

Tumour vascularization: sprouting angiogenesis and beyond

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Abstract Tumour angiogenesis is a fast growing domain in tumour biology. Many growth factors and mechanisms have been unravelled. For almost 30 years, the sprouting of new vessels out of existing ones was considered as an exclusive way of tumour vascularisation. However, over the last years several additional mechanisms have been identified. With the discovery of the contribution of intussusceptive angiogenesis, recruitment of endothelial progenitor cells, vessel co-option, vasculogenic mimicry and lymphangiogenesis to tumour growth, anti-tumour targeting strategies will be more complex than initially thought. This review highlights these processes and intervention as a potential application in cancer therapy. It is concluded that future anti-vascular therapies might be most beneficial when based on multimodal anti-angiogenic, anti-vasculogenic mimicry and anti-lymphangiogenic strategies.

Keywords Sprouting angiogenesis · Intussusceptive angiogenesis · Endothelial progenitor cells (EPCs) · Vessel co-option · Vasculogenic mimicry · Lymphangiogenesis · Angiogenesis inhibition

1 Introduction

Tumours can grow to a size of approximately 1–2 mm³ before their metabolic demands are restricted due to the diffusion limit of oxygen and nutrients. In order to grow beyond this

size, the tumour switches to an angiogenic phenotype and attracts blood vessels from the surrounding stroma. This process is regulated by a variety of pro- and anti-angiogenic factors, and is a prerequisite for further outgrowth of the tumour [1]. Next to sprouting angiogenesis, the process by which new vessels are formed from preexisting vasculature, several other mechanisms of neovascularization have been identified in tumours, including intussusceptive angiogenesis, the recruitment of endothelial progenitor cells, vessel co-option, vasculogenic mimicry and lymphangiogenesis (Fig. 1). Due to application for treatment of disease, these processes gained a lot of interest over the last years. This review summarizes the different mechanisms of tumour vascularization, the molecular players that are involved and their relevance in clinical practice.

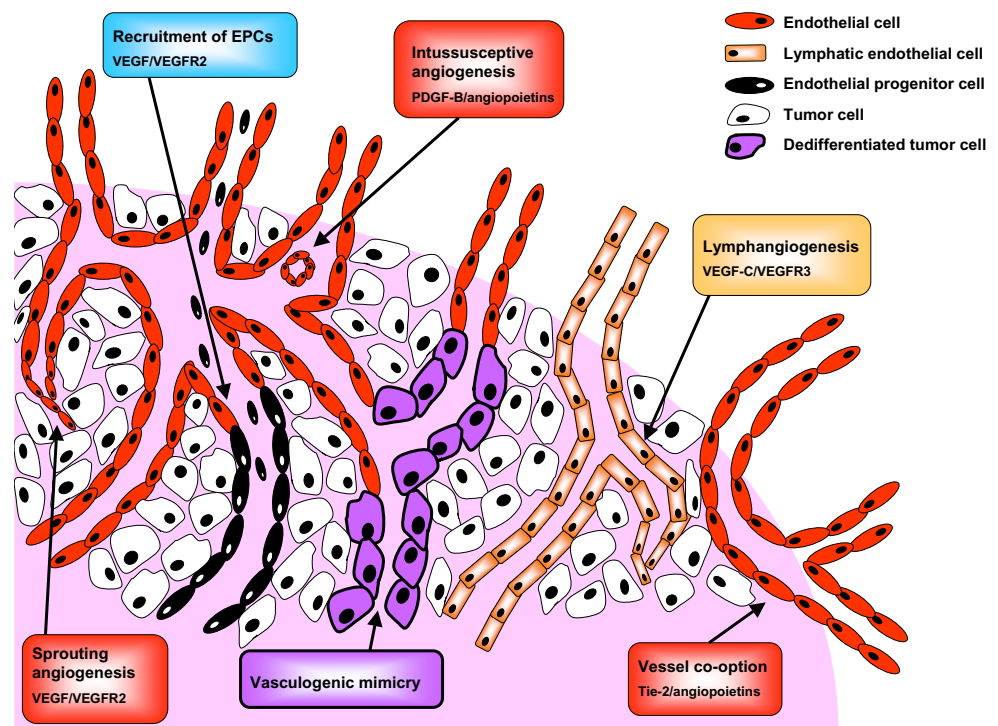
2 Sprouting angiogenesis

Sprouting angiogenesis is the growth of new capillary vessels out of preexisting ones. These blood vessels will provide expanding tissues and organs with oxygen and nutrients, and remove the metabolic waste. Angiogenesis takes place in physiological situations, such as embryonic development, wound healing and reproduction. It also plays an important role in many pathologies, like diabetes [2], rheumatoid arthritis [3], cardiovascular ischemic complications [4], and cancer [5]. In cancer, sprouting angiogenesis is not only important in primary tumours, it is also involved in metastasis formation and further outgrowth of metastases [6].

The process of sprouting angiogenesis involves several sequential steps. Tumour angiogenesis starts with the activation of endothelial cells by specific growth factors that bind to its receptors. As a result, the extracellular matrix and basement membrane, surrounding the endothelial cells,

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Fig. 1 Different mechanisms of tumour vascularisation. This diagram represents the six different types of vascularisation observed in solid tumours, including sprouting angiogenesis, intussusceptive angiogenesis, recruitment of endothelial progenitor cells, vessel co-option, vasculogenic mimicry and lymphangiogenesis. The main key players involved in these processes, if known, are indicated



are degraded locally by activated proteases. This allows the endothelial cells to invade into the surrounding matrix and, subsequently, to proliferate and migrate through the matrix. By polarization of the migrating endothelial cells a lumen is created, and an immature blood vessel is formed [7]. The stabilisation of the immature vessels is established by recruitment of mural cells and generation of extracellular matrix [8]. This process of sprouting angiogenesis is tightly controlled by positive and negative regulators, the balance of which determines the level of ongoing angiogenesis.

