

In pursuit of new anti-angiogenic therapies for cancer treatment

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1. ABSTRACT

Despite advances in surgery, radiation therapy, and chemotherapy, patients with cancer have a poor prognosis. Sustained aberrant tumor angiogenesis and metastasis is a major obstacle for effective cancer treatment. Just a few years ago, few would argue that one of the key success stories of the modern cancer medicine were the anti-angiogenic drugs targeting the vascular endothelial growth factor (VEGF) signaling pathway approved by FDA. This initial success inspired many researchers to search for new anti-angiogenic targets and drugs with the hope that one day, anti-angiogenic therapy might really become the panacea for cancer patients. Unfortunately, the limited clinical benefits achieved with anti-angiogenic drugs conflicts with the widely accepted notion that angiogenesis is a key event in tumor progression. Emerging data indicate that unique characteristics of the tumor vasculature within the tumor microenvironment may hold the key for success of anti-angiogenic therapy. In particular, the molecular and cellular alterations that sustain aberrant tumor angiogenesis in the face of angiogenic inhibitors represents novel targets for rationally designing and improving current anti-angiogenic strategies.

2. INTRODUCTION

Angiogenesis is a multi-step process of new blood vessel formation from pre-existing vasculature including the disruption of vascular basement membranes; activation, proliferation, and migration of endothelial cells; remodeling of the extracellular matrix of tissues; formation of vascular tubes and networks; recruitment of supporting cells, e.g. smooth muscle cells and pericytes; and anastomosis with the pre-existing vascular network (1). In the embryo, vasculogenesis and angiogenesis serve to provide the growing organs with sufficient nutrients and oxygen. After birth, it is angiogenesis that contributes to organ development. Although during adulthood, most blood vessels remain quiescent, vascular endothelial cells retain their ability to divide rapidly in response to hypoxia (2). A growing body of evidence indicates that carcinogenesis not only requires malignant events such as accumulation of DNA mutations, escape from endogenous cell-cycle control and DNA-damage checkpoints (3), but also a tumor microenvironment to nurture tumor angiogenesis (1).

3. TUMOR MICROENVIRONMENT AND TUMOR ANGIOGENESIS

3.1. Tumor microenvironment

Tumors are complex tissues that contain an expanding population of tumor cells surrounded by tumor stroma including extracellular matrix, fibroblasts, immune cells, pericytes, adipocytes, epithelial cells, glial cells, and vascular endothelial cells. Collectively, this tissue is referred to as the tumor microenvironment. It has become clear that those non-cancerous cells within the tumor microenvironment are not passive bystanders. Throughout tumor progression, they are engaged in a complex interplay with tumor cells. The tumor microenvironment contains activated fibroblasts, which provide a provisional matrix and a source of growth factors (4). Various types of immune cells have competing antitumorigenic surveillance roles as well as pro-tumor growth, pro-angiogenic, and pro-tumor invasion roles (5). Vascular endothelial cells are recruited to the tumor microenvironment to form new vasculature (tumor angiogenesis) to meet nutritional and oxygen requirements (1).

3.2. Tumor angiogenesis and tumor vasculature

Initially, tumor growth is sustained by nutrients and oxygen through passive diffusion from the host vasculature (6, 7). Then the cores of solid tumors gradually suffer from low oxygen levels (8) and nutrient deficiency (9). To counteract this process, tumor cells evolve a complex process of angiogenesis to induce new vessel growth towards them from the adjacent host vasculature (10, 11). Angiogenesis is dependent on the balance of activators and inhibitors (12). Members of the vascular endothelial growth factor (VEGF) family are major angiogenesis activators (3, 13). Indeed, the tumor microenvironment continuously produces VEGF at high concentrations over long periods of time, thereby generating tumor vasculature that is composed of a mixture of different disorganized vessels (14), of which some are newly formed and the others have been present for a long time. Interestingly, some of the vessels induced by VEGF require continuous VEGF expression for their maintenance and undergo apoptosis if VEGF levels fall below threshold level (15), while others, once induced by VEGF, persist indefinitely in the absence of exogenous VEGF and therefore have lost their dependence on exogenous VEGF (16, 17).

