hen's egg test on the chorioallantoic membrane (HET-CAM-) assay is an organotypic model which was initially designed to replace the highly discussed Draize-test to identify irritative reactions in the eye: haemorrhage, lysis and coagulation. These three reactions of the CAM are observed on the ninth day of embryonation. Test substances are applied directly onto the CAM and the membrane is scanned after five minutes for the above named reactions. Further the test allows analysis for angiogenesis of tumor-models growing on the CAM.

The CAMVA monitors the effects of potential eye irritants, drugs or other chemicals on the blood vessels of the CAM. It was developed by Leighton *et al.* [89] in 1985 and adjusted to industrial laboratory standards by Bagley *et al.* [90, 91]. The chorioallantoic membrane vascular assay is considered suitable as an option to replace animal trials to study vascular effects. The preparation of the test starts with an egg four days after fertilization in which a small window over the CAM is cut. By sucking out approximately 3 ml of albumen, best conditions are created for optimal growth of the CAM. Thereafter, the opening is resealed and the eggs incubated for another six days. In the course of incubation, test substances are applied directly onto the CAM, followed by an examination for vascular changes in the CAM. Changes observed are hyperaemia, haemorrhaging and the occurrence of ghost vessels.

4.3. Subcutaneous Air Sac model (SAS)

The rat subcutaneous air sac (SAS-) model was promoted by Lichtenberger *et al.* [92, 93] as a straightforward method to study anti-angiogenesis *in vivo*. A sac is produced by introducing air dorsally to anesthetized rats via subcutaneous injection. So that the wall of the air sac becomes progressively thicker in time, the air sac is re-inflated every fourth day. After 10-14 days an acceptable lining of cells has been grounded and an almost transparent avascular membrane has been created. Before treated another 10 days subcutaneously with various test compounds, a sponge is implanted into the cavity. It is important to choose a good injection site which eliminates the risk of interfering and irritating the membrane. To study tumor-

induced angiogenesis, tumor cells are inoculated subcutaneously on the membrane. Funahashi *et al.* modified the assay and use a tumor cell-containing chamber to determine the angiogenic effect. After the 10-day-treatment the animals are being sacrificed, the overlying skin of the air sac is removed and the transparent membrane is evaluated *in situ* for angiogenesis.

4.4. Matrigel plug assay

Most angiogenesis *in vivo* assays are very complex in both, the experimental setup and the surgical skills one needs. The matrigel plug assay is a simple *in vivo* angiogenesis assays. Derived from the engelbroth-holm-swarm (EHS) mouse sarcoma, the matrigel matrix is comparable to basement membrane proteins. It is "loaded" with either cells (mostly tumor cells) or angiogenesis-inducing substances (FGF, VEGF, etc.). Cold (and therefore liquid) matrigel is injected subcutaneously in nude mice. With increasing temperature the gel solidifies and forms a plug, which allows cell growth and vessel formation [94]. The plug is removed from the animal after 7-21 days inoculation, fixed with paraffin, stained and examined histologically.

The main benefit of the matrigel plug assay is that it is relatively easy and fast to perform, neither special experimental setup nor surgical skills are required and the test materials are available without difficulty. Nevertheless, the assay is limited to its specific organotypic location, i.e. subcutaneous. Further, matrigel is a reconstituted matrix, with a particular biochemical composition. In addition, Auerbach *et al.* reported considerable difficulties to maintain comparable three-dimensional plugs despite the fact that the gel volume is kept constant [95].

5. New *ex vivo* model for tumor angiogenesis

5.1. The Organotypic Glioma Invasion Model (OGIM)

The *Organotypic Glioma Invasion Model* (OGIM) is a recently developed method to study physiological angiogenesis and tumor-induced angiogenesis and allows researchers to address systematically molecular pathways of angiogenesis as well as tumor-vessel interactions [96, 97]. Furthermore, it is possible to monitor neuronal and tumor cell death, tumor proliferation and migration in time lapse with focus on specific tumor-host interactions and tumor microenvironment. The method bridges *in vitro* and *in vivo*-assays, since all cells of the brain (astrocytes, oligodendrocytes, neurons, ependymal cells microglia and vessels) are present in a natural environment.

Hippocampal brain slice cultures are acquired from postnatal rats and enhanced green fluorescent protein expressing rat glioma cells are implanted into slices in culture. After implantation cell death can be monitored in different regions of interest: The tumor bulk, the peritumoral invasion zone and the surrounding tissue. The brain slices can be kept in culture for several days after tumor implantation. Consecutively, various immunohistochemical staining for vessels can be performed. Beside the classical vessel marker, the platelet/endothelialmembrane cell adhesion molecule-1 (PECAM-1 or CD31), staining can also be per-

formed with antibodies against laminin, factorVIII (von Willebrand - factor), and smooth muscle actin (SMA). Anti-laminin is used to stain the basal membrane, anti-vWF for endothelial cells and anti-SMA for pericytes.

