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REVIEW

Microvascular endothelial cell heterogeneity: general concepts and pharmacological consequences for anti-angiogenic therapy of cancer

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Abstract Microvascular endothelial cells display a large degree of heterogeneity in function depending on their location in the vascular tree. The existence of organ-specific, microvascular-bed-specific, and even intravascular variations in endothelial cell gene expression emphasizes their high cell-to-cell variability, which is furthermore extremely adaptable to altering conditions. The ability of microvascular endothelial cells to respond dynamically to pathology-related microenvironmental changes is particularly apparent in tumor-growth-associated angiogenesis. An understanding of how they behave, how their behavior varies between and within tumors, and how their behavior is related to responsiveness to drugs is critical for the development of effective anti-angiogenic treatment strategies. In this review, we introduce some general issues concerning organ-imprinted microvascular heterogeneity and highlight the importance of studying microvascular endothelial cell behavior in an in vivo context. This is followed by an overview of state-of-the-art knowledge regarding the nature of the variation in microenvironmental conditions in pre-clinical and clinical tumors and their consequences for tumor endothelial behavior. We provide recent insights into the in vivo molecular activation status of the endothelium and, finally, outline our current understanding of the way that anti-angiogenic drugs affect tumor endothelial cells in relation to their anti-tumor effects.

Keywords Microvascular endothelial cells · Heterogeneity · Pharmacology · Cancer · Tumor angiogenesis

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Introduction

Endothelial cells line the interior surface of all blood vessels in the body, from the largest conduit vessels to the smaller resistance vessels and the capillaries in the organs. The microvasculature in the major organs exerts functions specific for each organ. For example, the microvasculature in the brain is an integral part of the tight blood-brain-barrier, whereas in the liver, the sinusoidal endothelial cells engage in the efficient clearing of numerous molecular entities from the body. In the kidneys, the glomerular endothelium acts as a semi-permeable membrane for the filtration of blood-borne components, and the descending and ascending vasa recta or peritubular capillaries engage in the re-absorption and excretion of components into, respectively from the blood circulation (Aird 2007). Furthermore, the smallest blood vessels of the body especially engage in disease-related processes such as the new formation of blood vessels that accompanies wound healing, tissue repair, and solid tumor growth (see below) and leukocyte recruitment during an inflammatory insult (Pober and Sessa 2007).

The microvasculature consists of endothelial cells and scarce support cells; hence, microvascular involvement in health and disease is strongly controlled by the behavior of the endothelial cells. At present, we are rather ignorant with regard to the molecular definition of the heterogeneity that

underlies organ-specific microvascular endothelial function and engagement in disease. Beyond any doubt, this functional heterogeneity is guided by variations in the biochemical and biomechanical properties of the local environment. For a pharmacologist, an understanding of the molecular control of microvascular endothelial cell function in normal and pathological conditions is of essential importance to be able to interfere successfully with a disease without affecting normal vasculature.

In the current review, we will briefly introduce some general considerations regarding microvascular endothelial heterogeneity in adult organs. From there on, we will focus on tumor-growth-related angiogenesis and review the state-of-the-art knowledge hitherto generated with respect to microenvironmental heterogeneity between and within tumors. We provide a concise, though not absolute, inventory of what is known about the responses of tumor endothelial cells to local tumor and host conditions and about our current understanding of the way that anti-angiogenic drugs affect endothelial cells. The focus will be on anti-vascular endothelial growth factor (VEGF) therapeutics as these have been most extensively studied in preclinical and clinical settings. In the general conclusion, we will briefly touch on a few issues that have not been addressed here in detail, because of space limitations, but that should be taken into account in our quest to therapeutically address cells that, despite being discovered almost 400 years ago, remain elusive, even today.

Heterogeneity of endothelial cells

The mesoderm is the exclusive source of endothelial cell precursors during embryogenesis. The close co-localization of endothelial and hematopoietic precursor cells within the embryo and the finding that these cells both bear universal molecular markers have given rise to the concept that both lineages arise from the hemangioblast as a common precursor (Patterson 2007). In the adult body, endothelial cells in quiescent vasculature are proliferative inactive, with a life-span of >100 days in the main organs, as reported more than two decades ago by Denekamp and colleagues (Hobson and Denekamp 1984). Pro-inflammatory and pro-angiogenic activation as a consequence of physiological stimuli or trauma activates the endothelium. Upon resolution of the inciting stimuli, the cells tend to regain a quiescent phenotype, among others via the expression of protective genes A20 and A1 (Ferran 2006), phosphatases, and other molecular inhibitors of pro-inflammatory signal transduction (Winsauer and de Martin 2007). Under certain conditions, including both small disturbances and larger insults, the induction of endothelial cell death takes place, as has been observed, for example, in antineutrophil cytoplasmic antibody-associated vasculitis (de Groot et al. 2007), in renal ischemia (Horbelt et al. 2007), and in solid tumor growth (de Jong et al. 2006). The potential role of circulating CD34+ hematopoietic and endothelial precursor cells in microvascular repair (de Groot et al. 2007) suggests the intriguing possibility that microvascular endothelial replacement can take place in the absence of endothelial proliferation. Hence, the life span of the endothelial compartment may be significantly shorter than previously estimated, with concurrent variability in the general status of the endothelial cells.

For many pre-clinical and clinical applications, the availability of molecular antigens to identify the endothelial cells in a tissue is of great importance. A large variety of endothelial marker genes have been proposed for this purpose (Table 1), a few of them being truly endothelial-specific, whereas the majority can be categorized as endothelial-restricted. In addition to the existence of species differences in the expression patterns of these markers, microvascular subset-restricted expression, organ-dependent microvascular bed-restricted expression, and patchy marker gene expression indicative of cell-to-cell variability within a microvascular bed have been reported (Muller et al. 2002; Samulowitz et al. 2002; Pusztaszeri et al. 2006). The highly heterogenic presentation of endothelial cells throughout the body invites one to pose the (rhetorical) question of what makes an endothelial cell an endothelial cell? It should not only look like an endothelial cell, but should also behave like an endothelial cell and communicate like an endothelial cell. *All* endothelial cells have the common characteristic that they line the vessels of the blood circulatory system that range from centimeters in diameter in the aorta to a few micrometers in diameter in the smallest capillaries in the organs. They are all thus in direct contact with the blood and all exert pronounced anti-coagulant activity via, among others, the expression of tissue factor pathway inhibitors, heparan sulphate proteoglycans that interfere with thrombin-controlled coagulation, and thrombomodulin. By this means, whole body homeostasis and hemostasis is secured, until (patho)physiological stimuli disrupt the status quo.

We recently investigated the expression patterns of a number of well-accepted endothelial marker genes, viz., CD31, vascular endothelial (VE)-cadherin, plasmalemmal vesicle (PV)-1, Endomucin, and von Willebrand factor (vWF), and the cell adhesion molecules E-selectin, vascular cell adhesion molecule (VCAM)-1, and intercellular adhesion molecule (ICAM)-1 in the five main organs of 10-week-old C57Bl/6 mice. Using immunohistochemistry, we showed that, even with this small number of molecular entities, a remarkable heterogenic endothelial signature became visible (Fig. 1; J. Kułdo and G. Molema, unpublished). This supports the idea that, although all

Table 1 Endothelial cell (*EC*)-restricted genes used to identify microvasculature in tissues. Expression patters of some of the markers in human tissues may be found in the Human Protein Atlas (at http://www.proteinatlas.org/index.php)

