

## Complexity of tumor vasculature and molecular targeting therapies

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## 1. ABSTRACT

Malignant solid tumors require blood supply for their uncontrollable progression. Angiogenic blood vessels generally sprout from preexisting vascular cells. In addition, various types of precursor cells also participate in tumor neovascularization. They include endothelial progenitor cells, hematopoietic stem cells and mesenchymal stem cells that are stimulated and attracted into tumor lesion, in which a wide variety of proinflammatory factors are involved. Among key molecules, vascular endothelial growth factor (VEGF) works as a mastermind regulator. Humanized monoclonal antibodies targeting VEGF-mediated signaling pathways are currently used as the most pervasive drugs in several types of progressive tumors. Adverse effects of these drugs include hypertension and proteinuria. Such symptoms are widely observed in preeclamptic patients whose blood contain high amount of natural VEGF antagonist. Vasoactive G protein-coupled receptors (GPCRs)-mediated signalings such as renin-angiotensin system and chemokine axes are also noticed that they may become effective therapeutic targets. In this review, we discuss general view of angiogenic microenvironment in solid tumors, and highlight several key signaling molecules and inhibitory effects of them on the whole system.

## 2. INTRODUCTION

Pro- and anti-angiogenic activities in solid tumors are complicated, and a wide variety of humoral factors and signaling molecules are involved in the process. Tumor angiogenic activities are supported by both local microenvironment and systemic reaction stimulated by tumor derived factors. Soluble factors activate the bone marrow (BM) and recruit several progenitor subpopulations that can differentiate into endothelial cells (ECs) and other vascular components. These progenitor cells include BM-derived endothelial progenitor cells (EPCs) and hematopoietic stem cells (HSCs). In addition, mesenchymal stem cells (MSCs) and immature myelomonocytic cells expressing endothelial markers also potentially show ECs commitment. It was at first expected that these progenitor cells arrived at angiogenic sites and differentiated into ECs. Later studies have added new insight into the notion; some of them may exist as heterogeneous cells of uncertain differentiation states and they seem to support tumor angiogenesis and hematogenous metastasis. Immature myelomonocytic cells also contribute to tumor progression by skewing host defense mechanisms of immune cells and by increasing refractoriness to anti-angiogenic therapies.

Currently, antiangiogenic drugs are widely available for the treatment of several types of advanced malignancies. Among angiogenic cytokines, vascular endothelial growth factor (VEGF) acts rather more selectively on ECs than other cytokines do, and VEGF works as a master switch of angiogenesis. Humanized monoclonal antibodies targeting VEGF, and receptor tyrosine kinase (RTK) inhibitors targeting VEGF receptors (VEGFRs)-mediated and platelet-derived growth factor receptors (PDGFRs)-mediated signaling pathways suppress tumor growth by pruning angiogenic vessels. Although these antiangiogenic agents are expected to work specifically on tumor neovessels, they may disturb normal physiological and homeostatic activities such as cardiovascular functions and immune responses. The common adverse effects of these drugs include hypertension, proteinuria, impaired wound healing and cardiac ischemia (1). Patients are also at increased risk of life-threatening complications such as thromboembolic diseases and gastrointestinal perforation (2).

We present an overview of recent progress in our understanding of tumor vasculature in this review. The contribution of heterogeneous precursor cells and tumor cells to neovessels, and targeting of VEGF, placental growth factor (PlGF) and some other related factors are discussed. Renin-angiotensin system and chemokine signalings that belong to angiogenic G protein-coupled receptors (GPCRs)-mediated cascades are also highlighted.

### 3. TUMOR VASCULATURE

Tumor angiogenic vessels are irregular in morphology, and they are leaky due to increased permeability and proteolytic activities (3, 4). These vessels are generally derived from preexisting vasculature. In addition, BM-derived cells and tissue-resident progenitor cells are recruited to angiogenic sites and potentially become components of tumor vessels. Furthermore, tumor cells themselves may form blood passages. Such unique blood pathway formation is also observed *in utero*-placental junction during trophoblastic invasion into endometrium.

