Review Article

Angiogenesis in cancer: anti-VEGF escape mechanisms

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Abstract: It is now widely accepted that tumor-angiogenesis plays a crucial role in tumor growth, tumor propagation and metastasis formation. Among several angiogenic activators, the vascular endothelial growth factor (VEGF) and its receptors represent one of the major inducers of tumor angiogenesis. Thus, this system has become the focus of therapeutic interventions, which led to the approval of the anti-VEGF blocking antibody bevacizumab and the VEGFR-2 pathway inhibitors pazopanib, sorafenib and sunitinib. However, not every cancer patient benefits from such treatment or finally becomes resistant to anti-VEGF approaches; others are suffering from adverse effects. Thus, there is an urgent need for a better understanding of VEGF-independent mechanisms leading to angiogenesis in cancer. This review focuses on anti-VEGF escape mechanisms of tumor cells and its microenvironment.

Keywords: Vascular endothelial growth factor (VEGF); angiogenesis; cancer; endothelial cells

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Introduction

Angiogenesis, the growth of new blood vessels, is important in the pathogenesis of malignant, infectious, fibro-proliferative, and inflammatory diseases. Angiogenesis in tumors was first described 100 years ago (1) and 1971 J. Folkman proposed that tumor growth and metastasis are angiogenesis-dependent and blocking angiogenesis could be a strategy to block progress disease (2). Meanwhile, it is widely accepted that pre-cancerous tissues acquire angiogenic capacities on the way to become cancerous. Thereby, the net balance between pro-angiogenic and anti-angiogenic molecules is tipped to favor angiogenesis (the 'angiogenic switch'). In fact, tumors produce pro-angiogenic growth factors. Among them the vascular endothelial growth factor, VEGF, is thought to be the most important as VEGF acts pro-angiogenic by augmenting all steps of angiogenesis: vascular permeability, which allows exudation of plasma proteins that lay down a provisional scaffold for detached endothelial cells, endothelial cell proliferation, endothelial cell migration or invasion into the surrounding tissue and finally capillary-like tube formation. Thus, VEGF-A is the major regulator of physiological and pathological angiogenesis. While VEGF under physiologic conditions is a hypoxia-regulated gene via binding of HIF to its promoter (3), VEGF is highly over-expressed in a variety of tumors (4). VEGF-A specifically binds to endothelial cells either via VEGFR-1 (flt-1) or VEGFR-2 (flk-1), but was recently described also to bind to neuropilin-1, or-2, whereby the latter ones are thought to mediate VEGF signaling leading to enhanced endothelial cell migration (5). Thus, various inhibitors interfering with VEGF/VEGFR have received FDA approval and are currently in clinical use; beside the monoclonal antibody that blocks human VEGF bevacizumab (Avastin; Genentech) (6), also small-molecule inhibitors of the VEGFR-2 tyrosine kinase such as sorafenib, sunitinib, and pazopanib are approved for certain tumor types (7). The introduction of anti-angiogenic therapies have been demonstrated to prolong progression-free survival (PFS) and/or overall survival (OS), and improves objective tumor responses, in patients with advanced malignancies such as in renal cell cancer, non-small-cell lung cancer (NSCLC), ovary and colorectal cancer (8-10). However, not all patients benefit from such anti-angiogenic therapy, and those tumors that initially respond to treatment will finally become resistant to VEGF-based therapies and relapse (11). Therefore, the development of more durable anti-angiogenic therapies requires an improved understanding of the cellular and molecular mechanisms that mediate resistance to anti-angiogenic agents.

There is increasing evidence that blockade of the VEGF/VEGFR-2 signaling pathway leads to an upregulation of

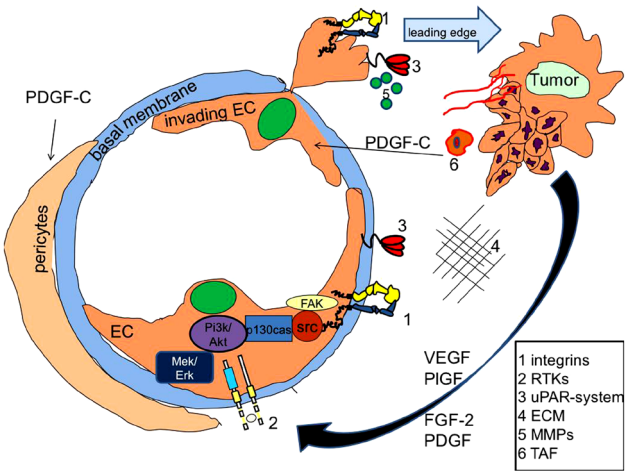

Figure 1 Cartoon illustrating tumor-induced endothelial cell activation. Growth factors secreted by tumor cells bind to receptor-tyrosine kinases, which lead to induction of intracellular signaling. As a consequence, endothelial cells become polarized and start to transmigrate through the basal membrane into the surrounding matrix. Endothelial cells express integrin adhesion molecules in newly formed focal adhesions as well as the proteolytic enzymes at the leading edge as a prerequisite for efficient cell motility. RTK, receptor tyrosine kinase; uPAR, urokinase-type plasminogen activator receptor; ECM, extracellular matrix; MMP, matrix metalloproteinases; TAF, tumor-associated fibroblasts.