The first angiogenic growth factor, fibroblast growth factor (bFGF), also known as FGF-2, was discovered in the early 1980s [9]. The FGF family consists of 23 members, of which FGF-2 and FGF-1 (aFGF) are the best known, and four FGF tyrosine kinase receptors have been described. bFGF stimulates all major steps in the angiogenesis cascade and is produced by many cells, among which are macrophages and tumour cells. Although FGF does not have a signal sequence that allows regular secretion, it is released in the extracellular matrix after which angiogenesis is initiated. bFGF is a pleiotropic mitogen for growth and differentiation, known to be involved in endothelial cell proliferation, extracellular matrix degradation, endothelial cell migration and modulation of junctional adhesion molecules. Moreover, the intricate interaction with other growth factors can result in many synergistic activities in endothelial cell functions [10]. In both mouse and human tumours, the role of bFGF in tumour growth and neovascularization has been demonstrated [11]. Neutralizing antibodies and siRNA techniques

have been described to inhibit tumour growth and neovascularization in mouse models [12, 13].

Vascular endothelial cell growth factor (VEGF) or vascular permeability factor, is another important player in the stimulation of angiogenesis. VEGF is a general activator of endothelial cell proliferation and mobility. It is the most potent factor that induces vasodilatation of the existing vessels and increases permeability of the vessel wall [14]. Moreover, it increases the expression of matrix metalloproteinases and plasminogen activators for the degradation of the extracellular matrix and subsequently endothelial cell migration [15]. The VEGF family of growth factors consists of six members (VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placental growth factor) that interact differentially with three cell surface receptor tyrosine kinases, the VEGFRs, or a second class of non-signalling co-receptors, the neuropilins. To date, the VEGF-A/VEGFR2 interaction appears to play a major role in sprouting angiogenesis [7]. In tumours, higher levels of VEGF are detected and many tumour cell lines were found to be inhibited *in vivo* by antibody targeting methods or the use of small-molecule inhibitors of VEGF or VEGFR2 [14].

Placental growth factor (PLGF), a member of the VEGF family that only binds VEGFR1, is also a mediator of the angiogenic switch, though its role was underestimated. However, activated endothelial cells are known to produce large amount of PLGF and thereby regulating the VEGF-mediated angiogenic switch. Moreover, other cell types like smooth muscle cells, inflammatory cells and tumour cells

can also produce PLGF when activated [16, 17]. Importantly, PLGF seems to play a role in vascular development but does not affect the functionality of physiological vessel formation during development and reproduction [17].

The angiopoietin family, another important growth factor family in angiogenesis, includes three members (in humans), angiopoietin-1, angiopoietin-2 and angiopoietin-4, that all bind to the endothelial tyrosine kinase receptor Tie-2. The most remarkable characteristic of this family is the opposing effect of the different ligands binding to the same receptor. Angiopoietin-1 activates the Tie-2 signalling while angiopoietin-2 inhibits this activation. Angiopoietin-1 is involved in endothelial cell migration, adhesion and the recruitment of pericytes and smooth muscle cells, while angiopoietin-2 is vessel destabilizer [18, 19].

Besides the above described angiogenic factors, tumour cells can produce other factors like transforming growth factor- β , which stabilizes newly formed vessels and suppresses the immune system [20], platelet-derived growth factor, which is a chemoattractant for pericytes [21], epidermal growth factor, which promotes tumour angiogenesis by upregulating VEGF [22] and interleukin 8 that specifically enhances endothelial cell migration [23].

Recent studies have shown similarities in the molecular regulation of guidance of neural and endothelial cells. Specialized endothelial cells, resembling axonal growth cones, are located at the tips of growing capillaries. These tip cells extend and retract their filopodia continuously to explore the environment and to define the direction in which a new vascular sprout grows [24]. Both axon growth cones and endothelial tip cells seem to use a repertoire of molecular ligand/receptor signalling systems including the family of Ephrins, Semaphorins, Slits, Netrins and Notchs. Most of these molecules seem to play a role in tumour angiogenesis. The injection of soluble Ephrin receptors was found to successfully inhibit tumour angiogenesis in an animal model [25]. Also semaphorins are hypothesised to have tumour suppressor characteristics since overexpression has been shown to inhibit metastasis in melanomas and highly metastatic melanoma cells showed a downregulation of expression [26]. On the other hand, Sema4D, a pro-angiogenic factor released by tumour cells, promoted invasion and metastasis [27]. Likewise, the Slit/Robo signalling seems to promote tumour angiogenesis. Neutralization of Robo1 reduced the microvessel density and the tumour mass of human malignant melanoma *in vivo*. Moreover, there is evidence of molecular crosstalk between cancer cells and endothelial cells [28]. Furthermore, the implication of netrins and their receptors has been studied. The positive signalling pathway of netrins that normally activates apoptosis, seems to be inactivated in tumours. Binding of netrin-1 to its receptors inhibits the tumour suppressor activity of p53 [29]. There is increasing evidence that Notch signalling is also involved in

tumour angiogenesis, although it seems to have both oncogenic and tumour suppressive roles [30]. It is obvious that the specific role (stimulatory and inhibitory effects) of these molecules in angiogenesis needs further research.