Nevertheless, tumor angiogenesis markedly facilitates rapid tumor growth rate and increased metastatic potential. An intravital microscopy study revealed that normal vasculature with an appropriate ratio of surface area to volume is able to optimally provide oxygen and nutrients by diffusion to all normal cells (19). Unfortunately, the disorganized tumor vessels alter the ratio of the surface area to volume, which impairs nutrient and oxygen supply to tumor cells (18). Arteriovenous shunts existing throughout tumor vasculature also make tumor nutrient and oxygen supply inadequate (14). Furthermore, overall blood flow (perfusion rate) in many tumors is found to be an order of magnitude lower than that in normal tissues, owing to the abnormality in tumor vasculature (20, 21). Thus,

lower blood flow in the tumor compromises clearance of carbon dioxide and other metabolites. This is coupled with high tumor cell glycolytic activity and results in acidosis and further oxygen and nutrient deprivation. Thus oxygen deficiency and nutrient deprivation become two key stressors closely associated with tumor progression and tumor angiogenesis in the tumor microenvironment.

4. ANTI-ANGIOGENESIS STRATEGY IN CANCER TREATMENT

4.1. Anti-angiogenic therapy

Tumor angiogenesis is linked to a switch involving a change in the local balance between activators and inhibitors, and mainly depends on the release of endothelial-targeted angiogenic growth factors or the down-regulation of natural angiogenesis inhibitors (3). The long-standing proposition of inhibiting angiogenesis as a potential therapeutic intervention in cancer treatment has changed the landscape in contemporary cancer medicine, as documented in a substantial body of preclinical studies involving in a growing list of molecules with anti-angiogenic activity over the past 15 years (22). These diverse molecules act either in the extracellular space, at the cell surface or within the cell, and can be divided into several broad mechanistic categories based on their targets (Table 1): (a) growth-factor and growth-factor-receptor, (b) adhesion molecules/ECM protein and derived fragments/morphogenic and guidance molecules, (c) proteinases, and (d) signaling molecules/transcription factors.

4.2. Anti-angiogenic drugs

Numerous clinical trials testing these molecules have led to a number of anti-angiogenic drugs being approved as cancer therapeutics (Table 2). Most notably these include drugs targeting VEGF (Bevacizumab, VEGF-trap) or its receptors (Sunitinib, Sorafenib, Pazopanib, Cediranib, Motesanib). Here we briefly describe the main anti-angiogenic drugs.

4.2.1. Bevacizumab (Avastin)

Bevacizumab is a humanized monoclonal antibody directed against free VEGF in the circulation and extracellular milieu which functions to prevent VEGF attachment to the VEGF receptors (VEGFRs) and activation of pro-angiogenic stimuli (23). The combination of Bevacizumab and chemotherapy resulted in an increased progression-free survival (PFS) or overall survival (OS) of patients with a variety of solid tumors as compared with chemotherapy alone (24-26). In 2008 Bevacizumab became the first anti-angiogenic agent to be approved for treating patients with advanced colon cancer, non-small-cell lung cancer, renal, and breast cancer.

4.2.2 Aflibercept (VEGF trap)

Aflibercept, also known as VEGF-Trap, is constructed from the fusion of domain two of VEGFR1 and domain three of VEGFR2 with the Fc region of human IgG1 (27). This fusion protein, that has exceedingly high affinity to all isoforms of the VEGF family, can block the activity of even low levels of VEGF. This in turn more