protein expression of secondary molecules in order to sustain the neo-vascularization response (12), whereby in particular components of the EGFR and FGFR pathways were shown to be upregulated in the tumor stroma (13). Further suggested mechanisms include genetic alterations of p53, which uncouples tumor dependency on a vascular blood supply (14). Other studies have shown that tumor cells may alter their pattern of growth when challenged with anti-angiogenic therapy by meeting their metabolic requirements via residing in close proximity to preexisting blood vessels (15). The key systems beyond VEGF/VEGFR-2 responsible for angiogenic endothelial cell behavior are discussed here, which will give an overview about most prominent mechanisms of escaping VEGF-targeting approaches in tumor angiogenesis.

Growth factors

FGF-2

The family of fibroblast growth factors (FGF) affects neurogenesis, organ development, branching morphogenesis, angiogenesis, but is also involved in the pathogenesis of cancer. Especially for tumor growth and angiogenesis, FGF-2 by binding to its receptor FGFR-1 (*Figure 1*), is thought to be the major family member as gene silencing by antisense targeting of FGF2 and FGFR-1 in models of human melanoma caused a dramatic reduction in the size of tumors (16). Other studies have given first evidence that FGF-1 and FGF-2 are able to affect angiogenesis in a VEGF independent manner, as both molecules were upregulated in tumors treated with anti-VEGFR-antibodies in a RIP-Tag model. Thus, together with an FGF-trap, anti-VEGF treatment was able to decrease tumor growth and lessen tumor angiogenesis (17,18). This was further supported by findings derived from clinical trials with FGF-2 affecting agents such as thalidomide or suramin, which demonstrated a therapeutic benefit in advanced prostate as well as renal cell cancer (16).

PDGF

The platelet derived growth factor (PDGF) is frequently upregulated in tumors and has recently been shown to synergistically with FGF-2 promote tumor angiogenesis and pulmonary metastasis. While overexpression of PDGF-BB alone in tumor cells resulted in dissociation of VSMCs from tumor vessels and decreased recruitment of pericytes, FGF-2 triggered PDGFR-α and-β expression at the transcriptional level in ECs, which acquire hyper-responsiveness to PDGF-BB (19).

However, increased expression of PDGF-BB has been demonstrated to reduce colorectal as well as pancreatic cancer growth via increasing the pericyte content of the tumor microenvironment, thus single-agent therapy targeting PDGF receptor might fail to show any benefit in cancer (20). This might explain the fact that tyrosine kinase inhibitors, which are characterized to be of broader substrate specificity, such as pazopanib (targeting PDGFR-a/b, but also the VEGFR-family and c-kit), is effective in some cancers.

Delta/Jagged family

Delta l-like ligand 4 (Dll4) is a member of the Delta/Jagged family of transmembrane ligands binding to Notch receptor. The delta-Notch pathway may not only have an effect on the regulation of artery-vein differentiation but is also able to stimulate blood vessel formation. Furthermore, it mediates cell-cell communication and controls cell determination, thus it is a major player in regulating proper vascular development (21). Recent studies have demonstrated that Dll4 knock-out mice have abnormal vessel sprouting, while blockade of Dll4-Notch signaling in tumors results in excessive, non-productive angiogenesis with resultant inhibitory effects on tumor growth, even in some tumors that are resistant to anti-VEGF therapies (22). Dll4 inhibitors are entering clinical trials and might provide novel therapeutic tools in VEGF/VEGFR inhibitor resistant tumors.