Gene	Proposed ligand; expression pattern	Reference
General endothelial expression[a]		
Angiotensin converting enzyme (ACE)	Angiotensin; lung capillary EC and EC of larger arteries and arterioles	Stevens 2007
αvβ3	RGD-containing ligands; extra-alveolar and alveaolar capillary EC, mild expression in hepatic portal vein	Singh et al. 2000
CD31	CD31 on EC, leukocytes; glycosaminoglycans; pan-endothelial marker	Pusztaszeri et al. 2006; Feng et al. 2004
CD34	L-selectin	Pusztaszeri et al. 2006
CD141 (thrombomodulin)	Thrombin; pan-endothelial marker	Boffa et al. 1987
CD144 (VE-cadherin)	CD144 homotypic interaction; pan-endothelial marker	Prandini et al. 2005
Endomucin	Unknown ligand	Samulowitz et al. 2002
CD105 (endoglin)	Transforming growth factor-β1 (TGF-β1) and –β3 in association with TGF-β receptor type II	Fonsatti et al. 2001
Endothelin-1 (ET-1)	ET receptors ET_AR and ET_BR	Nelson et al. 2003
Ephrin B2	EphB4; preferred, not selective expression on arterial EC	Gale et al. 2001
EphB4	Ephrin B2; preferred expression on venule EC	Taylor et al. 2007
Fli-1	Ligand unknown	Pusztaszeri et al. 2006
Plasmalemmal vesicle-1 (PV-1)	Ligand unknown; expressed in stomatal and fenestral diaphragms of a subset of EC in the smaller capillaries of some organs	Stan 2007
Tie-2	Angiopoietins	Wong et al. 1997
Vascular adhesion protein (VAP)-1	Unknown ligand; expressed on EC in a subset of blood vessels	Salmi et al. 1993
Vascular endothelial growth factor receptor 2 (VEGFR-2)	VEGF; expressed on the majority of EC	Jakeman et al. 1992
von Willebrand Factor (vWF)/ factor-VIII-related antigen	Factor VIII	Pusztaszeri et al. 2006
Disease-induced endothelial expression		
αvβ3 integrin	RGD sequence containing (poly)peptides	Schnell et al. 2008
CD54 (ICAM-1)	LFA-1 integrin	van Meurs et al. 2008
CD62P (P-selectin)	Carbohydrate determinants on selectin ligands, e.g., PSGL-1	Carvalho-Tavares et al. 2000
CD62E (E-selectin)	Sialyl-Lewis-X antigen and other carbohydrates	Asgeirsdottir et al. 2007
CD105 (endoglin)	TGF-β1 and –β3 in association with TGF-β receptor type II; overexpressed on angiogenic EC	Fonsatti et al. 2001
CD106 (VCAM-1)	VLA-4 integrin	Inoue et al. 2006
Endothelin $receptor_B$ (ET_BR)	ET-1	Buckanovich et al. 2008
PV-1	Ligand unknown; upregulated in brain tumors	Carson-Walter et al. 2005
R-AGE (receptor for advanced glycation end products)	AGEs	Soulis et al. 1997
Tie-2	Angiopoietins	Fathers et al. 2005
VAP-1	Leukocyte extravasation support	Jalkanen and Salmi 2008
VEGFR-2	VEGF	Brown et al. 1997

[a] References in this section refer to published work in which in vivo microvascular or organ-specific heterogeneity in the expression of the gene was specifically addressed. For the majority of markers summarized, publications regarding their original identification can be found in Garlanda and Dejana (1997)

endothelial cells have a number of characteristics in common, they are under the control, in each microvascular bed, of a combination of genes unique for that specific vascular bed (Aird 2006). From this, one has to conclude that *the* endothelial cell does not exist, and that each cell needs to be appreciated with regard to its own identity and functionality in relation to its location in the vascular tree.

Flexibility of endothelial cells to adapt to local conditions

The behavior of endothelial cells in the (micro)vasculature is intricately controlled by the microenvironment. Biological factors including extracellular matrix (ECM) components and locally produced growth factors, interactions

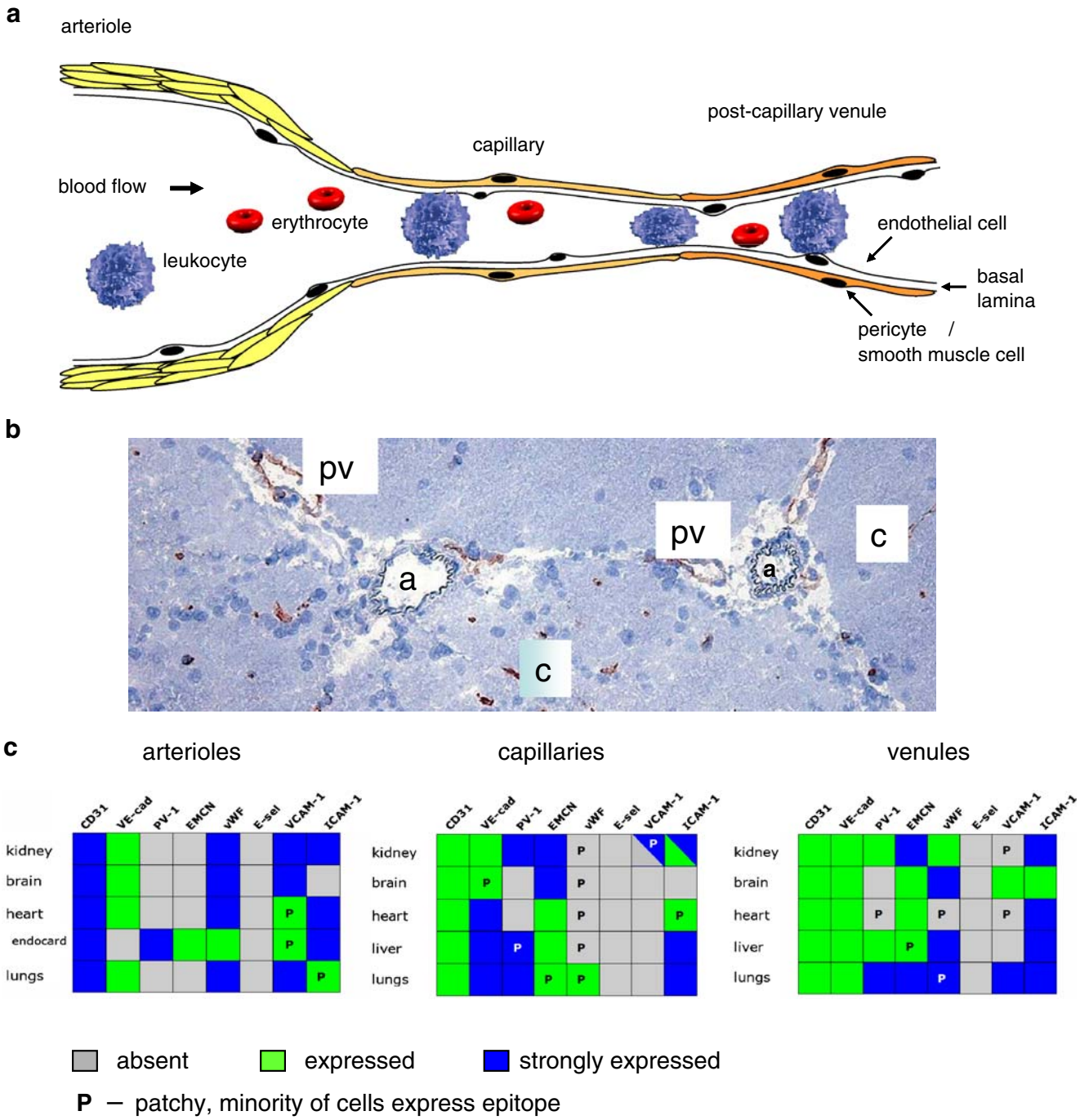

Fig. 1 Microvascular heterogeneity in perspective. **a** Representation of the cellular make-up and dimensions of the microvasculature in relation to the size of blood-borne cells. Whereas the last feeding arterioles are covered by a few layers of smooth muscle cells, capillaries and the first segment of the postcapillary venules are only covered by sparse pericytes. During an inflammatory insult, leukocytes mainly transmigrate from the blood into the tissue in the capillary and the postcapillary segments of the microvasculature. Angiogenesis is thought to take place mainly in the first segments of the postcapillary venules. For clarity, the collagen layer surrounding the arterioles (visible in **b**) was left out. **b** Immunohistochemical detection of Endomucin in mouse brain showing a clear demarcation between Endomucin-negative arterioles (*a*) and Endomucin-positive capillaries (*c*) and postcapillary venules (*pv*). Note that, in the postcapillary venules, Endomucin expression is not equally distributed among endothelial cells, thereby representing an additional level of endothelial heterogeneity. **c** Summary of expression of endothelial marker genes and adhesion molecules in different microvascular segments in 10-week-old C57Bl/6 mouse organs as assessed by immunohistochemistry (*endocard* endocardium). The kidneys have two capillary segments with specific functions, i.e., the glomerular microvasculature and the peritubular or vasa recta microvasculature. Although some peritubular endothelial cells expressed VCAM-1 under normal conditions, glomerular expression is not detectable. ICAM-1 is strongly expressed by all peritubular endothelial cells and to a lesser extent in the glomeruli (unpublished)

with neighboring cells, leukocytes, erythrocytes, platelets, and other constituents of the blood, and mechanical forces all influence general cell performance. The readiness of these cells to adapt to local changes was elegantly demonstrated in a mouse model for inter-positioning a venous segment into the arterial circulation, as occurs during coronary artery bypass surgery in the clinic. Upon connecting the external jugular vein to the common carotid artery, the endothelial cells covering the venular wall phenotypically shifted toward arterial endothelial behavior. This was accompanied by an increase in smooth muscle cell layers and microvessel ingrowth in the outer vascular wall, and the loss of vascular permeability function (Kwei et al. 2004).

