Molecular characterization of tumor vascular phenotype and pharmacology of antiangiogenic therapy
Langenkamp, Elise
Chapter 1

Introduction & Aim of the thesis
INTRODUCTION

Despite considerable advances in cancer treatment, cancer still represents a leading cause of death around the world. It accounted for 7.4 million deaths worldwide in 2004 (constituting 13% of total deaths) and this number is predicted to continue rising to an estimated 12 million deaths in 2030 [1]. In addition to conventional therapeutic options, such as surgical removal of the tumor, radiotherapy and chemotherapy, targeting the tumor blood supply has increasingly become in focus as a potential antitumor therapeutic strategy. This concept found its origin almost 40 years ago, when Dr. Judah Folkman discovered that tumors depend on angiogenesis for their growth [2]. In his landmark paper ‘Tumor angiogenesis, therapeutic implications’ he reported that tumor cells release a diffusible factor that is capable of stimulating neovessel formation, the so-called tumor angiogenesis factor (TAF), and that provides a promising target for therapy, as ‘…withdrawal of TAF is followed by disappearance of newly formed capillaries..’ [3]. Identification of vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) by Dr. Harold Dvorak and his team as one of the most potent tumor angiogenesis-inducing factors [4] has fuelled the development of agents that target the VEGF-VEGF receptor system as cancer therapy.

Blocking angiogenesis has since been considered a promising approach in cancer therapy, culminating in the FDA approval of the VEGF-neutralizing antibody Bevacizumab for colorectal cancer patients in 2004. This was followed by approval of the tyrosine kinase inhibitors sorafenib in 2005 and sunitinib in 2006. The therapeutic potential of these antiangiogenic compounds is illustrated by the survival benefit that was observed in a number of clinical trials [5-9]. Nevertheless, a subset of patients does not respond to anti-VEGF therapy and the overall survival benefit is relatively short, averaging 1-5 months [10-12]. Development of resistance in response to antiangiogenic therapy, for example by upregulation of alternative pro-angiogenic signaling pathways, by switching to different modes of acquiring a vasculature—as described below—, or by increasing tumor cell invasion into the surrounding tissue, contributes to reduced therapeutic efficacy [13]. Another factor that plays an important role in the limited efficacy is heterogeneity of the tumor vasculature. As will be explained in the following section, endothelial cells engaging in neovascularization go through different angiogenic activation stages that may render them differentially responsive to antiangiogenic therapy. Moreover, the tumor type, the microenvironment, the growth stage and site of tumor outgrowth determine endothelial behavior, and thus theoretically can influence responsiveness to therapy.
Angiogenesis and the tumor vascular phenotype

For growth, tumors require recruitment of an adequate blood supply, that provides them with nutrients and oxygen, a means to deposit waste products, and a systemic route for metastasis. Angiogenesis is one of the main processes through which a tumor acquires a vasculature, and involves the activation, proliferation and migration of endothelial cells, assembly into vascular tubes, formation of a lumen, recruitment of vascular support cells and perfusion of the newly formed vessel. The process of angiogenesis is regulated by a large array of growth factors, growth factor receptors, adhesion molecules, proteases and signaling molecules. A brief description of the major processes and regulators thereof is provided in the next paragraphs.