Angiopoietin system

The signaling system initiated by angiopoietin in endothelial cells,

which express its receptor Tie2, advocates the PI3K-pathway resembling the survival signal. Ang2's role in angiogenesis generally is considered as an antagonist for Ang1, thereby inhibiting Ang1-promoted Tie2 signaling, which is critical for blood vessel maturation and stabilization. Ang2's role in tumor angiogenesis, however, is controversial. While upregulation of Ang2 correlates with malignancy of various types of human cancers, because Ang2 overexpression augmented tumor angiogenesis and growth in mice, other studies have reported that specific induction of Ang2 in gliomas, mammary carcinomas, and lung carcinomas inhibited tumor growth and metastasis (23). Pre-clinical trials have revealed controversial results: while treatment of mice bearing xenografts of human A431 epidermoid tumor and Colo205 colon cancer with neutralizing anti-Ang2 antibodies led to growth reduction and regression of large established tumors by suppressing cell proliferation and inducing apoptosis of tumor-derived ECs, overexpression of Ang2 attenuated VEGF expression, impaired pericyte coverage of the tumor vasculature, inhibited EC proliferation, and induced EC apoptosis, resulting in a massive regression of tumor vasculature and tumor growth (24). Thus, a better understanding of Ang2 functions in tumor angiogenesis and progression is mandatory for future advances in effective anti-angiogenic approaches.

Subcellular systems

The proteolytic system

Activated endothelial cells emigrate from their residential site, a process which requires polarization of endothelial cells and transmigration through the basal membrane into surrounding matrix. Especially for the latter step, proteolytic degradation of matrix proteins is essential, whereby the plasminogen system, the matrix-metalloproteinase (MMP) system, as well as the heparanase and chymase families are thought to be important. Thus, inhibition of MMP-2 binding to integrin αvβ3 by the non-catalytic MMP-2 fragment PEX was shown to inhibit tumor angiogenesis (25).

A large body of experimental evidence from in vitro and *in vivo* data as well as from the clinics indicates an important role of the urokinase (uPA)/plasminogen system in angiogenesis and cancer. E.g., it was demonstrated that inhibition of functional activity of the receptor of urokinase-type plasminogen activator, uPAR, significantly decreased the invasive potential of endothelial cells (26-28), and the absence of the host plasminogen activator inhibitor-1 (PAI-1) prevented cancer invasion and metastasis (29). Consistently, PAI-1 correlates with poor prognosis in cancer patients, probably by preventing excessive proteolysis or other not yet defined mechanisms (30). In this context we and others could recently reveal that this system is essential for degrading surrounding matrix proteins at the leading edge, but also coordinates the redistribution of proteolytic as well as adhesive proteins to newly formed focal adhesions. Especially migrating cells continuously form focal contacts at the leading edge by new integrin-matrix interactions. The cell matrix contacts persist until they reach the trailing end, where integrins have to release their ligands in order to allow cell locomotion (31,32). Thereby, integrins become internalized and recycle back to the leading edge during cell migration (33). Although it is still unclear how integrins are internalized, the involvement of clathrin-coated vesicles has been suggested (34). It was suspected that the NPXY motif in the cytoplasmic tail of beta subunits might be responsible for integrin signaling and internalization (35,36); however, the internalization process of integrins was not affected by point mutations of NPXY (37). We and others observed that uPAR, which interacts with the fibronectin receptors α3β1 and α5β1 integrins (38) or with integrin αvβ5 or αvβ3 (39) tracked integrins into the endocytotic compartment via clathrin coated pits (40). In detail, we revealed a mechanism by which in endothelial cells VEGF-A and VEGF-E rapidly induced pro-urokinase (pro-uPA) activation on the surface of endothelial cells (41). This involved a phosphatidylinositol 3-kinase (PI3-kinase)-dependent change in integrin affinity, leading to activation of proMMP-2 and pro-uPA, when pro-uPA is bound to its surface receptor uPAR. As a consequence, this VEGF-induced pro-uPA activation on endothelial cells was responsible for VEGF-dependent local fibrinolytic activity and might be one of the initial steps in matrix degradation during the angiogenic process. Furthermore, active uPA forms complexes with its inhibitor PAI-1, which-when bound to uPAR-can be internalized and degraded. Internalization is performed via a member of the LDL receptor family (28,42), involving clathrin-coated vesicles formation. Thereafter, uPAR itself can recycle back from the endocytotic compartment to the cell surface (43). In VEGF-stimulated endothelial cells we were able to show that pro-uPA activation not only led to extracellular matrix degradation, but-as a consequence-led to a coordinated internalization of uPAR by an LDL-receptor like molecule. Data obtained from PAI-1-/-cells indicated that uPAR internalization in response to VEGF is PAI-1-dependent, which is consistent with the prerequisite of an uPAR/uPA/PAI-1 complex formation. As a consequence we were able to show that uPAR recycles back to the cell surface via a coordinated process leading to focusing of uPAR to newly formed focal adhesions at the leading edge (28). Internalization and target oriented recycling of uPAR to the leading edge plays a role in growth factor-induced endothelial cell migration, because cleavage of the GPI-anchor of uPAR, via which uPAR is fixed to the cell surface, diminished the migratory response significantly. This mechanism is not limited to VEGF165, but is induced by a variety of different growth factors; however, it might also get bypassed such as by placental-like growth factor (PlGF), which did not induce pro-uPA activation on the endothelial cell surface and consequently led not to uPAR internalization and recycling to the leading edge (28).