Sprouting angiogenesis can also be negatively regulated. Thrombospondin-1 was among the first naturally occurring angiostatic agent to be discovered [31]. Later on, more endogenous molecules with angiostatic activity were described. Among these were the 16 kD fragment of prolactin [32], platelet factor-4 and interferon- α [33] and interferon- γ inducible protein-10 [34]. Other members of this class of endogenously produced anti-angiogenic proteins are angiostatin [35], endostatin [36], bactericidal/permeability increasing protein [37], tumstatin [38]. It is interesting to note that many of these molecules are proteolytic fragments of endogenous macromolecules. Although for several of the currently described angiogenesis inhibitors receptors have been described, detailed mechanisms of action, in most cases, are still obscure [39].

Next to anti-angiogenesis approaches with endogenous inhibitors, several blocking strategies of the above described angiogenic factors have been reported. Strategies that block the VEGF-A/VEGFR2 signalling are the most abundant ones in the clinical field of anti-angiogenic therapy. A lot of attention is focussed on the approval of the first anti-angiogenic agent, Avastin, by the Food and Drug Administration [40, 41]. Avastin in combination with chemotherapy demonstrated a survival benefit in patients with metastatic colorectal cancer of several months [42]. Although a beneficial clinical effect is present, in some patients gastrointestinal perforations, thromboembolic events and impaired wound healing was observed [42]. Moreover, recent warnings about possible visual and neurological long-term problems in patients administrated with Avastin, will probably delay the FDA approval for more applications [43, 44]. Besides Avastin, several other VEGF inhibitors are being clinically implicated. The most advanced receptor tyrosine kinase inhibitors that target VEGF receptors are SU11428, BAY 43-9006 [41].

Next to the reported side effects of anti-angiogenic inhibitors, also induction of resistance against these agents must be acknowledged. There is emerging evidence that VEGF-A may be replaced by other angiogenic pathways and other members of the VEGF family [45]. Other mechanisms that can participate in resistance are the selection of more hypoxia resistant cells that are less dependent on angiogenesis [46] and the normalization of tumour vessel that become less responsive to anti-angiogenic therapy [47]. Moreover, the hypothesis that endothelial cells are more genetically stable than tumour cells (and thus less sensible to develop resistance) is now questioned, especially after several reports on genetic abnormalities in endothelial cells of tumour vessels [48, 49].

Although a lot of mediators and pathways that are involved in sprouting angiogenesis have been identified, it is clear that the inhibition of this process is very complex. Clinical trials in patients with less advanced stages of cancer, and the long-term effects of approved compounds will guide us to the use of angiostasis in the clinical management of cancer. However, already now, it seems very likely that efficient cancer therapy will be composed of combination of chemotherapy and anti-angiogenic strategies that target multiple angiogenic pathways.

3 Intussusceptive angiogenesis

A variant of angiogenesis, different from sprouting, is intussusceptive angiogenesis. This process was first observed in postnatal remodelling of capillaries in the lung [50]. In the third week of rat life and during the first 2 years in humans, the volume of the lungs increases by more than 20 times. In this developmental process, a new concept of vessel formation was found where preexisting vessels split in two new vessels by the formation of transvascular tissue pillar into the lumen of the vessel.

Intussusceptive microvascular growth is a fast process that can take place within hours or even minutes, because it does not need proliferation of endothelial cells. In this process endothelial cells are remodelled by increasing in volume and becoming thinner. Intussusception is believed to take place after vasculogenesis or angiogenesis to expand the capillary plexus, in a short time and with a little amount of energy. Transmission electron microscopy revealed four consecutive steps [51]. First, the endothelial cells of opposite walls make a “kissing contact”, by which a transluminal bridge is formed. Secondly, a reorganisation of the interendothelial junctions and perforation of the endothelial bilayer is executed. In the third phase, the interstitial pillar is formed and pericytes and myofibroblasts invade and cover the newly formed interstitial wall. In this stage, transluminal pillars have a diameter of $\leq 2.5 \mu\text{m}$. It is hypothesised that pericytes, with their contractile characteristics, are the main stimulator in this phase. During the final phase, the pillars grow in diameter and the endothelial cells retract and two separated vessels are formed. Pillar formation and remodelling is not only observed in capillary plexuses but also within smaller arteries and veins [52].

In 1993, the first *in vivo* intussusceptive microvascular growth was demonstrated by video microscopy in a chick chorioallantoic membrane [53]. This process has now been detected in various organs, tissue repair processes and also in tumour angiogenesis. Tissue pillars were detected in a colon carcinoma xenograft model. At the growing edge both sprouting and intussusceptive angiogenesis were ob-

served, in the stabilised regions mostly intussusception was detected [54]. Patan et al. [54] also hypothesised that intravascular blood flow patterns or changes in shear stress are parameters that regulate pillar formation. In mammary tumours of c-neu transgenic mice, smaller tumour regions exhibited numerous sprouts, while in larger tumours regions frequently pillar- and mesh formations were observed. Very often, these two forms of angiogenesis were seen in parallel in the same nodule. There are some indications that absence of VEGF is important in the induction of intussusceptive angiogenesis in fast growing tumours [55]. Also in human melanomas a high number of intraluminal tissue folds and a correlation between VEGF and intussusceptive angiogenesis has been observed [56].