Angiogenesis is rapidly initiated in response to hypoxic or ischemic conditions, through hypoxia inducible factor-1α (HIF-1α)-mediated upregulation of VEGF. VEGF exerts a plethora of effects on endothelial cells. Besides induction of vasodilation via stimulating endothelial NO production, VEGF induces vascular permeability and local fibrin formation, stimulates endothelial cell proliferation and migration, and induces expression of proteases and receptors that are important in cellular invasion and tissue remodeling. The activated endothelial cells produce Angiopoietin-2 that antagonizes activation of the vascular stability-inducing system formed by Angiopoietin-1/Tie2. As a consequence, the vasculature loses coverage with pericytes, which renders the endothelium sensitive to angiogenic factors. In addition to VEGF, fibroblast growth factor (FGF)-2 is a potent mitogenic factor for endothelial cells, and also transforming growth factor (TGF)-β signaling through its receptors activin receptor-like kinase ALK1 and endoglin (CD105) stimulates endothelial cell proliferation. Degradation of the basement membrane surrounding the vasculature is facilitated by matrix metalloproteinases and plasmin, allowing endothelial cells to invade the surrounding tissue. Plasma proteins, particularly fibrinogen, extravasate and form a provisional matrix that is used for endothelial cell migration and tube formation. Adhesion of endothelial cells to these extracellular matrix molecules is mediated by integrins, and provides a means to cell survival signaling. Integrin αvβ3 is minimally expressed on quiescent endothelium, but significantly upregulated on the cell surface once endothelial cells are angiogenically activated. Upon assembly of the endothelial cells into a vascular tube, the newly formed sprout starts to recruit pericytes to support the vessel wall. This is accomplished via the synthesis and secretion of platelet-derived growth factor that functions as a chemoattractant for pericytes and smooth muscle-like cells. Stable interactions between endothelial cells and pericytes require furthermore Angiopoietin-1-mediated activation of Tie2, TGFβ-ALK5 signaling, and activation of the adhesion molecule N-cadherin [14-19]. Additionally,
ligand/receptor systems such as the family of Ephrins, the Slit/Robo system and the Notch family, that were originally identified as neuronal guidance cues, have now been demonstrated to play a role in neovascularization, either by shaping the vasculature, or by promoting vascular maturation [20-23]. Physiological angiogenesis leads to the formation of functional blood vessels, yet pathological angiogenesis, as occurs in tumors, is characterized by a dysbalance in the regulation of these processes, resulting in the formation of abnormal blood vessels. Tumor blood vessels differ highly from a normal vasculature, as illustrated by their more dilated and tortuous architecture, lack of artery-capillary-vein hierarchy, uneven diameters and chaotic flow patterns, and by their increased permeability that can be partly explained by abnormal coverage with pericytes [24-26].

In addition to angiogenesis, tumors can acquire a blood supply via several other mechanisms. These include cooption of pre-existing vasculature [27], the recruitment of endothelial progenitor cells, and vasculogenic mimicry, i.e., the formation of tube-like structures that are lined by tumor cells next to, or instead of, endothelial cells [28]. The diversity in mechanisms of neovascularization and regulation thereof contribute to variations in the tumor vascular phenotype. Heterogeneity among the vasculature of tumors has been described in various tumor types, in tumors from the same origin growing in different host environment, in different stages of tumor outgrowth and even within the vasculature of one tumor at a given moment, as will be reviewed in Chapter 2 of this thesis. Clinical relevance for the occurrence of variations in vascular behavior of tumors is illustrated by variable expression patterns of endothelial markers and angiogenesis-regulating genes in e.g., human gliomas and head and neck squamous cell carcinomas [29, 30]. Spatiotemporal variations in tumor vascular behavior determine the responsiveness of endothelial cells to antiangiogenic therapy. For example, the efficacy of angiogenesis inhibitors can vary with tumor growth stage [31] and even among different vascular phenotypes within the same tumor [32]. We hypothesized that tumor vascular heterogeneity is brought about by variations in the transcriptome of endothelial cells in relation to tumor growth stage and morphological appearance. Identification of the molecular status of the tumor vascular phenotype is essential for drug regimen design aimed to affect multiple neovascularization stages.