Table 1. Angiogenic molecules

Groups	Examples of molecules
Growth factors	Vascular endothelial growth factor-A (VEGF-A)
	Platelet-derived growth factor (PDGF)
	Acid and Basic Fibroblast growth factor (FGF-1 and -2)
	Angiopoietin-1 and -2 (Ang-1 and -2)
	Transforming growth factor beta (TGF-β)
Receptor tyrosine kinases	Placenta growth factor (PlGF)
	VEGF receptor-1 and -2 (VEGF-1 and -2)
	PDGF receptors (PDGFRs)
	FGF receptors (FGFRs)
	Tie-1 and -2
Adhesion molecules	αvβ3 integrin
	αvβ3 integrin
	VE-cadherin
	N-cadherin
	immunoglobulin superfamilies (e.g. JAM-C)
Extracellular Matrix (ECM) proteins	fibronectin
	Collagens
remodeling and guidance molecules	Ephrin/Eph
	Notch/Delta
	Robo/Slit
	Netrin/UNC-DCC
	Semaphoring/plexin ligand/receptor families
Matrix-degrading proteases	Matrix metalloproteinase (e.g. MMP2, and 9)
	MMP inhibitors (i.e. TIMPs)
Plasminogen activators/inhibitors	uPA and PAI1
Plasminogen receptors	uPAR
Signaling molecules	Raf
	MAPK
	PKA
	Rac
	PKB
	mTOR
	HIF1α
	NFκB
Transcription factors	Id1 and 2
Transcription inhibitors	HoxD3
Homeobox gene products	HJobB3

completely abolishes the dependency of the tumor vasculature on VEGF and the co-dependency between the endothelial cells and the adjacent stromal cells (28). Aflibercept also displays extended pharmacological half-life, allowing long-term blockage (29). In pre-clinical studies, Aflibercept caused significant tumor regression and a substantial decrease in tumor vascularity (30, 31).

4.2.3. Sunitinib (SU11248)

Sunitinib is a small molecule, multi-targeted kinase inhibitor, which acts as an ATP-competitive inhibitor of VEGFR1, VEGFR2 and VEGFR3, PDGFRs, c-Kit, FLT3, and RET (32). Subsequent inhibition of the downstream signal transduction then impacts on tumor growth, progression, metastasis, and angiogenesis. Preclinical data suggest that Sunitinib may facilitate the regression of tumors formed from several malignant cell lines as a single agent (33). Furthermore, in combination with chemotherapeutic agents Sunitinib shows additive or synergistic effect in cancer therapy. Sunitinib has been approved for treating renal carcinoma and gastrointestinal stromal tumors (34).

4.2.4. Sorafenib (BAY 43-9006)

Sorafenib inhibits the phosphorylation of the MAP kinase pathway in several solid tumor cell lines, whether or not mutant K-Ras, mutant B-Raf, or wild-type Ras or Raf are present (35). Furthermore, cell-based assays

show that Sorafenib can inhibit several pro-antigenic tyrosine kinase receptors such as VEGFR2, VEGFR3, PDGFRs, c-Kit, FLT3, and FGFR1 (36, 37). There is evidence for decreased nuclear protein levels of extracellular signal-regulated kinase, and this has been suggested to be a possible mechanism by which Sorafenib exerts its anti-tumor activity (38). Pharmacokinetic studies confirm that Sorafenib distributes evenly throughout tissues and there is even some passage across the blood-brain barrier (39). Sorafenib has been approved for treating highly vascularized renal cancers (40) and hepatocellular carcinomas (41).

4.2.5 Pazopanib (GW-786034)

Pazopanib is a second-generation small molecule tyrosine kinase inhibitor, which highly selectively inhibits phosphorylation of VEGFR1, VEGF2 and VEGFR3, PDGFR, and c-Kit. It also has modest activity against FGF receptors, which leads to reduced cellular proliferation (42). Its anti-tumor effect has been demonstrated on human tumor xenografts (43). Specifically, it has resulted in a significant increase in progression-free survival in patients with metastatic renal cell cancer (RCC). Pazopanib has been approved for treatment of metastatic RCC (44). Moreover, Pazopanib has shown promising preliminary results in breast and thyroid cancers (45).