Almost similarly effortless is their adaptation to in vitro culture conditions. Many primary endothelial cell cultures and cell lines have been phenotyped for gene expression under normal culture conditions (Muller et al. 2002; Mutin et al. 1997; Chi et al. 2003). However, their in vitro behavior is not a perfect reflection of the in vivo situation. For example, glomerular endothelial cells gain expression of vWF upon culturing (Satchell et al. 2006), whereas vWF is almost absent in this part of the microvascular tree in vivo (Pusztaszeri et al. 2006). Recently, Liu et al. (2008) demonstrated that in situ human umbilical artery and human umbilical vein endothelial cells (HUVEC) exerted dramatic differences in tumor necrosis factor (TNF)α-mediated E-selectin expression capacity. In situ in the umbilical cord, transcription factor and p300 coactivator recruitment to the enhancer sequence in the E-selectin promoter was reduced in the artery endothelial cells compared with vein endothelial cells. Within 72 h of in vitro culture, however, the artery and vein endothelial cells became almost indistinguishable in this molecular control of their response to TNFα (Liu et al. 2008). *Vice versa*, Bcl-2-transduced HUVEC can become an integrated part of arterioles, capillaries, and venules in vivo with accompanying vascular subset-specific responses to TNFα. This implies that endothelial cell behavior is not based on cell fate decisions but a context-driven phenomenon (Enis et al. 2005).

Tumor angiogenesis is an exquisite circumstance in which microvascular endothelial cells can demonstrate the full potential of their adaptability to continuously changing conditions. In the remainder of this review, we will describe the nature of these conditions and how they depend on a large variety of factors, both in preclinical animal models and, where possible, in patient material. We will discuss the consequences of changing tumor conditions for endothelial behavior, and the pharmacological challenges associated with both tumor endothelial heterogeneity and tumor endothelial adaptability.

Tumor endothelial heterogeneity and its consequences for anti-angiogenic therapy

Angiogenesis is one of the main processes by means of which a tumor creates its own oxygen and nutrient supply and a route for systemic metastasis (Folkman 1971). It is tightly regulated and starts with the activation of (post-capillary) endothelial cells in pre-existing blood vessels, followed by the induction of vasodilation and an increase in endothelial cell permeability. ECM-degrading proteinases next degrade the endothelial basement membrane to allow the proliferating endothelial cells to penetrate into the tumor mass. The proliferation and migration of the endothelial cells result in the formation of endothelial tube structures. The newly formed vasculature matures upon interaction with ECM and mesenchymal cells, with mural cells (or pericytes) being recruited to form a surrounding support layer. Once new vessels have assembled, the endothelial cells become quiescent, and the vessels turn resistant to, for example, VEGF withdrawal (Conway et al. 2001; Auguste et al. 2005; Benjamin et al. 1999). The different angiogenic stages of the vasculature, from newly formed pre-mature sprout to fully stabilized mature new blood vessel, are precisely regulated by microenvironmental balances of pro- and anti-angiogenic molecules (Griffioen and Molema 2000).

The mechanistic repertoire that tumor cells use to regulate new vessel formation is diverse and may alter for a given tumor type or host environment. In addition to angiogenesis, other mechanisms have been recognized to contribute to tumor vascularization. These include recruitment of angioblasts, co-option of pre-existing blood vessels, and vasculogenic mimicry, the presence of blood-filled channels being lined by tumor cells rather than endothelial cells (Auguste et al. 2005; Hillen and Griffioen 2007). These different mechanisms may exist at the same time in the same tumor or may be selectively active in a specific tumor type or host environment (Auguste et al. 2005). For instance, uveal melanoma establishes its vasculature partially through vascular mimicry in the eye (Maniotis et al. 1999) but through both vasculogenic mimicry and sprouting angiogenesis when implanted subcutaneously (s.c.; Hendrix et al. 2003). Although the occurrence of vasculogenic mimicry in these uveal melanomas has been questioned by McDonald and Foss (McDonald and Foss 2000), who have shown clear endothelial lining of the blood vessels, this example nevertheless clearly illustrates the variety in mechanisms employed by tumors to acquire their vasculature.

Tumor blood vessels are often abnormal, being characterized by increased permeability, tortuosity, excessive random branching, and intratumoral variations in vascular lumen size. Many tumor vessels have abluminal endothelial sprouts that

penetrate deep into the perivascular tumor tissue and show aberrant patterns of pericyte investment, with pericytes loosely attached to the vessel wall and extending away from the vessel surface. Furthermore, they lack the defining structural features of arterioles, capillaries, or venules (Pasqualini et al. 2002; McDonald and Foss 2000; Abramsson et al. 2002; Morikawa et al. 2002). Heterogeneous vascular morphology has been described in various tumor types, in tumors from the same origin growing in different host environment, and in different stages of tumor progression, and even zonal vascular heterogeneity within one tumor stage has been observed. In addition to differences in vascular morphology, the endothelial fenestration pattern, pericyte association, and gene expression profile of the respective vasculatures are often variable, as will be discussed below.

A landmark study of the therapeutic efficacy of anti-angiogenic drugs with various molecular targets and administered at different stages of tumor outgrowth published by Bergers et al. in 1999 demonstrated that the anti-tumor efficacy of the angiogenesis inhibitors was tumor-stage-specific (Fig. 2). Whereas some inhibitors were more effective in reducing tumor growth when administered at the early stage of tumor progression, others showed better anti-tumor activity in late-stage disease. Until now, neither the underlying molecular mechanisms of (lack of) responses of tumor and tumor endothelial cells to the different anti-angiogenic therapies nor their relationships to anti-tumor activity have been elucidated. Moreover, in many animal and human tumors, these issues have been poorly addressed. As they are critical for the successful development of anti-angiogenic drugs, we will provide an overview on what is currently known about the variation in microenvironmental conditions that exist in tumors and its consequences for tumor endothelial behavior and anti-angiogenic drug effects.

Variations in tumor microenvironment that affect the angiogenic status of a tumor

As briefly referred to above, microvascular heterogeneity exists at different levels. This heterogeneity is brought about by variations in tumor cell dependency on the blood supply, the host environment in which the tumor grows, the tumor growth stage, and ill-defined local spatiotemporal differences in angiogenic gene expression by tumor, stromal, and infiltrating immune cells (Fig. 3).

Not all tumor cells depend in a similar way on the blood supply

As has been known for many decades, tumor cells within a tumor are highly heterogeneous with regard to genotypic and phenotypic characteristics such as proliferation rate, survival mechanisms, and capabilities to form metastases (Fidler and Hart 1982; Heppner 1984). Furthermore, the degree to which individual tumor cells rely on the blood supply varies, resulting in the presence of subpopulations of tumor cells with different angiogenic activities within one tumor (Fig. 3a). Isolation and subsequent subcutaneous or intradermal inoculation of these subpopulations into mice gave rise to tumors with different microvascular densities (MVD), apoptotic rates, and growth characteristics (Achilles et al. 2001; Yu et al. 2001). Tumors resulting from injection of cell populations originally located distal from the tumor vasculature had enhanced tumor growth rates and a lower number of vWF-positive blood vessels, whereas cell populations proximal to the vasculature gave rise to less aggressive tumors with higher numbers of vWF-positive blood vessels (Yu et al. 2001). Similarly, high-grade human renal cell carcinomas were associated with a different angiogenic pattern than low-grade tumors, with greater endothelial cell proliferation, larger and more immature vessels, but a lower MVD (Baldewijns et al. 2007). Thus, variation in the dependency of tumor cell subpopulations on their blood supply partly causes the variability in structure and density of the vasculature that is acquired by the different tumors.

Tumor vascular behavior depends on the host environment

Both in mice and man, neovasculature in identical tumor types can be drastically different with regard to vascular architecture, MVD, permeability, and gene expression when the tumors are grown in different locations in the body. The vasculature of tumors tends to acquire characteristics similar to those of the host environment. For example, the microvasculature of murine mammary carcinoma, rhabdomyosarcoma, and human glioblastoma implanted s.c. in nude mice became extensively fenestrated, with a large population of caveolae and a relatively high permeability, similar to the host endothelium in the subcutaneous space (Roberts et al. 1998). In contrast, the same tumors implanted in the brain acquired a microvasculature that is considerably less fenestrated, resembling more closely the brain microvascular phenotype. Similar host environment-induced variations in vascular morphology and/or permeability have been described in mouse models for mammary fat pad carcinoma (Monsky et al. 2002) and colon carcinoma (Fukumura et al. 1997). In addition to affecting blood vessel permeability, the tumor host environment also influences MVD and vessel distribution. Human renal carcinoma cells implanted into the kidney of nude mice became highly vascularized, as revealed by immunohistochemical staining for factor-VIII-related antigen, whereas the s.c. growing tumors did not (Singh et al. 1994).