**Molecular aspects of antiangiogenic therapy**

The vast majority of antiangiogenic treatment strategies in current clinical trials target VEGF signaling. VEGF exerts a broad array of cellular effects through activation of its main signaling receptor, VEGFR2. Binding of VEGF to its receptor leads to VEGFR2 dimerization, autophosphorylation of the receptor on a number of intracellular tyrosine
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Figure 1: Schematic representation of VEGFR2 intracellular signaling, and molecular targets of angiogenesis inhibitors. Binding of VEGF to the receptor induces dimerization and autophosphorylation of specific intracellular tyrosine residues. Phosphorylation at tyrosine 1175 recruits Shb that activates PI3K. Subsequent activation of Akt/PKB promotes cell survival via inhibition of the pro-apoptotic proteins BAD and CASP9, and stimulates permeability via activation of eNOS. Shb also activates the Rac-Rho pathway, leading to actin reorganization that induces permeability and migration. Phosphorylation of Tyr1175 also induces PLCγ-ERK1/2-mediated proliferation. Furthermore, phosphorylation of Tyr1214 activates p38 MAPK that is involved in actin reorganization via HSP27 activation. Many of the currently available antiangiogenic drugs target VEGF, VEGFR2 or a downstream player of VEGFR2 signaling. Small molecule tyrosine kinase inhibitors exert their effect at the ATP-binding pocket in the intracellular domain of the receptor. The majority of small molecule tyrosine kinase inhibitors also target related growth factor receptors, such as VEGFR1, EGFR or PDGFRβ. Note that while VEGFR2 is predominantly expressed by the endothelial cells, PDGFRβ is primarily expressed by pericytes. For graphical simplicity, these molecules are depicted in the same cell. Abbreviations: BAD, Bcl-2-associated death promoter; CASP9, caspase 9; EGFR, epidermal growth factor receptor; eNOS, endothelial nitric oxide synthase; ERK1/2, extracellular signal-regulated kinase 1/2; HSP27, heath-shock protein 27; MEK, MAPK/ERK kinase; p38 MAPK, p38 mitogen-activated protein kinase; PDGFRβ, platelet-derived growth factor receptor-β; PI3K, phosphoinositide 3-kinase; PKB, protein kinase B; PKC, protein kinase C; PLCγ, phospholipase Cγ; Shb, SH2 domain-containing adapter protein B [33-35].
residues and subsequent activation of downstream signaling pathways (Fig 1). VEGFR2 induces proliferation via activation of the extracellular signal-regulated kinase (ERK)1/2 pathway. Activation of phosphoinositide-3-kinase plays a role in cell migration and vascular permeability, and provides a survival signal via induction of protein kinase B/Akt. Furthermore, VEGF-induced actin remodeling via the activation of p38 mitogen-activated kinase (p38 MAPK) also contributes to vascular permeability [33, 34].

VEGF-targeted therapies interfere with VEGF signaling at various levels (Fig 1). By neutralizing VEGF, the antibody Bevacizumab prevents VEGF from activating its receptor. Small molecule tyrosine kinase inhibitors with selectivity for the VEGFRs prevent binding of ATP to the ATP-binding pocket of the receptor and thus autophosphorylation and downstream kinase activation. Owing to their mode of action at the ATP binding pocket, these tyrosine kinase inhibitors are considered selective rather than specific. This means that in addition to VEGFRs, they have affinity for other growth factor receptors, such as EGFR and PDGFR, or for kinases downstream from VEGFR2 such as Raf [35]. Although the affinity of these drugs for their different target receptors is quite well established in vitro, little is known about the effect of VEGFR2 inhibitor treatment on the kinetics of phosphorylation of VEGFR2 and its downstream kinases, in vitro and in vivo. Moreover, endothelial cells in a tumor are likely present in different stages of angiogenic activation, characterized by engagement in different steps of the VEGF signal transduction cascade. As such, it is likely that the pharmacological efficacy of VEGFR2 inhibitors is different when administered to endothelial cells that are already engaging in VEGF-activated angiogenic signaling.

Several mechanisms of action have been attributed to VEGF-inhibitor therapies, including inhibition of neovessel formation, induction of endothelial cell apoptosis, and blockade of incorporation of circulating endothelial progenitor cells in the tumor vasculature [36]. In vitro, inhibitors of VEGFR2 signaling reduce endothelial cell proliferation, migration and capillary sprout formation, and in vivo, VEGFR2 inhibitor treatment may reduce tumor microvascular density and inhibits tumor growth in mice [37-39]. A landmark study in patients with metastatic colorectal cancer demonstrated that VEGF-targeted therapy can induce antivascular effects [40]. Yet, the exact molecular effects that VEGFR2 inhibitors exert on the vasculature, the consequences for endothelial cell behavior, and their relation with the eventual antitumor outcome remain elusive (Fig 2). Design of effective antiangiogenic treatment strategies for cancer and identification of biomarkers for efficacy requires detailed knowledge of these underlying molecular events in the tumor microenvironment in vivo, as mimicking of the complex in vivo condition in vitro is not feasible [41].
The research described in this thesis aimed to identify the molecular nature of the tumor vascular phenotype, and to gain more insight into the changes therein in vivo in response to antiangiogenic therapy (Fig 3). We investigated how angiogenic activation of endothelial cells influences the efficacy of VEGFR2 inhibitor treatment, and how VEGFR2 inhibitor therapy changes the tumor vascular phenotype in relation to its antitumor effect. Our ultimate goal is to contribute to the design of more efficient antiangiogenic therapies, for example based on combination strategies that attack multiple aspects of vascular behavior in the tumor.