Table 2. Anti-angiogenic agents

Molecular Targets	Examples of agents	Mechanisms of actions
VEGF ligand	Bevacizumab (Avastin)	Humanized anti-VEGF monoclonal antibody
	Aflibercept (VEGF Trap)	Fusion protein of VEGFR 1 and 2 with immunoglobulin G1 Fc fragment
VEGF receptor		
Extracellular domains	IMC-1121B	Anti-VEGFR 2 monoclonal antibody
Cytoplasmic domains	AEE788	VEGFR 2 and EGFR inhibitors
	Axitinib (AG-0137736)	VEGFR and PDGFR inhibitors
	BMS-582664	VEGFR 2 and FGFR inhibitors
	Motesanib (AMG-706)	VEGFRs, PDGFR and c-Kit inhibitors
	Erlotinib	VEGFR 2 and c-Kit inhibitors
	Pazopanib	VEGFR 2 inhibitor
	Tandutinib	Flt-3, PDGFR and c-Kit inhibitors
	Vatalanib	Multikinase inhibitors
	Sorafenib	Multikinase inhibitors
	Sunitinib (SU11248)	Multikinase inhibitors
	Cediranib	VEGFR inhibitor
	Vandetanib	VEGFR and EGFR inhibitor
	XL-184	VEGFR 2, Met, c-Kit, Flt-3 and Tie 2 inhibitors
	XL-999	VEGFRs, FGFR, PDGFR and Flt-3 inhibitors
	dasatinib	Src and multikinase inhibitors
	Indolinones	Multikinase inhibitors
	Semaxanib (SU5416)	VEGFR 2 inhibitor
Other	EMD 121974 (Cilengitide)	$\alpha_v\beta_3$ integrin receptor inhibitor
	ATN-161	$\alpha_v\beta_1$ integrin receptor inhibitor
	Volociximab	$\alpha_v\beta_1$ integrin receptor inhibitor
	Vitaxin	Anti- $\alpha_v\beta_3$ integrin receptor antibody
	AMG-386	Angiopoietin inhibitor
	Thalidomide	Immunomodulatory agent
	AGN-745/SIRNA07	siRNA against VEGFR 1 mRNA
	Indolinones (BIBF 1000/1120)	VEGFR, PDGFR, FGFR inhibitors
	Menatetrenone	Vitamin K2 inhibitor
	Perindopril	Angiotensin-converting enzyme inhibitor
	Celecoxib	Cox-2 inhibitor
	Everolimus	mTOR inhibitor
	ATN-224	Analogue of ammonium tetrathiomolybdate (copper chelation)
	4-(N-(S-cysteinyglycylacetyl)amino) phenylarsonous acid (GCAO)	Synthetic mitochondria poison
	Enzastaurin	PKc beta inhibitor
	BAY 43-9006	Raf inhibitor
	PD 184352/0325901/ARRY-142886	MEK1/2 inhibitors
	BAY 12-9566	MMP inhibitor
	Bortezomib (PS-341)	Proteasome inhibitor

4.2.6. Cediranib (AZD2171)

Cediranib is a potent oral inhibitor of VEGFR2 with some degree of activity against VEGFR1 and VEGFR3, c-Kit, and PDGFRs (46). Human lung tumor xenografts show regression following treatment with Cediranib and immunohistochemical assessment of the tumors demonstrates a reduction in microvessel number (47). Surprisingly, clinical studies indicate that Cediranib is generally well tolerated, and compatible with once-daily oral dosing (48). Since the VEGFR1 signaling pathway can be activated by the other two members of VEGF family, PlGF and VEGF-B, Cediranib may provide additional benefits compared with those that only inhibit VEGF-A (49).

4.2.7. Motesanib (AMG-706)

Motesanib is a small molecule oral inhibitor of VEGFR1, VEGFR2, PDGFRs, and stem cell factor receptor (50). Preclinical studies have shown a broad dose-dependent antitumor activity of Motesanib against breast cancer-derived xenografts in combination of chemotherapy (51). In recent monotherapy studies, motesanib has shown acceptable toxicity and promising antitumor activity in patients with advanced solid tumors (52).