That this system is not only required for endothelial cell

migration, but also endothelial cell survival was shown in uPA (-/-) endothelial cells. Only when uPA was expressed, growth factor activated endothelial cells were protected against apoptosis, which was provoked via transcriptional up-regulation and partially by mRNA stabilization of inhibitor of apoptosis proteins, most prominently the X-linked inhibitor of apoptosis protein (XIAP). Thereby, the antiapoptotic activity of uPA was dependent on its protease activity, the presence of uPAR and LRP, but independent of the PI3kinase pathway, whereas VEGF-induced anti-apoptosis was PI3kinase dependent (44).

The uPAR-system itself is tightly regulated on a transcriptional level. Thus, Michael S. Pepper described first that pro-angiogenic growth factors led to a transcriptional upregulation of uPAR (45). In this context, we recently observed that uPAR expression on the surface of endothelial cells is only up-regulated when cells were in a sub-confluent state, but was down-regulated when cells reached confluence. Thereby, the receptor-like protein tyrosine phosphatase DEP-1 (density enhanced phosphatase-1/CD148), which is abundantly expressed in confluent endothelial cells, inhibited activation of MEK/ERK1/2 pathway, which is the major signaling pathway for transcriptional upregulation of uPAR expression. Consistently, overexpression of active ERK1 rescued the DEP-1 effect on uPAR. Hence, DEP-1 plays a biologic role in angiogenic endothelial cell behavior, such as in endothelial cell migration, proliferation, and capillary-like tube formation (46).

Integrin adhesion molecules and their role in angiogenesis

Integrin adhesion receptors are heterodimeric molecules, which mediate cell-matrix interaction, thereby affecting cellular behaviors such as cell migration, proliferation, differentiation and cell survival. Thus, the biological role of integrin over-expression and activation in human cancer was elucidated: via induction of major signal transduction pathways, which lead to cancer cell invasion, metastasis as well as enhanced angiogenesis, integrins play a central role in all phases of tumorigenesis (47). Thus, a coordinated regulation of integrin expression and activation mediates the functional role adapted to specific conditions. During embryonic development the accurately patterned network of blood vessels in addition depends on the superior regulation of integrin activation, which is reduced by diverse chemo-attractants, for example growth factors that regulate angiogenesis. A disruption of this precision adjustment is expected to occur in cancer (48).

Blood vessel formation is decisively reliant on extracellular signaling as well as their connection with the extracellular matrix (ECM) proteins, such as fibronectin, fibrinogen, vitronectin, laminin as well as collagens. Consistently, endothelial cell migration as well as cell survival is regulated by integrins (49,50). The attachment to the ECM is adjusted by integrin adhesion receptors; integrins operate as bidirectional transducer molecules by matching signals from both, the outside to the inside (outside-in) of the cell, or from the inside to the outside (inside-out). Both signaling directions are tightly regulated in focal adhesion to support cell adhesion, spreading and motility (51).

Integrin interaction partners

As heteromeric adhesion receptors integrins consisting of an α and a β chain, and are expressed in most of human cell types. 24 different αβ combinations can be formed by 18 α subunits and eight identified β subunits and are paired to function as distinct receptors (52). These integrin subunits have a large extracellular domain, a single transmembrane domain and a short cytoplasmic domain (53). The α and β subunits of integrins are associated via non-covalent bonds. Integrins are also able to affect cell behavior by interfering with receptor tyrosine kinases (RTKs) such as fibroblast growth factor receptor (FGFR) (54). Many ligands of integrins are recognized via their Arg-Gly-Asp (RGD) sequence motif such as for αvβ3 and αvβ5, whereas α4β1 recognizes Glu-Ilu-Asp-Val (EILDV). They may also interact with cell surface receptors, like Vascular Cell Adhesion Molecule-1 (VCAM-1) (55). In the cytoplasmic tail of β-integrins interaction with the FERM domain of talin led to high-affinity conformation of integrins, which enables ligand binding and linking the ECM to the cytoskeleton (56).

Adhesion-dependent signal transduction via integrins

Integrins have to interact with several kinases as well as related adaptor proteins since they have no intrinsic kinase activity. Subsequently, the β tail is adjuvant as the major site in the arrangement of focal adhesions. By the recruitment of signaling components such as pp125 Focal Adhesion Kinase (FAK), integrins can activate "outside-in" signaling thereby creating and communicating signals that can induce cell migration or cell proliferation (57). Hereby the non receptor protein kinase FAK is phosporylated at tyrosine 397, leading to activation of src family kinases (SFK) which control not only the activity of Rho GTPases but also downstream kinases such as AKT, thereby affecting endothelial cell proliferation, migration and survival (58,59).