In Chapter 2, we provide an overview of the current knowledge of the nature of heterogeneity in microvascular behavior in pre-clinical and clinical tumors. We furthermore review recent insights into the in vivo molecular activation status of the
Figure 3: Schematic representation of the experimental design employed in the research described in this thesis. We analyzed the in vivo tumor microvascular phenotype of B16.F10 melanoma growing in C57bl/6 mice by comparing gene expression in the tumor endothelium to that in non-angiogenic endothelium of healthy kidney venules, and we zoomed in on the molecular basis of differences in vascular morphology. Furthermore, we investigated the effect of VEGFR2 inhibitor treatment on tumor vascular gene expression in B16.F10 melanoma and Lewis Lung Carcinoma models. Vascular cells were isolated from their surrounding tissue by laser microdissection respectively by enzymatic digestion that was followed by incubation with magnetic beads conjugated with antibodies to CD31 (αCD31 + bead), to separate the endothelial (oval shaped) cells from the tumor (round shaped) cells. Expression of a variety of genes involved in endothelial cell behavior was quantified in a real-time reverse transcriptase-PCR set up. EC = endothelial cell.
tumor endothelium and outline the current understanding of the way that antiangiogenic drugs affect tumor endothelial cells in relation to their antitumor effects.

We hypothesized that variations in the angiogenic activation status of endothelial cells \textit{in vivo} would be brought about by spatiotemporal differences in the local expression patterns of angiogenesis- and vascular maturation-related genes during tumor outgrowth. In Chapter 3, we quantified the expression of angiogenesis-regulating genes in three different growth stages of a subcutaneously growing orthotopic B16.F10 mouse melanoma model. We furthermore zoomed in on the tumor vascular compartment by laser microdissection to obtain a more detailed view on the localization of angio-gene expression in the complexity of the \textit{in vivo} microenvironment, and related this to vascular morphology, pattern of pericyte association and proliferation status.

In a tumor, endothelial cells are continuously exposed to dynamically changing levels of VEGF, leading to angiogenic activation of the endothelial cells. In Chapter 4, we mimicked angiogenic activation \textit{in vitro}, and investigated the consequences of VEGFR2 inhibitor treatment \textit{after} activation by VEGF for its pharmacological efficacy. We furthermore analyzed the underlying kinetics of phosphorylation of VEGFR2 and its downstream kinases, ERK, p38, and Akt in response to VEGF stimulation and VEGFR2 inhibitor treatment.

In Chapter 5, we analyzed the compartmentalized effects of treatment with the VEGFR2/EGFR inhibitor vandetanib (ZD6474) on morphology and gene expression of the vasculature of a subcutaneously growing Lewis Lung Carcinoma. Based on the observed changes in gene expression, we focused on the effects of VEGFR2 inhibitor therapy on endothelial-pericyte adhesion and the pattern of pericyte coverage. In order to understand whether the observed changes in vascular behavior were a direct result of antiangiogenic treatment, or secondary to treatment, we assessed the immediate changes in gene expression that occur upon a single dose of vandetanib. In Chapter 6, we compared the effect of vandetanib on vascular architecture and angio-gene expression in Lewis Lung Carcinoma with that in B16.F10 melanoma. Based on the observed differences in response to vandetanib between these two models, we examined the effects of vandetanib on target protein expression level and localization. Furthermore, we assessed a possible role for a newly identified VEGFR2-controlling microRNA, miR-296, in the observed changes in VEGFR2 expression upon vandetanib treatment.

Finally, the results of the research described in this thesis are summarized and discussed in a broader context, and the future perspectives of our studies for further development of antiangiogenic cancer therapy are delineated in Chapter 7.
REFERENCES

1. World Health Organization. 2010. Internet Communication