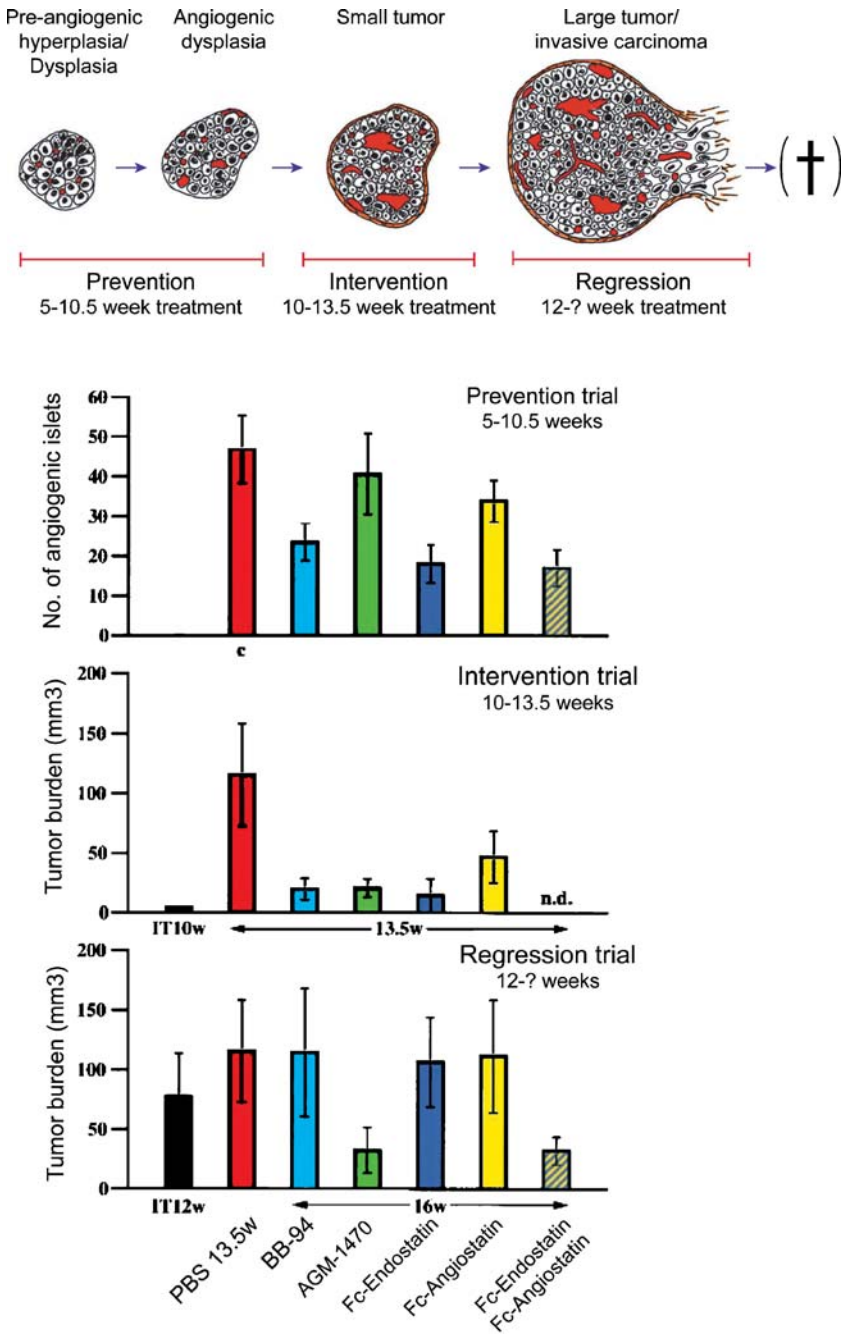

Fig. 2 Efficacy of anti-angiogenic drugs is tumor-growth stage-dependent. Rip-Tag2 mice develop pancreatic islet carcinomas in a multistage process, which starts with the formation of hyperplastic islets that, upon angiogenic switching, give rise to angiogenic islets. Vessel sprouting facilitates the formation of solid tumors that progress into large, intensely vascularized and invasive carcinomas. Four different anti-angiogenic drugs were administered to mice at three different stages of tumor progression. In the prevention trial, the inhibitors were tested for their ability to block the onset of angiogenesis. The intervention trial addressed whether the inhibitors were able to slow down or stop tumor growth (*n.d.* not done). In the regression trial, the drugs were tested for their ability to induce tumor regression. Each of the treatment modalities exhibited a different efficacy profile in the various stages. *IT* (initial tumor burden) represents the size of the tumor at the beginning of the trials (10 weeks for the intervention trial and 12 weeks for the prevention trial). Adapted from Bergers et al. 1999, with permission from AAAS

Similarly, the vasculature of mouse B16.F10 melanoma growing intracranially had a higher density, but a smaller diameter, than the vasculature in s.c. growing tumors (Kashiwagi et al. 2005).

Differential patterns of expression of angiogenic genes accompany and are probably responsible for these host-environment-induced differences in vascularization. Renal cell carcinoma growth in the kidney resulted in a higher