Two cytokine-related pathways affect integrin-dependent angiogenesis. One is either induced by fibroblast growth factor (FGF) or tumor necrosis factor α (TNF-α) and needs the function of integrin αvβ3, whereas the other one requires αvβ5 and is initiated by vascular endothelial growth factor (VEGF) or transforming growth factor α (TGF-β) (60). The distinction is given by the fact that β3 and β5 can affect the activation of Ras/Raf/MEK/Erk pathways in the vasculature in different manners. Thus, the αvβ5 related pathway downstream from VEGF activates focal adhesion kinases (FAK) and Src kinases, however αvβ3 integrins is able to activate p21 kinases (61). In

addition αvβ3 induces activation of Raf at tyrosine 340/341 and Mek dependent protection from extrinsic mediated apoptosis, which is initiated by pro-apoptotic ligand binding to receptors, including TNF-α and Fas (62). On the other hand, αvβ5 affected pathways activate Raf on serine 338/339, which leads to Raf-1 mitochondrial translocation and shelter from the intrinsic pathway of apoptosis (62).

Interfering with angiogenesis via integrin family members

A range of integrins control endothelial cell functions such as cell proliferation, survival, and migration and invasion during angiogenesis, whereby various integrins have been functionally related, such as α1β1, α2β1, α4β1, α5β1, α6β1, α6β4, α9β1, αvβ3 and αvβ5 (63). A major regulator for developmental angiogenesis and blood vessel formation is resembled by fibronectin (64). Its receptor, the β1 integrin is an essential mediator in angiogenesis, as the genetically knock out of β1 integrins led to embryonic lethality or blocked blood vessel development in teratomas (65,66). Another fibronectin binding partner, α5 is also crucial, because its loss led to developmental deviations and vascular deformation (67). α5β1 integrins have been described to be overexpressed in endothelial cells of malignant tumors, suggesting their functional role in tumor angiogenesis (68,69), which was supported by the fact that targeting α5β1 was able to block tumor-angiogenesis (68). Even for normal vasculature development α5 is thought to be essential as its genetical knock out was embryonic lethal at day E10-E11 with abnormalities in the vascular development (70). In contrast, patients suffering from the Glanzmann thrombasthenia, which is characterized by null mutation in integrin β3 subunit, do not have any aberrations in angiogenesis and vascular development. However, knockout studies of αv, including αvβ3, αvβ5 and all other αv combinations, revealed a deficiency in vascular integrity (71). It was hypothesized that αvβ8 is the key αv integrin as vasculature abnormalities aroused only in β8 integrin knockouts, but not in β5 or β3 knockout mice. β3 knockout as well as α3/β5 double knockout mice revealed improved tumor angiogenesis (72), probably via upregulation of receptor tyrosine kinases.

Antagonists for α5β1 are already under investigation for cancer therapies (73). αvβ3 integrins are mainly expressed by proliferating and activated vascular endothelial cells and is consequently a main molecule for the arrangement of vasculature, supporting migration and survival of endothelial cells (74). The activation can either be induced by cytokines or within the influence of a malignant tumor whereas blocking αvβ3 integrins restrains tumor angiogenesis in addition to blood vessel formation of *in vivo* models (60,75,76). Subsequently, αvβ3 might represent a potential target in anti-angiogenic therapy. Integrins α1β1 and α2β1, which bind to laminin and collagen, seemed to be indispensable for angiogenesis (77), because functional blocking α1β1 and α2β1 integrins inhibited angiogenesis in vitro, as well as it reduced tumor growth and angiogenesis *in vivo* (78-80). Current concepts of anti-integrin therapeutics are focused on targeting directly or indirectly the receptor binding sites, although new functional insights propose alternative approaches such as targeting most upstream integrin signaling (81).

Reduced toxicity has been recognized for various antagonists of αvβ3 integrins as they have been seen to be only expressed on activated or remodeling tissues such as tumors. Among these antagonists, abergrin (vitaxin, Medi-522) is a humanized antibody against αvβ3 integrins, and was the first anti-integrin approach introduced into clinical trials for malignant tumors (82). This antibody blocks binding to vitronectin and fibrinogen, prevents cell adhesion, migration, proliferation and integrin mediated cell signaling in a dose dependent manner (83). Tumors of patients with a high expression of αvβ3 had a poorer prognosis than patients with lower expression levels. Consistently, high expression of αvβ3 in tumors was accompanied with an aggressive phenotype and augmented metastatic potential and invasive traits. Furthermore, association of αvβ3 with Src promoted attachment-independent autonomous cell growth while αvβ3 integrin mediated FAK signaling fostered the survival of cancer stem cells (84).