5. LATEST DEVELOPMENTS IN ANTI-ANGIOGENIC THERAPIES

The clinical achievements of anti-angiogenic therapy increasingly become part of our armamentarium, eliciting survival benefits for many patients with aggressive tumors, but there is an unfortunate limitation. Many of the demonstrable clinical benefits of anti-angiogenic drugs are not only modest in the form of tumor dormancy, tumor vasculature regression and in some cases increased survival, but these effects are transient and typically measured in months (53-56). Furthermore, the anti-tumor activity of most anti-angiogenic drugs only becomes clinically significant in combination with conventional chemotherapeutic regimens (57, 58). Thus, there is a clear need to elucidate the mechanistic basis of this apparent defect in the current therapeutics targeting tumor angiogenesis. This information can then be used to improve existing anti-angiogenic drugs or to devise new anti-angiogenic therapies. Here we elaborate several major associated difficulties based on recent laboratory data and emerging clinical data and propose possible explanations.

5.1. Indifference to anti-angiogenic drugs

Evidence for many cancer patients showing primary resistance to anti-angiogenic therapy was revealed

during current trials involving the FDA approved VEGF pathway inhibitors. For example, in phase III trials metastatic pancreatic cancer patients were randomly assigned to receive chemotherapy (Gemcitabine, Erlotinb) and Bevacizumab or chemotherapy and placebo (59). Of the 301 patients enrolled, median overall survival (OS) was 7.1. months and 6 months in the bevacizumab and placebo arms, respectively (95% CI, 0.7.4 to 1.0.7, $p=0.2.087$). This indicates that the pancreatic cancer patients did not receive survival advantages from anti-angiogenic therapy. A model of pre-existing resistance to anti-angiogenic therapy has been proposed and the supporting evidence has been discussed in depth in a review by Bergers and Hanahan (60).

However, current evidence for a heterogeneous tumor vasculature may provide us with an alternative explanation. Unlike normal blood vessels, tumor vasculature commonly follows a tortuous course with irregular branches of uneven diameter (61). It is becoming increasingly accepted that the tumor vasculature is composed of a chaotic mixture of abnormal, hierarchically disorganized vessels. Current experimental evidence suggests that there are at least six distinctly different tumor vessel types. Four of them develop through angiogenesis, and include mother vessel (MV) (62), capillary (63), glomeruloid microvascular proliferation (GMP) (64), and vascular malformation (VM) (65), while the remaining two, feeder arteries (FA) and draining veins (DV) (66), develop by remodeling of preexisting arteries and veins. A study of neovascularization revealed that these different types of tumor vessels can develop sequentially in normal mouse tissues injected with an adenoviral vector expressing VEGF A_{164} (Ad VEGF- A^{164}) (67). In response to Ad VEGF- A^{164} , MV formed from preexisting venules and/or capillaries. The response was rapid (1-5 days) and followed the typical steps of angiogenesis. Notably, the newly MVs were large, thin-walled, lightly fenestrated and hyperpermeable with poor pericyte coverage. It took four weeks for GMP tumor vessels to develop from MV in the presence of high tissue VEGF A_{164} levels. First, poorly differentiated endothelial cells are deposited in the endothelial lining of MV and formed the nascent GMP. Then these cells proliferated rapidly, extending inwardly into MV lumens and outwardly into the surrounding extravascular matrix, and divided single large MV lumens into multiple small channels. Finally, vascular lumens were covered by pericytes and double layers of basement membrane. The term glomeruloid microvascular proliferation derives from the fact that these poorly organized vascular structures resemble renal glomeruli. Ad VEGF- A^{164} is also able to induce new capillary formation within several weeks, either from MV through a process that endothelial cells project cytoplasmic processes across MV lumens to form transluminal bridges, or as result of devolution of GMP. However, it takes the longest time for VM to evolve from MV (>1 year) by acquiring a stabilizing coat of smooth muscle cells. The inappropriate large size of VM resembles certain features of the benign vascular malformation. Immediately adjacent to highly vascularized areas around the injection sites of Ad VEGF- A^{164} , small numbers of feeder arteries and draining veins are found. Concurrent