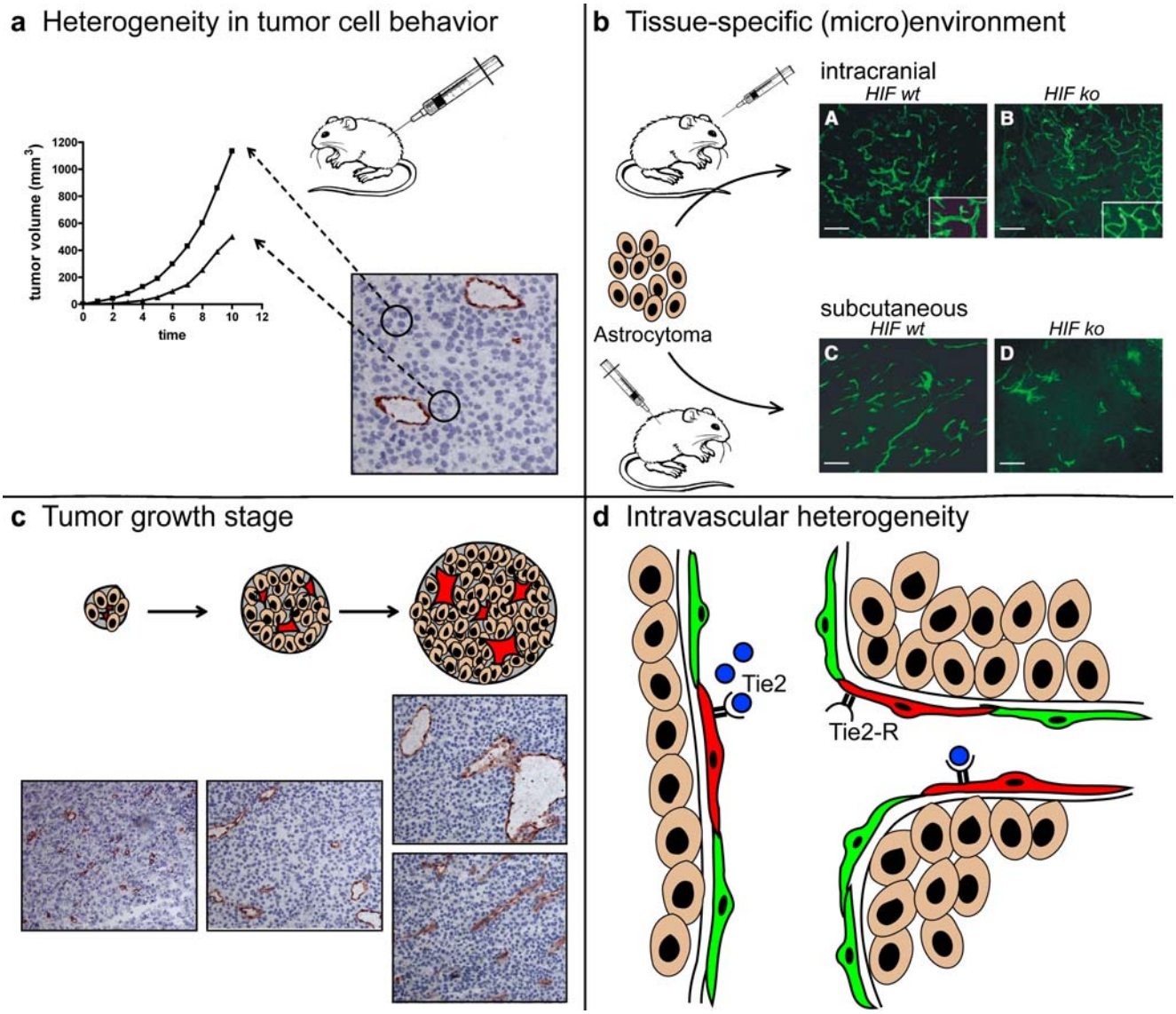

Fig. 3 Tumor endothelial cell heterogeneity finds it origin at different levels. Tumor endothelial heterogeneity occurs among different tumor types or tumor cell subpopulations (**a**), among tumors grown in a different host environment (**b**), in different stages of tumor progression (**c**), and even within the same tumor vessel segment (**d**). **a** Tumor cells are heterogeneous in their dependence on the vasculature. Isolation of tumor cells based on their proximity to the blood vessels followed by inoculation of these cells into mice results in tumors with different growth characteristics. The tumors resulting from cells originally localized proximal to the vasculature have a low growth rate, whereas tumors originating from cells distal from the vessels grow more aggressively, as they are less angiogenesis-dependent (Achilles et al. 2001; Yu et al. 2001). **b** The functionality of a gene and thus the consequences of its dysfunctionality for vascular behavior depend on the tumor host environment. Knock-out (*ko*) of hypoxia inducible factor-1α (*HIF*) results in enhanced microvessel density (MVD) in intracranially growing astrocytomas, but in decreased MVD when tumors are growing in a subcutaneous microenvironment, as shown by fluorescein-isothiocyanate-labeled tomato lectin perfusion (*wt* wild-type). Reprinted from Blouw et al. (2003), with permission from Elsevier. **c** As tumor growth progresses, the vasculature goes through a repeated cycle of angiogenic stages, with concurrent changes in vascular morphology throughout tumor progression. Immunohistochemical staining for CD31 in B16.F10 melanoma growing subcutaneously in C57bl/6 mice. The tumors were harvested at ~25 mm^3 (small volume), ~180 mm^3 (intermediate), and ~520 mm^3 (large). Small tumors showed small vascular profiles, predominantly without a lumen, whereas in intermediate and large tumors, vessels with increasing lumen size existed next to small vessels that did not contain a lumen. Large tumors exhibited strong zonal variations in vascular diameter (unpublished; E. Langenkamp et al., manuscript in preparation). **d** Intravascular heterogeneity for Tie2 expression exists in human tumors; although some endothelial cells within one vessel segment express Tie2, others do not (Fathers et al. 2005)

expression of fibroblast growth factor-2 (FGF-2) as compared with subcutaneous growth of the same tumor cells (Singh et al. 1994). Likewise, the decrease in fenestration pattern and permeability in (glioblastoma) tumors growing in the brain as compared with those growing s.c. was accompanied by an elevated expression of the receptors for VEGF, whereas expression of VEGF itself did not differ per tumor location (Roberts et al. 1998).

Human ductal pancreatic adenocarcinoma grown in the pancreas of nude mice exhibited enhanced expression of VEGF with concomitant higher growth rate compared with ectopic tumors in the abdominal wall (Tsuzuki et al. 2001), and colon cancer xenografts grown in their orthotopic location in the cecum wall produced higher levels of interleukin (IL)-8, carcinoembryonic antigen, and multidrug resistance protein-1 than their s.c. growing counterparts (Kitadai et al. 1995).

In addition to influencing angiogenic gene expression, the host microenvironment can also determine the functionality of genes (Fig. 3b). SV40 T-transfected murine astrocytoma cells grown either s.c. or in the brain of nude mice give tumors with approximately similar vascular densities. However, upon knocking out the gene encoding hypoxia inducible factor (HIF)-1α, the intracranially growing tumors showed a 50% increase in vessel density, compared with the wildtype tumor, accompanied by a 30% increased tumor cell proliferation. In contrast, in the s.c. growing astrocytomas, a 50% reduction in vessel density and a 30% decrease in tumor cell proliferation rate had been observed upon HIF-1α knock-out (Blouw et al. 2003). Thus, the molecular and cellular consequences of dysfunctional HIF-1α, and probably also of other proteins, are highly dependent on the microenvironment. Together with the observation that the vasculature in HIF-1α-deficient tumors growing in the brain resembled those of the normal brain parenchyma, this lead to the conclusion that HIF-1α-deficient astrocytomas were impaired in their ability to induce angiogenesis. Instead, they switched to the mode of co-opting pre-existing brain vessels; this presented as an increased vessel density, as the brain had a higher MVD than the tumor. In the avascular subcutaneous space, this scenario of co-option was unfeasible.

The clinical relevance of host-environment-driven tumor endothelial behavior was recently demonstrated by Morrissey and colleagues (2007). They assessed patient biopsies from prostate carcinoma metastasized to bone, liver, and lymph node. The resulting tumors differed in MVD and expression of angiogenic factors in a location-dependent manner. Bone metastasis displayed the highest MVD, which correlated with increased expression of factor XIII, plasminogen activator inhibitor (PAI)-1, hepsin, and urokinase plasminogen activator (u-PA), but with decreased expression of the pro-angiogenic growth factor Angiopoietin (Ang)-2, as compared with liver and lymph node metastases. In contrast, a study of vascularity in human primary invasive mammary carcinomas and their respective metastases in axillary lymph node failed to demonstrate a local tissue-environment-induced difference in vascular density and angiogenesis (Edel et al. 2000). Possibly, the number, nature, and level of angiogenic genes active in a tumor determines its capacity to overrule the host-environment-driven control of tumor vascular behavior.

Spatiotemporal changes in angiogenic gene expression during tumor outgrowth

As tumor growth progresses, the vasculature goes through a repeated cycle of angiogenesis. Thus, the morphology of the vasculature (Fig. 3c) and the accompanying angiogenic make-up of the endothelium varies dynamically at any given moment during tumor progression. Tumor growth-stage-dependent heterogeneity in the expression of angiogenesis-regulating molecules has been well documented. For example, the distribution and the intensity of expression of VEGF, FGF-2, and IL-8 has been shown to differ in small tumors versus large tumors. In orthotopic KM12SM colon carcinoma, the zonal expression of these pro-angiogenic molecules demonstrated intralesional variation in which FGF-2 and IL-8 are predominantly expressed in small tumors, and at the periphery of large tumors. At the same time, VEGF was present in all zones, but with the highest intensity in the center of large tumors (Kumar et al. 1998). In contrast, in a rat glioma model, VEGF was expressed at equal levels in both small and large tumors at 2 weeks after tumor implantation into the brain, whereas after 4 weeks, its expression was markedly induced at the tumor rim (Holash et al. 1999). The importance of such spatiotemporal variations in gene expression for tumor vascular behavior has been nicely shown for VEGF. A high dose of VEGF results in extensive remodeling of the vasculature, leading to aberrant vessels with features of destabilization and with pericytes loosely associated with the endothelial cells (Pettersson et al. 2000; Ozawa et al. 2004). On the contrary, low levels of VEGF induce the growth of vessels that are morphologically normal and stable (Ozawa et al. 2004).

Zonal differences in transcriptional activity of the VE-cadherin promotor has been documented in s.c. implanted murine Lewis Lung carcinoma. VE-cadherin was absent in a variety of vessels throughout the tumor, but intensely expressed by the endothelium at the tumor periphery (Prandini et al. 2005). A center-versus-periphery distribution of proteins has also been described in human tissues. For example, clinical specimens of prostate carcinoma metastases in liver and lymph node have been found to display a heterogeneous distribution of fibulin-1; this ECM-derived protein forms a component of the blood vessel wall and was more intensely present at the periphery than in the center of the secondary tumors (Morrissey et al. 2007).

In addition to intralesional heterogeneity, phenotypic heterogeneity exists even between tumor endothelial cells within one blood vessel segment (Fig. 3d). Fathers and colleagues (2005) demonstrated by immunohistochemistry that Tie2 expression in human colorectal carcinoma and human melanoma grown s.c. in immune-compromised mice was patchy, with Tie2-positive vessels existing next to

Tie2-negative and Tie2-composite vessels. Examination of clinical specimens of malignant melanoma and colorectal carcinoma confirmed that Tie2-heterogeneity was common in certain types of human cancers (Fathers et al. 2005).

These studies suggest that a widespread heterogeneity occurs among tumor vascular profiles because of local differences in growth factor production by tumor cells and/or inflammatory infiltrates and stromal cells. As a consequence, local differences exist in endothelial cell activation status.