α5β1 integrin antagonists are under development for treatment of malignant tumors. Volociximab is a chimeric human-mouse monoclonal antibody binding to human α5β1 integrins (85). Volociximab is able to induce cell death and prevents capillary tube formation *in vitro*, thereby inhibiting signaling cascades. Because there is no effect on cell proliferation when combined with a VEGF blocking antibody, it was suggested that volociximab induced blocking of α5β1 is downstream of the VEGF induced pathway. *In vivo* volociximab showed also anti-tumor as well as anti-angiogenic capacities (86). In a Phase II study, volociximab showed promising potential efficacy in prolonging disease progression and overall survival. In clear cell renal cell carcinoma, volociximab delayed disease progression up to 22 months. Additional Phase II and III trials, where volociximab is used as single agent as well as in combination regiments, are currently ongoing to test its effect in advanced melanoma, non-small cell lung cancer and peritoneal cancer (85).

Because integrin αvβ3 and αvβ5 both regulate angiogenesis a human monoclonal antibody against both integrins was developed, called CNTO 95. It showed preclinical activity by reducing angiogenesis and tumor growth in human melanoma xenografts in nude mice and rats (87). CNTO 95 was recently tested in a clinical Phase I/II trial for the treatment of melanoma (88). αv integrin targeting is able to inhibit both, tumor cell invasion and metastasis as well as tumor angiogenesis (89,90).

During adulthood the majority of blood vessels remain quiescent, and angiogenesis occurs only during embryonic development. Whereas in many diseases angiogenesis processes are activated, resembling tumor growth and metastasis as well as ocular and inflammatory diseases. Huge advances have been

made over the last two decades in finding and engineering integrin targeting therapeutics, as integrin expression or functions are altered in many disorders. Progress in the structural characterization of the integrin-ligand complex led to the development and design of powerful and selective blocking agents for a variety of integrins, thereby targeting angiogenesis, metastasis and tumor growth.

The tumor microenvironment

Tumor cells have long been thought to be the main source of angiogenic factors (4,91). However, growing evidence now indicate that the tumor microenvironment, which consist of fibroblasts, pericytes, mesenchymal-stem cells and inflammatory-immune cells also contributes to angiogenesis via secretion of angiogenic molecules as well as via modulation of the immune system (92). In this context Bv8, also known as prokineticin 2, has recently been shown to be a mediator of myeloid cell-dependent tumor angiogenesis. In detail, myeloid cells defined by the expression of CD11b+ and Gr1+ have been associated with refractoriness to anti-VEGF therapy (93,94). Thus, the bone marrow derived CD11b+ and Gr1+ cells are frequently increased in the tumors and the peripheral blood of tumor bearing animals as well as in humans (92), thereby promoting angiogenesis (95). In several xenograft models as well as transgenic cancer models, it was demonstrated that CD11b+ and Gr1+ cells produce Bv8, which led to an enhanced peripheral mobilization of myeloid cells and local stimulation of angiogenesis (96,97). Thereby G-CSF, which is a well known inducer of granulocytic-progenitor cell differentiation and mobilization, was identified to be a strong inducer of Bv8 expression. Although discussed controversial, administration of G-CSF has been correlated to enhanced tumor angiogenesis and growth (98,99). Consistently, whenever G-CSF or Bv8 were targeted by administration of blocking inhibiting antibodies, reduced tumor angiogenesis and growth was observed. Notably, the effect was additive to anti-VEGF mAb in refractory tumors and treatment with G-CSF resulted in increased CD11b+ and Gr1+ cell numbers and reduced responsiveness to anti-VEGF therapy (94).

Fibroblasts in the tumor-microenvironment

Tumor associated fibroblasts (TAFs) have been shown to promote angiogenesis mainly via stromal cell-derived factor 1 (SDF-1) expression. Thereby, SDF-1 recruits endothelial progenitor cells (EPCs) into carcinomas and contributes to tumor promotion (100). That TAFs derived from anti-VEGF treatment resistant tumors can induce angiogenesis independent of VEGF was shown by Crawford Y, *et al*. (101), who demonstrated that platelet-derived growth factor C (PDGF-C) was upregulated in TAFs from resistant tumors, and PDGF-C-neutralizing antibodies blocked the angiogenesis induced by such TAFs *in vivo*. This demonstrated that PDGF-C does not require VEGF activity to fulfill its angiogenic function. A combination therapy using both anti-PDGF-C and anti-VEGF antibodies was more effective in inhibiting tumor angiogenesis than using anti-VEGF treatment alone. Notably, PDGF-C has direct effects on all three types of vascular cells, endothelial cells, pericytes and smooth muscle cells, by promoting their proliferation, survival and migration, but might also affect fibroblasts themselves as well as inflammatory cells (102). In this context, it is notably that tumors, which highly express PDGF-C, such as pancreatic cancer, do not respond towards VEGF targeting treatment regiments (4,103,104), thus, it is tempting to speculate whether targeting PDGF-C would lead to a therapeutic response. However, neither sorafenib nor imatinib has so far been demonstrated to be effective in pancreatic cancer (105,106). This might be explained by the observation that interfering with PDGF-C induced pericyte recruitment leads to pro-motion of metastasis (107).