Although the mechanism of intussusception is not fully understood, there are several key players that could influence pillar formation. Alteration in blood flow dynamics in arterial branches could stimulate this process, as observed in the chick chorioallantoic membranes [52]. Furthermore, changes in shear stress on the endothelial cells, and in wall stress on the pericytes, can activate a biochemical cascade which might result in cytoskeletal rearrangements and adaptations of gap junction complexes [51]. The changes in shear stress can be sensed by the endothelial cells and transduced by molecules such as CD31, resulting in increased expression of angiogenic factors, adhesion molecules and endothelial nitric oxide synthase [52]. Although many cells appear to play a role in the process of intussusception, such as the endothelial cells, pericytes, macrophages and blood cells, it is now widely thought that it is mainly mediated by endothelial cell-endothelial cell and endothelial cell-pericyte interactions. Factors, that are known to be involved in these interactions in sprouting angiogenesis, such as the angiopoietins and their Tie-receptors, platelet derived growth factor-B, monocyte chemoattractant protein-1, ephrins and EphB-receptors, are candidates for the mediation of intussusceptive angiogenesis [51]. Injection of platelet derived growth factor-B in a developing chick chorioallantoic membrane stimulated the process of intussusception [57]. Transgenic mice that overexpress VEGF-A and angiopoietin-1 developed blood vessels that showed small holes in the capillary plexus, representing transluminal pillar formation [58].

It can be hypothesized that inhibition of sprouting angiogenesis may stimulate the process of intussusceptive angiogenesis. Therefore, it could be a means of drug-resistance against anti-angiogenic agents. The fact that intussusception only involves migration of endothelial cells and vascular remodelling but not cell proliferation, makes it unlikely that anti-proliferative agents will be able to prevent intussusception. In order to develop effective anti-angiogenesis strategies, novel compounds should involve anti-migration characteristics as well.

4 Endothelial progenitor cells

Until 1997, the growth of new blood vessels in adults was considered to exclusively occur through the mechanism of sprouting and intussusceptive angiogenesis. This paradigm of vascular development changed after the discovery of CD34-enriched subpopulation of mononuclear blood cells [59]. These cells were able to adapt *ex vivo* to an adherent cell type with an endothelial phenotype. They were named endothelial progenitor cells or angioblasts. It is now generally accepted that new vessels can also grow through the recruitment of endothelial progenitor cells (EPCs) that are circulating in the blood. EPCs express several endothelial specific markers like CD34, CD31, VEGFR2, Tie-2 [59] and CD14 [60]. The first *in vivo* observations of incorporation of EPCs in blood vessels were evident from different mouse and rabbit bone marrow transplantation models. In these models, with heterologous, homologous and autologous transplantation/incorporation of CD34+, CD133+, VEGFR2+ mononuclear blood cells, EPCs incorporated exclusively in blood vessels of neovascularised ischemic limbs [59]. Moreover, transplantation of endothelial progenitor cells improved limb perfusion, increased capillary density and reduced the risk of limb loss [60]. In another setting, Lin et al. [61] showed incorporation of cultured mononuclear cells in blood vessels after a sex-mismatched bone marrow transplantation.

The mobilization and recruitment of EPCs is promoted by several growth factors, chemokines and cytokines, which are produced during processes such as physiological stress (tissue ischemia), physical exercise and tumour growth. Mobilization of endothelial progenitor cells starts with the activation of matrix metalloproteinase-9, which in turn promotes the transformation of membrane-bound Kit ligand to a soluble form. Subsequently, early c-kit positive progenitor cells will detach from the bone marrow niche, move to the vascular zone of the bone marrow and will be released in the circulation [62]. Angiogenic factors like PLGF and VEGF, which bind to the highly expressed VEGFR2 on EPCs, stimulate the release of EPCs from the bone marrow [63, 64]. Other factors that can elevate the release of EPCs are stromal cell-derived factor-1, which binds to CXCR-4 on the EPCs, and angiopoietin-1 [65]. A key player in the activation of matrix metalloproteinase-9 by VEGF and stromal cell-derived factor-1, was found to be endothelial nitric oxide synthase [66]. Furthermore, factors like granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor have identified as bone marrow stem cell mobilizing factors [67].

The recruitment and integration of EPCs implicates a complex multistep process, including chemoattraction, active arrest and homing within angiogenic vasculature, trans-

migration to the interstitial space, incorporation into the microvasculature and differentiation into mature endothelial cells. P-selectin, E-selectin and integrins are considered to be important in the adhesion of EPCs to the vessel wall and in transendothelial migration [68, 69]. A recent paper demonstrated a functional role of high-mobility group box 1 (HMGB1) in the homing of EPCs. The HMGB1-induced migration of EPCs could be inhibited by antibodies against β_1 and β_2 integrins [70]. During diapedesis CD31 and CD99 mediate the passage of EPCs [71, 72]. The differentiation of EPCs to mature endothelial cells is mainly mediated by VEGF [59, 73]. After differentiation to a mature endothelial cell, EPCs lose their progenitor properties and start to express endothelial markers like VE-cadherin, von Willebrand factor and endothelial nitric oxide synthase [61].