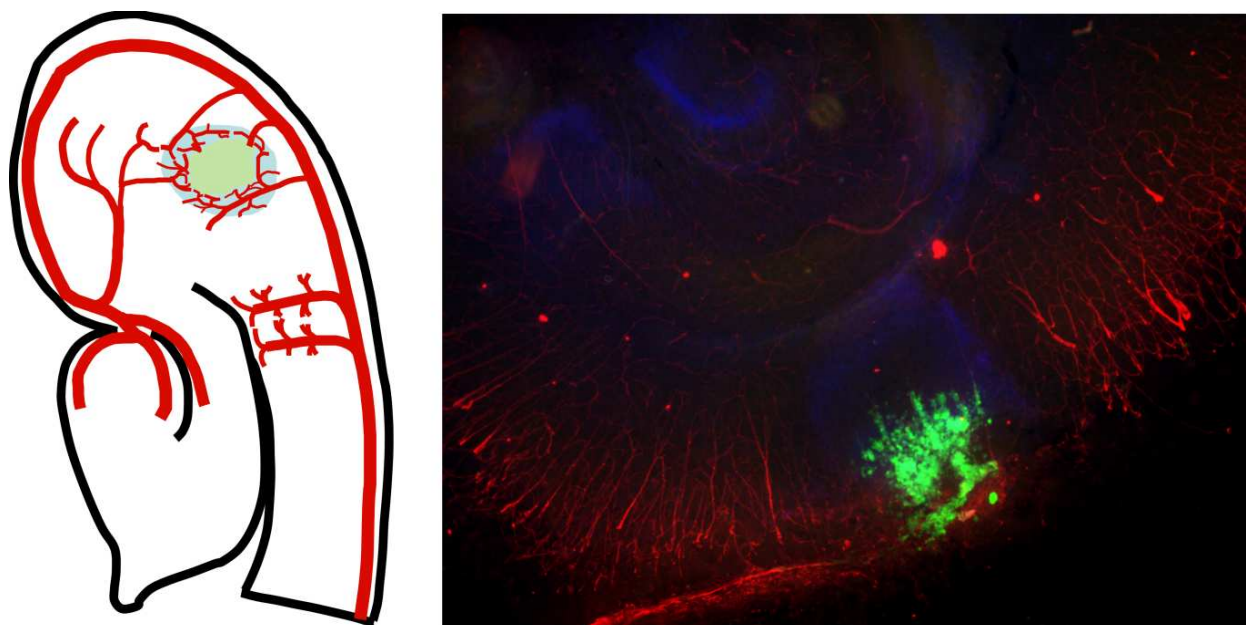


Figure 5. The Organotypic Glioma Invasion Model (OGIM) for angiogenesis studies. Left, Scheme of the brain slice and its vessels in the hippocampus and entorhinal cortex. The vessel architecture in the peritumoral invasion zone differs highly from that of the tumor zone and control area. Right, the fluorescent image shows a typical example of a GFP-tumor-implanted brain slice after immunohistochemistry performed for vessels. Green: glioma cells, blue: nuclei, red: laminin staining. The image is given as x4 magnification.

The OGIM measures vessel density (overlying grid method), branching as well as morphological vessel aberrations such as tortuous, disorganized vessels, blind-ends and auto-loops. It is also possible to monitor and analyze vascular mimicry in a time lapse mode. In our lab we established glioma vessel features over time. The regular vessel architecture ranging from big lumen vessels down to arteriols, metarteriols and capillaries is breached and replaced by a chaotic vessel structure with probable back-and-forth blood flow and non-functional vessels with blind ends in tumors. Moreover a high-density microvessel network is produced via tumor-induction. The organotypic brain slice model represents the tumor microenvironment *in vitro* in a three-dimensional culture [98], which is biologically more relevant, but technically more challenging than migration, proliferation and cytotoxic assays being an appropriate possibility to test and optimize anti-cancer compounds.

6. Conclusion

The current development of the tumor angiogenesis field with newly identified angiogenic factors craves for robust, viable and easy to perform assays which are also assessable for analysis. Although divergent opinions exist on what are the best methods for studying angiogenesis,

several *in vitro* bio-assays have been established and are in worldwide use. Here we described various established *in vitro* assays commonly used in the phase of expedition and confirmation. Since at least four key steps in angiogenesis exist, i.e. proliferation, migration and invasion, sprouting and vessel formation, and vessel modeling and reorganization (maturation, regression). Thus, *in vitro* bio-assays cannot reflect all key events in one. In principle, this is also the case for *in vivo* assays. For this reason, assays make certain key vascular events assessable in a short period of time leaving others not detected. So far, established endothelial cell culture assays have a high level of redundancy and utilize endothelial cells from different origin which have been preselected for their proliferative capacity. In addition, *in vitro* tissue assays have been established which also consider the microenvironment and represent higher cellular complexity. Further, regarding the amount of data *in vitro* tests can generate, it appears also necessary to spent efforts on the precise evaluation system to be used. Some *in vitro* assays such as the tube formation assay allow not only for one parameter to investigate, i.e. tube formation, but open the possibility to analyze also the dynamic and morphology of such tubes. *In vivo* assays are more demanding in terms of material, time and labor. Also, these assays often require surgical skills. With all the interactions present, evaluation of certain angiogenesis processes can be hard to perform accurately. On the other hand, *in vivo* assays reflect the organotypic environment and cellular components involved. When developing antiangiogenic drugs, assays are needed that are easy to perform in a short period of time and that consider certain aspects of the *in vivo* situation to prevent artificial phenomena. We describe here a bridge technique, i.e. the *organotypic glioma invasion model* (OGIM), which combines the advantages of *in vitro* assays with that of the organotypic brain environment as an *in vivo* situation [98]. The OGIM is a robust *in vitro* assay enabling the investigation of key events of tumor angiogenesis within the brain. This assay allows to analyze the impact of a therapeutic within the balance of concerted factors promoting and inhibiting angiogenesis.

Acknowledgements

We thank all members of our neurooncology lab for critical suggestions and valuable comments during developing new techniques. Our work is supported in part by the German Research Foundation (DFG grant Ey 94/2-1).

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The authors declare no competing financial interests.

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