studies show that only a subset of Ad VEGF- A^{164} -induced blood vessels (MV, GMP) require VEGF A_{164} for their maintenance, whereas capillary, VM, FA, and DV can be long lasting even after VEGF A_{164} expression has ceased. Thus, one can imagine that in a substantial number of cancer patients these VEGF- independent vessels become the predominant vessel types in tumors that have been growing over a period of many months or years. It seems more likely that the cancer patients with this particular developmental ontogeny fail to show even transitory clinical benefit to anti-VEGF therapy. This may be the reason behind the findings from an animal study that mouse tumors responded effectively to anti-VEGF treatment at early stages of transplant, but over time some the tumors gradually lost response to such treatment.

All tumor vessel types deserve further investigation and rigorous evaluation of their prevalence and significance, both in animal models of cancer and in man. We also expect that if anti-angiogenic drugs targeting tumor blood vessels are to be more effective, they will have to attack not only MV and GMP but also later angiogenic vessels (capillaries, VM) as well as the FA and DV. Additionally, it is important to clarify circumstances that elicit development of tumor vasculature in the context of chemotherapy and radiotherapy for different cancer types, recognizing that the likely future of most anti-angiogenic therapies will be used in such combinations. However, there remains the challenge to clinically identify new targets on different tumor vessel types (68). We predict that functional imaging studies, and collecting and analyzing tumor biopsies from late tumor and tumor-surrogate blood vessels will be instrumental. With the help of high molecular-weight fluorescent tracers (e.g. FITC-dextran) and intravital microscopy (IVM) it will be possible to dynamically measure vessel diameter, length, surface area and volume, branching, and permeability in tumors (69). Furthermore, red blood cell velocity monitored by IVM represents spatial and temporal blood perfusion in tumors (70). Thus, IVM imaging techniques have revealed that different organ niches influence tumor angiogenesis activity and vessel function. For example, B16 murine melanomas implanted into a cranial window (metastasis site) exhibit a higher vessel density and smaller vessel diameter than those grown in a dorsal skin chamber (*in situ* lesion) (71). An extensive search for new tumor endothelial targets from the late tumors has begun (72). However, technical obstacles hamper direct isolation of such antigens from human tumor tissues. To circumvent such technical difficulties, another promising avenue involving isolation of tumor surrogate blood vessels at late times from animal tissues is becoming a popular approach to identify proteins that are highly overexpressed on late tumor blood vessels (73).

5.2. De novo resistance to anti-angiogenic therapy

Current anti-angiogenic drugs are often effective at the initial stage of treatment. Then, inevitably the patients become refractory to the same drug, the tumors begin to grow again, and the disease progresses. A phase III study of Bevacizumab plus chemotherapy in metastatic colon cancer showed an initial 50% improvement in

progression-free survival from 6.2. to 10.6. months, but did not meet its primary endpoint for significant improvement of survival past 20-24 months, indicative of de novo resistance to Bevacizumab treatment (74). In theory, de novo resistance to anti-angiogenic drugs can be acquired by the activation of alternative ways to evade the blockade of specific therapeutic targets of the anti-angiogenic drugs, thereby facilitating revascularization. Possible evading mechanisms including switching to expression of alternative angiogenic factors, which raises the possible mechanisms of intrinsic and acquired resistance circumventing the impact of anti-angiogenic drugs, including (a) upregulation of alternative angiogenic factors (75), (b) recruitment of bone marrow-derived pro-angiogenic cells (76), (c) modification of vascular pericyte coverage (77), (d) selection of more aggressive tumor cells (78), and (e) vessel cooption (79). These mechanisms are described in depth and discussed in two reviews by Bergers and Hanahan (60), Grepin and Page (80).