Clinical aspects

Inhibitors against the VEGF/VEGFR-system are already in clinical use. As only a certain number of patients suffering from cancer respond to such therapy, efforts were attempted to targeted angiogenesis by novel strategies. Thus, a variety of compounds, which revealed promising pre-clinical results, were tested in clinical phase I to III studies, whereby the most important drugs, if not mentioned above, are discussed here:

Aflibercept

Aflibercept is a fusion protein with sequence homologies with native VEGFR-1 and VEGFR-2 and was specifically designed to bind several variants of VEGF (108). The soluble VEGF receptor fragment was tested in Phase II trials described minor single agent activity in platinum-and erlotinib-resistant adenocarcinoma of the lung and metastatic urothelial cancer, respectively (109,110). However, a recent Phase III trial (VELOUR trial) evaluated effects of aflibercept with irinotecan/5-FU as second line chemotherapy for metastatic colorectal cancer and experienced extended progression free survival and overall survival suggesting it for clinical approval (111). A subgroup analysis revealed that aflibercept was also effective in patients pretreated with the anti-VEGF antibody bevacizumab. This interesting finding might be explained by the fact that aflibercept does not only bind to VEGF165, but also to PlGF, and other splicing variants of VEGF-A.

Vandetanib

Vandetanib, a once-daily oral inhibitor of RET kinase (an abbreviation for "rearranged during transfection"), EGFR as well as VEGFR signaling, has previously shown antitumor activity in a Phase II study of patients with advanced hereditary medullary

thyroid carcinoma (MTC) (112). Furthermore, in a double-blind Phase III trial 231 patients suffering from advanced MTC who received vandetanib showed significant progression free survival (PFS) rates when compared with the placebo control group. The role of vandetanib was also investigated in the second-line treatment for advanced non-small-cell-lung cancer patients: A meta-analysis by Wei-Xiang Qi *et al*. (113) of randomized controlled Phase III trials, showed significant improvement of PFS and overall response rate (ORR) but no enhancement of overall survival. The superior response towards vandetanib may underlie the importance of the combined blockade of RET kinase and growth factor receptors, all involved in MAPK and PI3K/Akt signaling, which is not precisely disclosed yet, but gives hope for beneficial effects in other cancers. Furthermore, in patients suffering from advanced breast cancer, vandetanib in combination with docetaxel was already tested in a Phase II trial showing promising results (114).

Cilengitide

Another αvβ3 antagonist is cilengitide (EMD 121974), a cyclic RGD-peptide inhibitor of αvβ3 and αvβ5 integrins. Significantly enhanced progression free survival was observed in a Phase I/IIa clinical trial for glioblastomas (115). Cilengitide is now in clinical Phase III studies for glioblastomas and in Phase II studies for several other tumor identities including breast cancer, squamous cell cancer, non-small cell lung cancer and melanoma (116-119). Cilengitide is able to detach cells that have attached to the matrix at concentrations nearly equal to concentrations that inhibit cell attachment. Cilengitide decreases cell growth, cell proliferation and cell survival in vitro. Moreover, it blocks FAK-Src-Akt and Erk signaling pathways (120,121). Cilengetide is therefore able to influence embryonic as well as established tumor vasculature. As the communication of tumor cell with endothelial cells is still unclear, additional experimental advances have to be done to get more insights into this complex interplay (81). Other integrin inhibitors are discussed above.

Pazopanib

Since Phase I and II trials have shown partial responses and well tolerability, a randomized, double-blind, placebo-controlled Phase III study was assessed to investigate efficacy and safety of pazopanib monotherapy in patients with advanced renal cell carcinoma (RCC) (122,123). Results indicated significant prolonged PFS with pazopanib compared with placebo in the overall study population [median, PFS 9.2 *vs*. 4.2 months; hazard ratio (HR), 0.46; 95% CI, 0.34 to 0.62; P<0.0001](124). Therefore in 2009, the Food and Drug Administration (FDA) granted approval to pazopanib for the treatment of patients with advanced renal cell carcinoma.