EPCs also home in at the site of neovascularization in tumours. Asahara et al. [74] were the first to report the incorporation of β -galactosidase labelled progenitor cells in both tumour stroma and the endothelial layer of tumour blood vessels. These findings led to the hypothesis that EPCs not only incorporate into the vascular endothelium but also can secrete pro-angiogenic factors in the perivascular sites in the tumour stroma. Later on, the family of Id (inhibitor of DNA binding) proteins was shown to play an important role during incorporation of EPCs in tumour endothelium. Id 1/3 double-mutant mouse embryos had vascular malformations in the brain, leading to fatal haemorrhage [75]. Moreover, adult Id1^{+/-}/Id3^{-/-} mice could not support metastasis and growth of three different tumour cell lines, while transplantation of bone marrow cells of wild-type mice could restore this effect [76]. The contribution of EPCs to the actual vessel growth, however, is variable. In tumours there are reports of EPCs being the leading process in tumour angiogenesis, while others described a minimal contribution to tumour vasculature [76–82]. In studies with cancer patients similar mixed results were found. In breast carcinoma patients, a higher level of EPCs was detected in the peripheral blood and was suggested as a prognostic marker in tumour patients [83]. In contrast, the number of EPCs in the blood was not found to be increase in a patient group of 52 gastric cancer and 19 breast cancer patients in comparison to control patients [84]. These contradictory results on the contribution EPCs could be due to difference in methodology.

Although most clinical applications of EPCs are in the field of ischemic tissue recovery, inhibition of EPC mobilization from bone marrow has tremendous potential in cancer treatment. Some studies have demonstrated an impaired role of EPCs in angiogenesis after specific interventions. In Id mutant knock out mice with xenograft tumours impaired tumour growth was observed [75]. In a study by Capillo et al. [85], endostatin was described as a potent inhibitor of mobilization and clonogenic potential of

EPCs. Similarly, simultaneous inhibition of VEGFR2 and VEGFR1 demonstrated an effective inhibition of mobilization and incorporation of EPCs in tumour vasculature [76]. Another clinical application of EPCs is their use as a marker for validation of effectiveness of anti-angiogenic therapy. In 8 different mouse strains there was a striking correlation between bFGF- or VEGF-induced angiogenesis and the level of EPCs [86]. Alternatively, EPCs might be another source of tumour-homing cells to deliver toxins to the tumour. CD34⁺ cells that were transfected with a thymidine kinase gene showed a co-localisation with tumour vasculature. As expected, the recruitment of these transfected EPCs inhibited tumour growth [87]. However, the success of the use of EPCs in cancer treatment depends on the isolation of the proper CD34⁺, VEGFR2⁺ haematopoietic cells from the bone marrow or out of circulation. There is still controversy on the exact characterisation of EPCs and possible contamination of the EPC population with circulating endothelial cells [88]. Moreover, the exact molecular pathways that are involved in the mobilization and homing of EPCs to tumours, still have to be elucidated. Improvement of purification of these progenitor cells and study of their long-term effect to generate endothelial cells *in vivo* will clarify this embryonic field of cancer research. Nonetheless, it is obvious that the impact of EPCs in tumour vascularization cannot be neglected and the development of targeting strategies to prevent them from incorporating in regions of neovascularization in the tumour is a new challenge.

5 Vessel co-option

As stated above, it is generally accepted that growth of tumours and metastases start as an avascular mass and must induce the development of new vessels to grow beyond a few millimeters in size. However, it has been suggested that many tumours can grow in an avascular stage, mainly in well-vascularized tissue like brain and lung [89–91]. Tumour cells can grow along existing vessels without evoking an angiogenic response. This process was defined as vessel co-option.

The first evidence of this process was found during experiments for the search for the molecular players, like angiopoietins, that are involved in early angiogenic events [92]. After 1 or 2 week(s) after implantation of C6 glioma cells in a rat brain, the small tumours were already well vascularized with vessels that had characteristics of normal brain vessels. Moreover, no angiogenic response was observed. After 4 weeks, blood vessels had undergone a dramatic regression without any compensatory angiogenic response. In the center of the tumour, tumour cells were organised around the few functional vessels and massive

tumour cell death was detected. In the tumour periphery, in contrast, a robust angiogenic response was observed. These data showed that most malignancies and metastases originate as an avascular mass, co-opt with host vessels and are rescued. It can be hypothesized that the regression of the initial co-opted vessels is a host defence mechanism. Unfortunately these remaining tumour cells are rescued in a later stage, by robust angiogenesis at the outer rim of the tumour.

The finding that vessel regression was associated with the regression of endothelial cells, due to detachment of pericytes and smooth muscle cells, raised the hypothesis that angiopoietins could be involved in this process. Holasch et al. discovered high angiopoietin-2 expression in co-opted vessels of 2 weeks old tumours and in late-stage tumours with a necrotic core. The expression of VEGF, however, was rather low in early-stage tumours and increased later on. The expression of angiopoietin-1 did not change throughout tumour development. Angiopoietin-2 seems to be the key regulator in the regression of initially co-opted tumour vessels. While the expression of angiopoietin-2 in the absence of VEGF facilitated vessel regression, the co-expression of angiopoietin-2 and VEGF, induced the activity of VEGF and subsequently vessel sprouting. This operation between the two angiogenic factors is similarly present in developmental angiogenesis [19]. The same expression levels of angiopoietin-2 and angiopoietin-1 were found in human glioblastomas and not in normal brain vasculature [92]. Vessel co-option has now been observed in different tumour types like murine Lewis lung carcinoma, murine ovarian cancer, human melanoma and human Kaposi sarcoma [92–95]. The role of VEGF in vessel co-option suggests that anti-VEGF therapies may be considered not only for blocking angiogenesis but also to inhibit maturation of vessels in the process of vessel co-option. However, the systemic anti-angiogenesis treatment of a glioblastoma with an anti-VEGFR2 antibody was able to reduce tumour angiogenesis but led to an increased co-option of host vessels in the brain [96]. Thus, more potent VEGF-inhibitors are needed to prevent both angiogenesis and vessel co-option. Maybe targeting of VEGF, together with angiopoietins, could overcome the growth of tumours along existing vessels.