Over the past few years studies of the biological regulatory mechanisms operative in the tumor microenvironment have raised the exciting possibility that endothelial cells possess a potential for selective resistance because of their capability of altering gene expression based on physiological requirements and microenvironment stimuli. Evidence comes from a study of global gene profiling of endothelial cells derived from blood vessels of normal and malignant colorectal tissues (81). Although tumor and normal endothelial cell share many specific markers, the endothelium derived from tumors is qualitatively different from that derived from normal tissue of the same types. The tumor endothelium is found to express higher levels (>10-fold) of mRNAs for 46 endothelial-specific transcripts whereas 33 transcripts are at substantially lower levels than in the normal endothelium. Notably, these genes are expressed specifically in tumor-derived endothelial cells from several tissues, indicative of tumor endothelium being different from that in the surrounding normal tissues. More interestingly, some of these “tumor” genes are not detectable in physiological angiogenesis such as wound healing, further suggesting that there are discrete differences between tumor endothelium and their normal counterparts.

A suggestion of analogous alterations in endothelial gene expression in response to tumor-induced hypoxia has come from a study comparing the effect of pO₂ fluctuation with continuous hypoxia on the gene expression of tumor endothelium (82). Tumor-induced hypoxia is detected in a number of types of tumor tissues, as are pO₂ instability and fluctuation called cyclic hypoxia (83). These features originated from heterogeneities of red blood cell flux (84), indicating that tumor microvessels within a given tumor area are also influenced by fluctuations in pO₂. This hypothesis is supported by the findings that fluctuation in hypoxia can result in a preferential induction of hypoxia-responsive genes, such as cyclooxygenase-2 (COX-2) to an even larger extent than in response to continuous hypoxia (82). A concurrent study showed that the COX-2 expression actively participated in the assembly of new blood vessels (85).

An experimental study by our group shows that tumor-co-cultured endothelial cells increase expression of the molecular chaperone alpha-Basic-crystallin (CRYAB) (J. Cai, unpublished observations). Subsequent experiments and the work of others suggest that VEGF can activate the unfolded protein response (UPR), which in turn upregulates protein maturation machinery including CRYAB. CRYAB appears to have the potential to enhance the internal autocrine VEGF signaling pathway. In another study, our group demonstrated that enolase-1 (ENO1) is significantly upregulated in the microvascular endothelial cells co-cultured with breast cancers under hypoxic condition (J. Cai, unpublished observations). Furthermore, it is shown the tumor cells lead to the translocation of 60% ENO1 to the endothelial cell surface with a dramatic increase in cell surface ATP production. These results collectively suggest that endothelial cells associated with tumor cells may alter their phenotype by virtue of their own genetic instability, therefore rendering tumor endothelial cells less responsive to anti-angiogenic drugs.

Extrapolation from a clinical investigation of differential gene expression profile of breast cancer patients after treatment with Bevacizumab (86), we speculate that anti-angiogenic drugs may further differentially alter gene expression of the tumor endothelial cells in the context of influencing the tumor microenvironment. Those genes significantly expressed in response to Bevacizumab, including CD 31, PDGFR-beta, 26 gene ontology (GO) classes for VEGFR and mitosis, are downregulated in some patients refractory to the treatment. Larger prospective cohort clinical trials may be needed to confirm these data in order to assess the impact of anti-angiogenic drugs on gene expression of tumor endothelial cells.

5.3. Strategies for administration of effective anti-angiogenic treatments

Since disease palliation rather than cure is achieved by anti-angiogenic drugs in the majority of cancer patients (87) it has been proposed that angiogenesis inhibitors should be delivered to cancer patients for the rest of their lives (76). Support for long-term treatment with anti-angiogenic therapy comes from a clinical investigation involving a retrospective analysis of the patients with metastatic renal carcinoma treated with tyrosine kinase inhibitors (Sunitinib and Sorafenib) (88). There is a demonstrable risk of progression with new metastasis in the patients after discontinuation of the treatment. Provocatively, many of these patients responded to the reintroduction of the same drug. Further corroboration comes from animal studies showing that cessation of anti-angiogenic treatment could result in a rebound effect on tumor angiogenesis, leading to accelerated re-neovascularization and possible tumor invasion (89, 90). A recent molecular imaging experiment using a VEGF-based tracer, ^{99m}Tc-scVEGF, provides possible mechanistic evidence (91). ^{99m}Tc-scVEGF binds and is internalized by VEGF receptor. Autoradiography analysis revealed a 2.2. - and 2.6.-fold decrease in the VEGF-tracer uptake after Sunitinib treatment, but a rapid increase in tracer uptake once Sunitinib was withdrawn.