Because of its potent and selective multi-targeted receptor tyrosine kinase inhibitor characteristics for VEGFR-1, VEGFR-2, VEGFR-3, PDGFR-a/β, and c-kit (125), pazopanib might also effect the outcome of other tumors and was consequently tested in ovarian-and breast cancer as well as advanced soft tissue sarcoma in several trials suggesting further investigations and rising hope for new treatment indications (126-128).

Thalidomid and thalidomide-derived immunomodulatory drugs (IMiDs)

Initially used because of its sedative effects, thalidomid has long been known for its adverse properties in development such as phocomelia. Intense investigations, however, have revealed many immunomodulatory functions (129).

Additionally, in hematologic malignancies, thalidomid is an upcoming drug showing inhibition of endothelial cell activation via VEGF, FGF-2 and others (130). Especially in myeloma patients, thalidomide was shown to suppress VEGF secretion in bone marrow endothelial cells (129). In a Phase II clinical trial, 169 refractory myeloma patients were treated with thalidomide monotherapy (131). 30% of patients showed a partial response, reflected by 50% paraprotein reduction, and 14% achieved a nearly complete remission with two-year event-free and overall survival rates around 48%.

These findings resulted in many clinical trials of thalidomide, finally leading to FDA approval in 2006 for the treatment of newly diagnosed multiple myeloma in combination with dexamethasone. In a respective Phase III trial, the response rate in patients receiving thalidomide plus dexamethasone was 63% compared to 41% with dexamethasone alone (P=0.0017) (132).

Besides suppressing VEGF secretion, thalidomide is also known to inhibit NFkB signaling, an important pathway in prostate cancer. A randomized Phase II trial found remarkable PSA decline (53% *vs*. 37%)-and an overall median survival rates (68.2% *vs*. 42.9%) in 74 patients with metastatic androgen-independent prostate cancer when treatment with docetaxel plus thalidomide *vs*. docetaxel alone (133).

Angiostatin/Endostatin

Potent endogenous angiogenesis inhibitors like angiostatin, a plasmin fragment, or endostatin, derived from type XVIII collagen, are currently under investigations for clinical usage. Recent promising results in mice have shown that angiostatin is not only reducing angiogenesis in combination with bevacizumab but also decreases a possible bevacizumab-induced invasion of U87 glioma cells (134). Angiostatin shows multiple ways of inhibiting angiogenesis like diminishing of ERK activation by basic fibroblast growth factor and vascular endothelial growth factor but there is still uncertainty on its mechanism of action (135).

In a randomized, open-label, Phase II study, two different doses (15 *vs*. 60 mg) of rh-angiostatin were subcutaneously administered in combination with paclitaxel and carboplatin to patients with advanced NSCLC (136). Partial response showed favorable results for higher dosage of angiostatin (54.5% when administered 60 mg

and 25% with 15 mg). One-year survival data of 45.8% are higher than in treatments with caboplatin and paclitaxel alone, which is additionally suggesting further studies for angiostatin.

Endostatin has long been known for its inhibitory effects on normal-and lymphangiogenesis (137,138). Inhibiting the expression of VEGF-A, VEGF-C and VEGF-D besides others, opens a wide array of therapeutic potential (139). A randomized, double-blind Phase III trial of rh-endostatin in the treatment of advanced non-small cell lung cancer patients showed significantly improved results that the drug was consequently approved by the State Food and Drug Administration of China (SFDA) in 2005 (140). The 486 patients in this multicenter trial were treated either with vinorelbine and cisplatin plus endostatin or vinorelbine and cisplatin plus placebo. Patients included in the endostatin group had increased overall response rates (35.4% *vs.* 19.5%; P=0.0003) and also showed enhanced median time to progression (TTP) of around 3 months.

The promising results rose hope also to be beneficial in other tumors affected by lymphangiogenesis and metastasis. However, results could not be confirmed in a Phase II study of advanced neuroendocrine tumor patients as treatment with rh-endostatin did not lead to a significant tumor regression (141). Recently, endostatin was shown to suppress growth and metastasis in a mouse xenograft model of colon cancer (139) opening the door for further studies.

Conclusions

Accumulated studies on VEGF-independent angiogenesis and its therapeutic implications using models of cell culture, tumor xenografts, as well as clinical investigations, have depicted several candidates as potential targets to interfere with tumor growth and metastasis formation. As not every cancer patient benefits from an anti-VEGF treatment or finally becomes resistant to such therapeutic approaches, there is an urgent need for a better understanding of VEGF-independent mechanisms leading to angiogenesis in cancer.

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