6 Vasculogenic mimicry

In 1999, the term “vasculogenic mimicry” was introduced to describe the masquerade of tumour cells as endothelial cells. This process of cell plasticity occurs mainly in aggressive tumours in which tumour cells dedifferentiate to an endothelial phenotype and make tube-like structures. This mechanism provides tumour cells with a secondary circulation system of vasculogenic structures lined by tu-

mour cells, independently of angiogenesis [97]. This phenomenon was described for the first time in melanomas. Tissue sections of uveal and cutaneous melanomas and their respective liver metastases revealed patterned networks of interconnected loops of extracellular matrix, as identified by periodic acid-Schiff's reagent (PAS) staining. Importantly, the presence of PAS patterns was associated with worse patient outcome [98]. Further research suggested that these PAS positive networks might be in close connection with regular blood vessels and can be detected with markers for endothelial cells. Furthermore, endothelial cells could not be identified, strongly suggesting that these vessel-like structures are lined by tumour cells. The same patterned networks could be obtained *in vitro* in collagen and matrigel three-dimensional cultures with aggressive melanoma cell lines but not with poorly invasive melanoma cell lines [97].

Microarray analysis comparing highly invasive and poorly invasive melanoma cells from the same patient indicated a genetic reversion of aggressive melanoma cells to an undifferentiated embryonic-like phenotype [99]. Endothelium associated genes such as VE-cadherin, Ephrin A2 and tissue factor pathway inhibitors, CD34, tyrosine kinase receptor 1, neuropilin 1, E-selectin and endoglin (CD105) had a more than 2-fold increased expression in vasculogenic mimicry positive cells. Also several matrix related components had an increased expression such as laminin 5 γ 2, fibronectin, collagen IV α 2, collagen I. Genes related to a melanocytic phenotype, like Melan-A, microphthalmia-associated transcription factor (MTIF) and tyrosinase, were more than 20-fold downregulated.

The exact mechanism underlying vasculogenic mimicry still needs to be unravelled. Several molecules have been identified to have a functional role. For example, PI3 kinase (PI3K) was proposed as the key player in activating the transmembrane metalloproteinase MT1MMP [99]. This protease activates matrix metalloproteinase-2 that cleaves laminin 5 γ 2 into pro-migratory fragments used for tumour cell migration in vasculogenic mimicry [100]. There is also a role for VE-cadherin and Ephrin A2 since downregulation of these genes in melanoma cells resulted in an abrogation of their ability to form vasculogenic-like structures [101]. Both molecules are found to co-localize and VE-cadherin can regulate the expression of EphA2 through its receptor ephrin-A1. So far, several other molecules, mostly found by means of siRNA techniques or anti-body blocking techniques, have been described to play a role in vasculogenic mimicry. Tissue factor pathway inhibitor 2 (TFPI-2) was discovered to be necessary for vasculogenic network formation and is involved in the activation MMP-2. Furthermore, several recent papers reported on different molecules like focal adhesion kinase, cyclooxygenase-2, bone morphogenetic protein-4, insuline-like growth factor binding

protein 3 and Nodal and their role in promoting an aggressive melanoma phenotype [102–106].

Next to the above described mediators, genetic characterisation of cell plasticity of tumour cells revealed several molecules that are related to extracellular matrix like fibronectin, collagen IV α 2, collagen I. The importance of the extracellular matrix, as a component of the microenvironment, in vasculogenic mimicry was demonstrated by Seftor et al. [107]. Normal epidermal melanocytes, exposed for 4 days to an extracellular matrix conditioned by metastatic cutaneous melanoma, were reprogrammed to a genotype with specific genes that were associated with the ability to form vasculogenic-like networks. Importantly, these changes in gene expression were only transient, because gene analysis after 7 to 21 days revealed a normal melanocyte phenotype. Recent findings suggested that another microenvironmental component, oxygen, may be essential in melanocyte transformation. Low levels of oxygen or hypoxia, are known to promote melanoma cell invasion, metastasis and transformation [108, 109]. Moreover, hypoxia induces vasculogenic mimicry tube formation *in vitro* in a matrigel assay [110, 111]. In another paper, a B16 melanoma ischemic limb mouse model was used to mimic an hypoxic environment. Initially a decreased tumour growth was observed while later on there was no difference in size with the control tumours. However, the amount of vasculogenic mimicry channels and the gene expression of HIF-1 α , MMP-2, MMP-9 and VEGF was increased [112]. The role of several known tumour growth factors has also been studied, though with disappointing results. Several growth factors, such as basic fibroblast growth factor, vascular endothelial growth factor, transforming Growth Factor- β , platelet derived growth factor and tumour necrosis factor- α were found not to be able to induce formation of vascular networks when added to the poorly invasive melanoma cell lines [97]. This indicates that angiogenesis and vasculogenic mimicry, in contrast to the previous described tumour vascularization types, are not sharing the same signalling pathways. Moreover, anti-angiogenic targeting strategies do not inhibit the process of vasculogenic mimicry [111] and could even induce the formation of vasculogenic mimicry vessels as an escape mechanism of the tumour to keep on growing.