Molecular activation status of tumor endothelial cells

To date, scarce information is available concerning the endothelial activation status in human and animal tumors. Neither in-depth analyses of signal transduction activity status nor detailed gene expression profiles in tumor endothelial cells in varying conditions have been reported as yet. This may be attributable to the limited availability of antibodies specific for proteins in general and for phospho-kinases for use in immunohistochemistry or immunofluorescence applications, and of in situ hybridization protocols that can be widely applied for mRNA localization studies. As tumor endothelial cells are numerically under-represented in the tumor mass, whole tumor RNA or protein isolates are not likely to reveal the molecular signature of the endothelium. Many in vitro studies make use of primary endothelial cells or endothelial cell lines established from normal non-diseased blood vessels. These endothelial cell cultures do not represent the endothelial cells that are present in the local tumor environment and influenced by often unknown and rapidly changing concentrations of growth factors, cytokines, and other angiogenic molecules. Moreover, the biomechanics of blood flow and interactions between blood-borne cells and endothelial cells are not taken into account in cultures in vitro. Several studies have reported the isolation of endothelial cells from tumors by enzymatic digestion, followed by gradient centrifugation or magnetic bead cell sorting and culture (Bian et al. 2006; Miebach et al. 2006). These methods influence endothelial cell behavior in various ways, thereby inevitably inducing changes in kinome and transcriptome status. Rapid changes in antigen expression upon culturing, as evidenced by, for example, loss of vWF, VE-cadherin, and CD31 (Miebach et al. 2006), demonstrate the high plasticity of tumor endothelial cells similar to that of normal endothelial cells, as described in the Introduction. Moreover, information regarding their original location within the tumor is lost, thereby possibly complicating the interpretation of the experimental results even more.

Nevertheless, the isolation of tumor endothelial cells by enzymatic digestion can provide a valuable source of information regarding their transcriptome when immediately used for gene expression profiling. St. Croix and colleagues (2000) have identified markers specifically induced in endothelial cells from human colorectal carcinoma through a comparison of gene expression profiles of endothelial cells isolated from human colorectal carcinoma and normal human colorectal tissue. Using the same approach, the St. Croix group has recently identified genes that are differentially expressed during pathological angiogenesis in tumors grown in the liver in mice, on the one hand, and during physiological angiogenesis in liver regeneration, on the other hand (Seaman et al. 2007).

Application of laser microdissection enables the capture of tumor endothelial cells from their (patho)physiological environment, for subsequent in situ molecular profiling. By combining immunohistochemistry-guided laser microdissection with microarray transcriptional profiling of ovarian carcinoma vasculature, Buckanovich et al. have recently revealed the overexpression of a set of genes in ovarian-cancer-associated endothelium; these genes might be useful as biomarkers for diagnosis and may shed light on the molecular nature of angiogenic activation in this tumor (Buckanovich et al. 2007). Reverse-phase protein microarray in combination with laser capture microscopy has disclosed a reduction in phosphorylation of Akt in metastatic breast cancer as a result of treatment with the epidermal growth factor (EGF)-receptor inhibitor Erlotinib (Wulfkuhle et al. 2008). If such a technique were to allow the analysis of phosphorylated kinases from microdissected tumor endothelium, a more detailed view of the activation status of tumor endothelial cells and the heterogeneity thereof in the various tumor segments would come within reach.

Furthermore, by using in vivo or in vitro phage display, alternatively combined with laser dissection microscopy (Yao et al. 2005), differences in the composition and properties of the vasculature of different pathological lesions can be identified. Several studies have employed this technique to identify peptide sequences that specifically home to the vasculature of either pre-malignant hyperplasias or malignant solid tumors, but not to that of normal tissue (Hoffman et al. 2003; Joyce et al. 2003; Yao et al. 2005). Although these studies have established that different tumors express distinct repertoires of molecular markers in their vasculature, detailed insight is lacking with respect to the actual identity and meaning of such differentially expressed markers.

Taken together, these studies suggest the existence of extensive heterogeneity in the behavior of tumor endothelial cells. Pharmacological intervention with anti-angiogenic drugs targets the molecular control of microvascular behav-

ior, either directly by interfering with tumor endothelial cell signal transduction or indirectly by affecting angiogenic gene expression by tumor and stromal cells. As the variation in microvascular behavior is highly likely to find its basis in tumor endothelial cell heterogeneity, this latter phenomenon may be the underlying cause of the different responses to pharmacological interference with hitherto unknown consequences.

Efficacy of anti-angiogenic therapy

To date, anti-tumor therapies targeting the vasculature of tumors have concentrated on two different strategies: either they attack the endothelial cells in the tumor to disrupt the vasculature and cause a rapid and selective shutdown of the established tumor vascular network (the so-called vascular disrupting agents or VDAs), or they aim to prevent the processes that drive neovascularization (e.g., by using angiogenesis inhibitors). VDAs cause acute occlusion of existing tumor blood vessels, leading to rapid and massive tumor cell necrosis, while they leave the blood flow in normal tissues relatively intact. The largest group of VDAs is the tubulin-binding combretastatins, several of which are now being tested in clinical trials (Tozer et al. 2005). VDAs will not be further addressed because of space limitations.

Most well-known examples of anti-vascular agents are inhibitors of receptor tyrosine kinases (RTKs) and VEGF-blocking antibodies. Their primary effect is the blockade of new vessel formation, resulting in impaired tumor outgrowth. The exact biological consequence of treatment with these inhibitors is unknown, but anti-angiogenic therapeutic strategies have been documented to result in hypoxia (Casanovas et al. 2005; Shaked et al. 2006), endothelial cell apoptosis (Laird et al. 2002), and normalization of the vasculature (Jain 2005). In rectal carcinoma patients, a single infusion of the VEGF-specific antibody Bevacizumab decreased tumor perfusion, vascular volume, MVD, interstitial fluid pressure, and the number of viable circulating endothelial progenitor cells (EPC) and increased the fraction of tumor vessels covered by pericytes (Willett et al. 2004). In a combination treatment regimen with chemotherapy, this VEGF neutralizer prolonged the survival of patients with metastatic colorectal cancer (Hurwitz et al. 2004). In 2007, ten new drugs with anti-angiogenic activity have been approved by the FDA for the treatment of cancer and age-related macular diseases, and at least 43 drugs were in clinical trials in the USA (Folkman 2007).

Nonetheless, anti-angiogenic drugs have not yet lived up to their high expectations as a powerful new member of anti-cancer drugs for use in daily clinical practice to control tumor growth. While anti-VEGF-specific agents showed promising anti-tumor results in preclinical studies, they did not demonstrate an overall survival benefit when used as a mono-therapy in phase III clinical trials (Jain et al. 2006; Duda et al. 2007). Below, we will discuss why the limited efficacy of anti-angiogenic therapies in the clinic may find its origin in the under-appreciated heterogeneity of the molecular activation status of endothelial cells in the tumor vasculature.

Anti-angiogenic effects of anti-VEGF therapy

VEGF is considered one of the major regulators of angiogenesis. Its potency and its consistent overexpression in many tumor types together with the successful modulation of tumor growth with anti-VEGFR2 antibodies and small-molecular inhibitors of VEGFR2 in (pre)clinical studies validate its usefulness as a therapeutic target for anti-angiogenic therapy (Youssoufian et al. 2007). Activation of the VEGF-VEGF receptor signaling axis triggers multiple signaling cascades that result in vascular permeability, endothelial cell survival, proliferation, migration, and differentiation, and mobilization of endothelial progenitor cells from the bone marrow (Hicklin and Ellis 2005). Binding of VEGF to its receptors induces receptor dimerization, resulting in the activation of its kinase activity with consequent autophosphorylation. The phosphorylated receptors recruit interacting proteins and induce the activation of diverse signaling pathways. For example, the phosphorylated tyrosine residue 1175 on VEGFR2 binds phospholipase Cγ, which mediates activation of the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK)-1/2 cascade to induce proliferation of endothelial cells. Moreover, the adaptor molecule Shb binds to phosphorylated Tyr1175, thereby activating phosphatidylinositol-3 kinase, which in turn activates the serine/threonine kinase AKT/protein kinase B pathway that mediates survival of the endothelial cells (Olsson et al. 2006).