The strategy of applying the maximum tolerated dose of anti-angiogenic drugs for cancer patients is attractive, but the high frequency of adverse effects and cost of higher treatment undermines the maximal clinical benefits. One such clue has been described in a recent meta-analysis of four clinical trials of Bevacizumab for unresectable non-small-cell lung cancer (92). Although the trial designs did not involve dose optimization, it seems likely that high-dose Bevacizumab may increase 2-year overall survival rates, prolong progression-free survival and improve tumor response but at the high cost of treatment-related death. It is clinically challenging to clarify the relationship between dose and activity of each anti-angiogenic drug due to the lack of a suite of predictive, pharmacodynamic and response biomarkers to monitor patients' response to anti-angiogenic drugs (93). Nevertheless, long-term half-life of anti-angiogenic drugs appears to be particularly relevant to their clinical efficacy. In the case of Bevacizumab, a Phase I clinical trial revealed that Bevacizumab has a half-life in the range of 13-21 days (94), which implies a plasma concentration profile close to constant even with spaced administration. In separate clinical studies, Sunitinib and Sorafenib exert a half-life only up to 40 hours (95, 96). Interestingly, daily dosing (50-75mg/day) for 28 days can still result in 3-5 fold accumulation of Sunitinib and 7-15 fold accumulation of Sorafenib, which is sufficient to produce >50ng/ml of plasma concentrations. Preclinical studies show that a 50ng/ml plasma concentration is required to inhibit the kinase activity of VEGF receptors for both Sunitinib (97) and Sorafenib (98). A suggestion that the maximally tolerated dose does not guarantee the greatest clinical benefit comes from a clinical trial of non-small cell lung cancer patients being treated with Motesanib (99). At the maximal tolerated dose of 125 mg daily in combination with chemotherapy, Motesanib only results in 17% of patients achieving a partial response.

Continuous long-term treatment and maximum tolerated dose (MTD) with anti-angiogenic drugs may increase the magnitude of the adverse effects of the drugs. An intriguing clue to the dose of anti-angiogenic drugs by de-escalation comes from a clinical study of SU5416-inhibitor of VEGF receptors for advanced solid tumor which aimed to optimize the most efficacious dose based on their pharmacodynamic modulatory effect, rather than their adverse effects (100). The rationale of de-escalation strategy is that if the de-escalated dose seems similar in biological response to the MTD, it qualifies to be considered for the biological modulatory dose (BMD), entering phase II clinical trials; if the number of patients with the expected biological response at the de-escalated dose is equal or larger than the number seen at the MTD, a further de-escalated dose should advance to a phase II trials comparing it with the MTD (101). We foresee that the emerging regularly spaced (metronomic) dosing of anti-angiogenic drugs would seem to have particular promise to circumvent their dose-dependent adverse effects and reduce costs, and should be tested in clinical trials.

6. FUTURE CONSIDERATIONS

Over the past fifteen years great progress has been made in understanding the molecular basis of angiogenesis and this has led to the exciting development

of anti-angiogenic molecules in experimental cancer models. Many anti-angiogenic molecules have entered clinical trials and the VEGF signaling pathway has remained the main focus of therapeutic endeavors to date. We expect that a better understanding of the molecular mechanisms involved in the regulation of the VEGF signaling pathway should lead to the development of reliable biomarkers that allow accurate selection of responsive patients and monitor therapeutic efficacy. Moreover, a more detailed understanding of the complexity of the tumor microenvironment as well as the interactions between tumor and vascular compartments will help to identify novel therapeutic targets. Last, but not least, *in vitro* and *in vivo* angiogenesis assays are still great tools to select the best anti-angiogenic drugs from a list of potential inhibitors.

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