Although the functionality and the contribution of vasculogenic-like channels to circulation was criticised at first, several papers evidenced its functional role in tumour circulation. The contribution of vasculogenic mimicry patterns was first proven *in vitro*. Looping patterns, that were formed *in vitro* by highly aggressive melanoma cell lines, distributed fluid after microinjection [97]. Several groups tried to prove the fluid-conducting characteristic of vasculogenic mimicry channels *in vivo*. Clarijs et al. co-localised an intravenous injected tracer with both blood vessels and

matrix patterns in a uveal melanoma xenograft model [113]. Shirakawa et al. [114] reported on blood flow in areas of vasculogenic mimicry in a breast carcinoma model using MRI techniques. Another approach was used by Ruf et al. [115], where Doppler ultrasonography was used to show blood flow in these vasculogenic-like channels. The first *in vivo* demonstration of blood circulation in vasculogenic mimicry tubes in humans was observed with laser scanning confocal angiography in patients with a choroidal melanoma [116]. Up to now, tumour cell plasticity has been described in uveal [98], cutaneous [117] and oral [118] melanoma, breast carcinoma [114], prostatic carcinoma [119], ovarian carcinoma [120], hepatocellular carcinoma [121], bladder carcinoma [122], rhabdomyosarcoma and mesothelial sarcoma [123], osteosarcoma [124], astrocytoma [125], pheochromocytoma [126] and Ewing sarcoma [111].

The recent findings on the ‘plastic’ endothelial-like phenotype of melanoma and other tumour cells confused the field of cancer biology even more. The idea that these structures could form a functional secondary vascular network that provides the tumour of blood, independent from angiogenic growth factors, makes tumour growth inhibition even more complex. A variety of genes has been investigated concerning their role in tubular network formation of tumour cells. An option for therapy is the use of monoclonal antibodies to these molecules for drug targeting. However, the therapeutic functionality and the choice of the best targets still need to be elucidated. It is evident now that the microenvironment plays an important role in tumour progression and therefore is a novel target for therapy. An initial study to target MMPs was performed. The administration of a chemically modified tetracycline, COL-3, to aggressive melanoma cells in three-dimensional culture, inhibited MMP-2, MMP-9, MT1-MMP and VE-cadherin expression. Next to that, the cleavage of laminin 5 was inhibited and decreased vascular network formation was observed [127]. However, caution is warranted since administration of modified tetracyclines have reported serious side effects [128, 129]. In another paper, the addition of anti-angiogenic compounds TNP470, anginex and endostatin could not block the formation of networks [130]. Until now, only very limited data on targeting vasculogenic mimicry is available. Clearly, more investigation, on essential regulatory pathways of plastic tumour cells that do not overlap normal biological processes, is needed to develop new promising therapeutic approaches.

7 Lymphangiogenesis

Lymphatic vessels are also part of the vascular circulatory system. The lymphatic system is a network of capillaries, collecting vessels and ducts that drains most of the organs.

In contrast to the blood vascular network, the lymphatic network is an open ended, one way transport system, without a driving force, that drains extravasated fluid, collects lymphocytes and returns it to circulation [131]. Over the last years there is accumulating evidence for a role of the lymphatic system in tumour progression. Metastasis of malignant tumours to regional lymph nodes is one of the early signs of cancer spread in patients. In certain cancer types, such as breast cancer, lymphatic metastasis is one of the predominant routes of cancer spread [132]. From the lymphatic system, cancer cells can spread to other organs and tissues.

The lymphatic system has not received as much scientific attention as the blood vascular system, maybe due to a lack of specific markers and to the lack of knowledge about the molecular regulation of its development and function. The possibility and optimisation to isolate and culture lymphatic endothelial cells, however, has led to the identification of several markers that are specific for the lymphatic vasculature [133]. Vascular endothelial growth factor receptor-3 (VEGFR-3) was the first lymphatic marker that was identified [134]. Later on specific markers such as lymphatic vascular endothelial hyaluronan receptor-1 (LYVE-1) [135], podoplanin [136] and transcription factor Prox1 [137] were identified.

Similar to blood endothelial cells, lymphatic endothelial cells are quiescent under physiological conditions. Experimental evidence for a ‘lymphangiogenic switch’ is still lacking. Nonetheless, it seems likely that the formation of new lymphatic vessels is triggered in a similar way as angiogenesis of blood vessels. Already now, a range of lymphangiogenic factors/receptors that are produced by tumour cells and inflammatory cells have been identified.

After the identification of the lymphatic specific marker VEGFR-3, both VEGF-C and VEGF-D were cloned as unique ligands for this receptor [138]. In the development of the lymphatic system, the role of VEGF-D is dispensable [139], whereas VEGF-C null mouse embryos completely lack a lymphatic vasculature and die prenatally [140]. *In vitro*, VEGF-C stimulated proliferation, migration and survival of lymphatic endothelial cells [141]. To demonstrate the VEGF-C/VEGF-D/VEGFR-3 signalling pathway in tumour lymphangiogenesis, tumour cells expressing VEGF-C and -D were used in a mouse tumour model. Both the expression of VEGF-C and -D increased intratumoural lymphangiogenesis and metastasis. In addition, a blocking VEGF-D antibody could inhibit this lymphatic spread [142, 143]. Furthermore, there are indications that there is a cross talk between blood vessel angiogenesis and lymphangiogenesis. Angiogenic mediators are identified to play a role in lymphangiogenesis but their role is mostly studied in physiological situations. The VEGF-A/VEGFR-2 signalling pathway stimulates lymphangiogenesis. However, the new