The effects of VEGF on the vasculature are tightly regulated and vary depending on its microenvironmental concentration, as discussed above. Low levels of VEGF induce the growth of vessels that are morphologically normal and stable; however, a high dose of VEGF extensively remodels the vasculature, inducing enlargement of the vessels and formation of bulbous vascular structures resembling glomeruloid bodies (Ozawa et al. 2004). These enlarged thin-walled pericyte-poor vessels ("mother" vessels) are characterized by microvascular hyperpermeability, edema, clotting of extravasated plasma fibrinogen, and deposition of an extravascular fibrin gel matrix (Pettersson et al. 2000). At high VEGF concentrations, mother vessels evolve into distinct types of vessels, such as glomeruloid microvascular proliferations, vascular malformations, but also structurally normal capillaries (Dvorak 2007). The

permeability properties of these vessels are different, indicating that the response of vessels to VEGF can be variable. Whereas mother vessels and glomeruloid-like vessels become highly permeable in response to VEGF, capillaries and vascular malformations do not (Nagy et al. 2006). Furthermore, VEGF affects vascular architecture, as has been shown in gastric tumors in which the absence of VEGF resulted in impaired vascular lumen formation (Stoeltzing et al. 2004). In the quail chorioallantoic membrane assay, vessel diameter increased maximally at high VEGF doses, but MVD decreased (Parsons-Wingerter et al. 2006). In contrast, in human renal cell carcinoma, an increased MVD was correlated with an increased expression of VEGF and its receptors and of the VEGFR1-activating factor placental growth factor, when compared with renal cell carcinoma exhibiting a low MVD (Baldewijns et al. 2007). Thus, histological read-out of the effects of VEGF-blocking therapy is complicated and may differ for a given tumor type.

Treatment of HUVEC with the tyrosine kinase inhibitor SU6668, which has affinity for both VEGFR2 and platelet-derived growth factor receptor-β (PDGF-Rβ), resulted in the inhibition of VEGF-induced proliferation. In vivo, this compound significantly inhibited subcutaneous A431 epithelial carcinoma growth in nude mice, by preventing tumor expansion when administered at an early stage of tumor growth, and by reducing tumor size when administered to mice carrying late-stage carcinoma (Laird et al. 2000). Its effect on the tumor vasculature has been established to be a reduction in tumor vessel density; however, the underlying molecular events in the endothelium have not been addressed. Furthermore, inhibition of VEGFR2 with the tyrosine kinase inhibitor PTK787 in the same tumor model caused a reduced occurrence of microvessels in the interior of the tumors, whereas larger, more mature vessels forming particularly at the tumor periphery before initiation of the treatment remained unaffected (Wood et al. 2000).

Molecular effects of anti-VEGF therapy on tumor endothelial cells

Only a few studies have addressed the molecular events induced in tumor endothelial cells upon pharmacological treatment with angiogenesis inhibitors. In human colorectal carcinoma liver metastases in nude mice, tumor vessels and some tumor cells surrounding the vessels showed intense staining for phosphorylated ERK and Akt. Upon treatment of these mice with SU6668 or with the VEGFR2-specific tyrosine kinase inhibitor SU5416, levels of phosphorylated ERK and Akt in the tumor vasculature decreased (Solorzano et al. 2001). Thus, treatment with these inhibitors resulted in decreased signal transduction via at least Akt and ERK in endothelial cells in vivo. Furthermore, Sasaki et al. (2007) have demonstrated that in vitro treatment of murine mesenteric endothelial cells with the dual VEGFR2/EGF-R RTK inhibitor AEE788 diminished ERK1/2 and Akt phosphorylation induced by TGF-α alone and by TGF-α combined with VEGF. In vivo treatment of SW260CE2 orthotopic human colon carcinomas with AEE788 alone or in combination with conventional chemotherapy resulted in decreased phosphorylation of the receptors for EGF and VEGF in endothelial cells, with consequent reduction in the number and diameter of blood vessels, increased apoptosis of endothelial and tumor cells, and a decreased proliferation rate of the tumor cells (Sasaki et al. 2007). In this tumor model, VEGFR2 and EGF-R expression is restricted to the endothelial cells. However, many other studies have reported the expression of VEGFR2 and other RTK targets of anti-angiogenic drugs by tumor cells in vivo (Cimpean et al. 2008; Xia et al. 2006; Thaker et al. 2005; Kuwai et al. 2008). Therefore, the localization of the molecular effects on the respective cell types is critical for a proper understanding of the actual mechanism of anti-angiogenic therapy in relation to therapeutic success or failure.

Tumor endothelial heterogeneity and efficacy of anti-angiogenic drugs

Most pre-clinical successes with anti-angiogenic therapy have been achieved when treatment is initiated at a very early stage of tumor development, which is characterized by synchronized blood vessel outgrowth. The studies by Bergers et al. (1999, 2003) unambiguously showed that the efficacy of angiogenesis inhibitors in the Rip-Tag2 model of multistage pancreatic carcinoma is tumor-growth stage-dependent (Fig. 2). This transgenic mouse model serves as a prototype of spontaneously developing tumors in which different (angiogenic) growth stages sequentially arise. At 3–4 weeks of age, hyperplastic islets begin to appear, giving rise to angiogenic islets by switching on angiogenesis in the normally quiescent islet capillaries. This transformation from normal to angiogenic islets is accompanied by an increase in vessel diameter that precedes vessel sprouting and endothelial proliferation. Solid tumors emerge at week 10 and progress into large, intensely vascularized adenocarcinomas by week 13. These carcinomas display a higher vessel density and dramatic vessel heterogeneity as revealed by hotspots of neovascularization and irregular vessel diameters (Ryschich et al. 2002; Bergers et al. 1999). The four angiogenesis inhibitors exert different efficacies depending on the stage of carcinogenesis being targeted. The matrix metalloproteinase inhibitor BB94, endostatin, and endostatin combined with angiostatin perform best in inhibiting both early and mid-stage

disease, i.e., in preventing angiogenic switching in dysplastic lesions or blocking expansive tumor growth. In contrast, the inhibitor of endothelial cell proliferation TNP470 reduces the mass of bulky end-stage tumors but is ineffective in preventing angiogenic switching in the early stage of tumor growth. Of note, the SV40 transgene is not synchronically activated in all pancreatic β-cells, resulting in a temporal spectrum of developing neoplastic foci. This spatiotemporal heterogeneity of tumor development within the pancreas is of considerable value for assaying pharmacological interventions at different developmental stages of tumor growth, as it reflects the biological diversity of cancer development in humans.

The tumor growth-stage-specific efficacy of the drugs suggests that qualitative differences exist in the angiogenic vasculature at the different tumor growth stages. The contribution of the various kinases, molecular targets of anti-angiogenic drugs, to tumor growth may be different at the different stages of tumor vascular development. Hence, the fraction of the tumor vasculature that is affected by a specific drug given at one specific moment may vary for the different growth stages. Several studies have indicated that, for an efficient anti-tumor effect, at least ~90% of the tumor vasculature needs to be attacked. Targeting of a blood-coagulation-inducing coaguligand to the tumor vasculature could only induce long-term tumor regression upon affecting the complete tumor vascular network. Incomplete thrombosis of the tumor vasculature attributable to the absence of detectable levels of target epitope on the neovasculature permitted the local survival of tumor cells and the regrowth of the tumors (Huang et al. 1997). Moreover, for anti-angiogenic agents, a partial attack of the vasculature has been shown to be devoid of strong anti-tumor activity. A Tie2-antagonizing antibody effectively reduced MVD and tumor growth in a WM115 melanoma, a tumor of which 95% of the vasculature is positive for Tie2. In contrast, in the HCT116 colon carcinoma, of which only 70% of the vessels expressed Tie2, Tie2 inhibition had no effect (Fathers et al. 2005). A metronomic dosing schedule of anti-angiogenic drugs in which the drugs are administered at low(er) doses for a prolonged period of time (Kerbel and Kamen 2004) may provide a means of keeping the neovasculature under continuous pharmacological pressure to circumvent incomplete exposure.

The question arises as to whether the anti-tumor effects and also the development of resistance to treatment are predominantly attributable to an effect on the tumor endothelium, or whether the surrounding tumor cells are also involved. Evidence is emerging that VEGF may have an additional role in tumor growth through the stimulation of VEGF receptors on tumor cells (Hicklin and Ellis 2005; Thaker et al. 2005). Hence, VEGFR2 expression on tumor cells may contribute to the anti-tumor effects of VEGFR2-targeted anti-angiogenic drugs. Nevertheless, several other studies have established the efficient tumor-growth-inhibitory effect of anti-angiogenic therapy solely mediated through the targeting of epitopes selectively present on the tumor vasculature (Fathers et al. 2005; Sasaki et al. 2007).

Redundancy in angiogenic factors and neovascularization types provides routes for resistance

Although increasing evidence demonstrates the occurrence of genetic alterations such as chromosomal translocations and aneuploidy in tumor-associated endothelial cells (Streubel et al. 2004; Hida et al. 2004, 2008), these cells are still considered to be more genetically stable than tumor cells, as they are not oncogenically transformed. Therefore, anti-angiogenic therapy in theory can circumvent the problem of therapy-induced resistance. Indeed, the natural inhibitor of angiogenesis, endostatin, demonstrated anti-tumor activity and a lack of resistance upon repeated treatment after re-growth in three different mouse xenograft tumor models. After several treatment cycles, no tumors recurred upon discontinuation of therapy, indicating that resistance to endostatin has not developed in these tumors (Boehm et al. 1997). Unfortunately, emerging preclinical and clinical data show that resistance to anti-angiogenic therapy is a fact that we have to face. The presence of an array of different angiogenic molecules provides the tumor with a variety of redundancy pathways. When VEGF is inhibited, FGF-2 and other pro-angiogenic factors may be present to take over the control of neovascularization. For instance, 10 days of treatment with a VEGFR2-function-blocking antibody initially resulted in a significant impairment of tumor formation in the Rip-Tag2 model. This was associated with a marked decrease in vessel density, vascular dilation, and permeability. After a treatment period of 4 weeks, this phase of stable disease was followed by regrowth of the tumors, which was supported by a second wave of angiogenesis. This second wave was controlled by FGF and possibly also by other pro-angiogenic factors such as the Ephrins and angiopoietins, as suggested by their upregulation in both tumor and endothelial cells upon VEGFR2 blocking treatment (Casanovas et al. 2005). In concordance with this, FGF-2 and PDGF-BB have recently been described as having an important synergistic role in tumor neovascularization and metastasis, without any involvement of VEGF (Nissen et al. 2007).

The capacity of tumors to employ other mechanisms than VEGF- or FGF-driven sprouting angiogenesis to acquire a blood supply, such as intussusceptive angiogenesis, recruitment of endothelial progenitor cells, vessel co-option, or vasculogenic mimicry, provides even more

possibilities for evading anti-angiogenic therapy. Under the influence of the VEGFR2 inhibitor ZD6474, tumor angiogenesis in the brain was blocked with a concomitant decrease in vessel density, but tumor growth was not inhibited. Instead, tumor progression sustained via co-option of pre-existing vessels (Leenders et al. 2004). Interestingly, VEGFR2 was also present on the tumor cells (personal communication, Dr. W.P.J. Leenders), which may imply that the ZD6474-induced switch to vessel co-option partially involved a tumor-cell-mediated effect. Furthermore, cessation of treatment with the VEGFR2-inhibiting compounds AG-013736 and AG-028262 resulted in rapid regrowth of the vasculature in the Rip-Tag2 model of spontaneous pancreatic carcinoma and in s.c. implanted Lewis lung carcinoma. Both agents caused a 50%-60% loss of the tumor vasculature, as demonstrated by immunofluorescent detection of CD31, but empty sleeves of basement membrane were left behind. These sleeves and accompanying pericytes functioned as a scaffold for quick tumor vessel regrowth after treatment was stopped (Mancuso et al. 2006). In addition, recruitment of EPCs circulating in the blood may contribute to vessel formation under the pressure of anti-vascular therapy. Treatment of Lewis lung carcinoma- and human melanoma-bearing mice with the vascular disrupting agent Oxi-4503, a second generation derivative of combretastatin, induced the mobilization of EPCs to become incorporated into the vasculature (Shaked et al. 2006).

Efficacy of combination therapy to inhibit tumor angiogenesis

The redundancy in strategies acquired by the tumor to establish a vasculature suggests that anti-tumor therapy might benefit from a combination approach. Indeed, several studies have shown an enhanced anti-tumor effect when combining two vascular targeting agents. The VEGFR2 blocking agent SU5416 efficiently blocked the angiogenic switch in premalignant lesions in the Rip-Tag2 model but was incapable of inducing tumor regression in middle- or end-stage disease. On the contrary, SU6668, which has a high PDGF-Rβ inhibitory effect in addition to VEGFR2 blockade, has proved more effective in inhibiting end-stage tumor growth. Combining these two agents, and thereby targeting angiogenic switching of the tumor endothelium and PDGF-Rβ activity on pericytes, was highly efficacious against all stages of pancreatic islet carcinogenesis (Bergers et al. 2003). Inhibition of either VEGFR1 or VEGFR2 signaling in murine B16 melanoma alone had no significant effect on subcutaneous tumor growth and metastasis formation, whereas blocking both receptors successfully inhibited solid tumor progression and formation of lung metastasis (Gille et al. 2007). Similarly, the anti-tumor efficacy of Oxi-4503 increased when combined with an anti-angiogenic agent (Shaked et al. 2006), and combining anti-VEGFR2 therapy with an MMP inhibitor caused more extensive vascular regression in Rip-Tag2 tumor growth (Mancuso et al. 2006). Combination of anti-angiogenic therapy with conventional chemotherapy and radiotherapy can also give rise to synergistic tumor growth inhibition; these combinations have been and are still being investigated both in preclinical and clinical studies (Kerbel and Kamen 2004) but will not be addressed further as this subject is beyond the scope of this review.

In summary, although monotherapy aimed at blocking angiogenesis-associated tumor endothelial signal transduction can exert powerful effects in preclinical tumor models, resistance to therapy due to endothelial heterogeneity and/or by switching to a different angiogenic mode is a realistic threat. Combination therapies blocking multiple molecular pathways in tumor endothelial cells, tumor cells, and stromal cells concomitantly are hence warranted. For success, the dosing of the right drug(s) at the right time needs to be carefully considered and may be guided in the near future by phospho-kinase screening prior to treatment.

Concluding remarks

Microvascular endothelial cells and tumor endothelial cells display remarkable heterogeneity in their cellular and molecular characteristics, which are spatiotemporally controlled by their (patho)physiological microenvironment. In vitro studies have provided an enormous wealth of information regarding the way that endothelial cells respond to stimuli of various sorts, and in rather a short time, a significant number of drugs affecting pro-angiogenic signal transduction in cancer have reached the stage of clinical testing. The lack of comprehensive knowledge of the factual molecular status of tumor endothelial cells in their complex in vivo environment, which is dynamically changing under the influence of a plethora of ill-defined processes, now poses an important challenge for the biomedical field. Not only technological advances in analyzing the complex kinome, transcriptome, and epigenetics of tumor endothelial cells and the local molecular effects of anti-angiogenic drugs on these cells are essential prerequisites for progress (G. Molema, M. Mrug, E. Verpoorte, K. Schutze, R. Bischoff, H. Struijker-Boudier, manuscript in preparation), but also the identification of proper biomarkers and new methods for molecular imaging of anti-angiogenic effects on tumor vasculature will be instrumental for future developments (Iagaru et al. 2007).

A number of other issues could not be addressed here because of space constraints but should not go unnoticed as they may have importance for future research. They include

the widespread use of xenografted human tumors in immune-compromised animals and of rapidly growing mouse tumors implanted in avascular pockets. Extrapolation of data from these models to the clinical situation is difficult, if not impossible. More and more animal tumor models based on orthotopic "spontaneous" tumor outgrowth are becoming available to the research community, and these may overcome part of the extrapolation problems encountered with the artificial models. Furthermore, the majority of studies on the molecular behavior of tumor endothelial cells and anti-angiogenic drug effects have been performed in young mice that are otherwise perfectly healthy. Recently, Klement and colleagues (2007) have demonstrated that vascular aging and atherosclerosis are accompanied by retarded tumor outgrowth and concurrent diminished MVD, microvascular proliferation index, and TEM1 and VEGFR2 expression, whereas acute tumor hypoxia increases. Moreover, treatment with cyclophosphamide in a metronomic dosing schedule previously shown to exert anti-angiogenic effects is less effective in old atherosclerotic mice (Klement et al. 2007). Similarly, high glucose levels associated with aging but also with diabetes type-2 associated with a Western-style diet have been shown to impair some essential signaling pathways and general EPC functions (Chen et al. 2007). This may have repercussions for the day-to-day repair of small microvascular damage by progenitors and for EPC-related repair of larger cardiovascular insults and cellular processes in tumor angiogenesis.

The remarkable progress made in unraveling the molecular control of vascular development and tumor angiogenesis has paved the way into the next era. By expanding our knowledge regarding the way that microvascular endothelial cells molecularly control their function in the different organs, we may become able to fine-tune culture conditions to maintain their behavior ex vivo. This might create new opportunities for integrating the correct endothelial cells with the appropriate function in engineered tissue constructs and for creating valuable in vitro screening systems for use in, for example, drug development. For tumor angiogenesis, the newly aquired knowledge should definitely assist in the design of rational drug treatment schedules that will become an integral part of daily clinical practice.

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