lymphatic vessels generated by VEGF-A are functionally and structurally abnormal [144]. The group of Chang et al. [145] demonstrated that bFGF could induce both blood vessel angiogenesis and lymphangiogenesis and even lymphangiogenesis alone depending on the dose of bFGF that was administered on mouse cornea. In the same mouse cornea model, PDGF-BB was found to be the most potent of the PDGF family in stimulating lymphangiogenesis [146]. Above that, PDGFs are often found to be highly expressed in tumours that have increased incidence of lymphatic metastasis [147]. The first evidence of a role of angiopoietin-2 in lymphangiogenesis was suggested by the angiopoietin-2-null mice that displayed disorganized and hypoplastic lymphatic capillaries [148]. Importantly, the lymphatic but not the blood vessel phenotype could be rescued by genetic transfer of angiopoietin-1. In addition, Morisada et al. [149] were able to demonstrate the stimulation of both *in vitro* growth of lymphatic endothelial cells and lymphangiogenesis in the mouse cornea by angiopoietin-1. Similarly to angiopoietin-2-null mice, NRP-2 mutants showed absence or severe reduction of small lymphatic vessels and capillaries [150]. Also an *in vitro* and *in vivo* stimulatory role of hepatocyte growth factor [151] and insulin-like growth factor-1 and -2 [152] on the lymphatic vessel formation was observed.

Now that specific markers are available and some insight into the biology of lymphangiogenesis is available, it becomes evident that lymphangiogenesis is an important parameter in the process of tumour growth [153]. Nevertheless, there is still an ongoing debate on the role of lymphangiogenesis in tumour progression. It was previously thought that lymphatic metastasis occurred by preexisting lymphatic vessels that are present at the outer rim of the tumour. However, other papers report on the presence of peritumoural and/or intratumoural lymphatics, not only in mouse studies but also in human tumours. Nevertheless, intratumoural lymphatics are rare and their functionality and role in tumour metastasis is still discussed [154, 155]. There are also reports that lymph angiogenesis parameters such as lymph vessel density, lymph angiogenic growth factors [156], or the presence of tumour cells within lymph vessels or lymph nodes are valuable prognostic markers [157–161].

The high incidence of metastatic lymphatic spread and the knowledge of several lymphangiogenic markers urged researchers to investigate the inhibition of lymphangiogenesis as a strategy of tumour treatment. Stacker et al. [143] reported the reduction of lymphatic spread by blocking VEGF-D with a monoclonal antibody. The application of a VEGFR-3 fusion protein (called VEGF-C/D trap) was able to inhibit the growth of tumour-associated lymphatic vessels and inhibited tumour metastasis [162]. On the other hand, administration of VEGF-C seems to have therapeutic

potential for patients with lymphedema since lymphatic function ameliorated significantly [163]. However, the regulation of lymphatic vessel growth is more difficult because it is not only promoted by the VEGF-C, VEGF-D/VEGFR-3 system. Several other growth factors and molecules that are specific for lymphangiogenesis, of which the exact function has not been resolved yet, could play an important role. An efficient anti-lymphangiogenic therapy should target different lymphatic growth factors. Furthermore, additional information is needed on specific tumour lymphatic markers. A recent paper of Zhang et al. presented some promising results. In search for a lymphatic tissue specific signature, it was demonstrated that tumour development is associated with organ- and stage-specific changes in lymphatics [164]. Although clinical implementation will take years, cancer patients will benefit from anti-metastatic therapy that can decrease metastatic lymphatic spread.

8 Conclusion

Tumours depend on the growth of a vascular network, which is stimulated by a variety of angiogenic mediators, providing them with blood and oxygen. Inhibition of sprouting angiogenesis has gained a lot of progression. Several clinical trials, in which specific growth factors or receptors are being blocked, are currently being performed. Strategies that block the VEGF-A/VEGFR2 signalling are the most abundant ones in the field of anti-angiogenic therapy. After successful clinical trials, Avastin is now entering into the clinic. Because side effects are observed, the emphasis of such growth factor inhibition mediated treatment may shift towards other growth factors, e.g. PLGF [17], or to simultaneous targeting of multiple pathways.

Clinical success of anti-angiogenesis therapy is present but still limited. Since anti-angiogenic therapy alone seems not to be sufficient to improve patient survival, clinical studies are all in combination with conventional strategies, such as chemo- and radiotherapy. The successful combination of chemotherapy and anti-angiogenesis therapy may benefit from the normalization of the tumour vasculature by anti-angiogenic therapy and subsequently a better administration of chemotherapy [165].

It is clear now that tumour vasculature is not necessarily dependent of endothelial cell proliferation and sprouting of new capillaries. Several additional mechanisms can provide the tumour of oxygen and nutrients. The molecular players involved and their specific role in tumour development still need to be elucidated. The current knowledge that anti-angiogenesis therapy work best in combination with chemotherapy, should probably in the near future be extended to other types of vascularization as well. There is still a long way to go before we fully understand the different mech-

anisms of tumor vascularization. But we anticipate that combination of a multimodal anti-vascular approach, representing anti-angiogenesis, anti-lymphangiogenesis and vasculogenic mimicry targeting, together with chemotherapy may become the best possible strategy in the fight against cancer.

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