#### 3.1. EPCs, HSCs and Gr-1<sup>+</sup> CD11b<sup>+</sup> BM-derived cells

Several types of progenitor cells such as EPCs, HSCs, and immature myelomonocytic populations contribute to tumor vessels. These cells are now widely accepted as important components of tumor vessels in addition to angiogenic constituent cells sprouting from preexisting vessels. Earlier studies focused on the potential of these BM-derived cells to become ECs. Later studies have further revealed non-endothelial population among these cells. For example, a study on the trafficking of BM-derived cells revealed the existence of NG2<sup>+</sup>, CD11b<sup>+</sup> (Mac-1<sup>+</sup>), CD45<sup>+</sup> cells that contacted with ECs and pericytes but not differentiated into either of them (5).

EPCs are generally defined by several cell surface markers such as CD34, VEGFR2 and CD133 (also named Prominin 1 and AC133) (6). EPCs can be mobilized from the BM by hypoxic stimuli, VEGF upsurge, and so

on. Studies on the contribution of EPCs to angiogenic vessels in ischemic and neoplastic diseases showed considerably varied results. It might owe in part to the diversities of angiogenic and proinflammatory milieu among rodent models. The study on human subjects who developed malignancies after BM transplantation from the opposite gender revealed that the majority of ECs (90% or more) are composed of non-BM-derived cells (7). On the other hand, in some rodent tumor models reconstituted with BM cells labeled with GFP, significant number of VEGFR2<sup>+</sup>, CD133<sup>+</sup>, VE-cadherin<sup>+</sup>, CD105<sup>+</sup>, CD31<sup>low</sup>, CD11b<sup>-</sup>, CD45<sup>-</sup>, GFP<sup>+</sup> cells luminally incorporated into tumor vessels (8).

The origin of embryonic HSCs is attributable to the specialized cell clusters attached to ventral aortic ECs. The hematopoietic site is subsequently shifted to other organs, and adult mammalian hematopoietic site is established in the BM. HSCs in vascular niche of the BM are further subdivided into lymphoid and myeloid cells. In Id-deficient mice, BM-derived VEGFR1<sup>+</sup> HSCs were shown to cooperate with VEGFR2<sup>+</sup> EPCs for promoting tumor neovascularization (9). Among immature BM-derived cells, Gr-1<sup>+</sup> CD11b<sup>+</sup> immature BM-derived cells have been studied intensively. They are heterogeneous myelomonocytic cells preferentially recruited to tumor lesion by tumor derived factors (TDFs) such as interleukin (IL)-6, IL-10, VEGF and colony-stimulating factor (CSF)-1. They include immature macrophages and dendritic cells (DCs). A small number of VEGFR2<sup>+</sup> subsets in Gr-1<sup>+</sup> CD11b<sup>+</sup> cells potentially form luminal surface of blood vessels in tumor-bearing mice (10).

The difficulties in distinguishing EPCs from HSCs and other BM-derived cells should be carefully considered. Some HSCs, if not all, can be detectable in the fraction of CD31<sup>+</sup>, CD45<sup>-</sup>, VEGFR2<sup>+</sup>, CD133<sup>+</sup> BM-derived cells. Culture-expanded EPCs from human peripheral blood may express both VEGFR2 and VEGFR1. Thus, the expression profiles of cell surface markers may not be sufficient to define their features as “EPCs” or “HSCs”. Further functional characterization such as Ulex lectin binding and Dil-labeled acetylated LDL (Ac-LDL) uptake might estimate cell nature as ECs-like or not. It should also be considered that the fate of BM-derived cells is not promised and that differentiation process may be altered depending on tumor microenvironment. Some of these cells may maintain pluripotent properties and adapt themselves flexibly to pathological events. The contribution of recruited cells to tumor progression, therefore, might be more critical for supporting highly angiogenic microenvironment than for becoming vascular cells in a direct manner. The properties of Gr-1<sup>+</sup> CD11b<sup>+</sup> cells include that they secrete VEGF and matrix metalloproteinase 9 (MMP9) to accelerate vessel remodeling (11), and that they skew immune responses by suppressing the development of cytotoxic T cells. Another study suggests that Gr-1<sup>+</sup> CD11b<sup>+</sup> cells may contribute to tumor resistance to anti-VEGF treatment (12).

Several subgroups of characterized BM-derived cells have been reported so far. “Recruited bone

marrow-derived circulating cells (RBCCs)” are defined as CD45<sup>+</sup>, CXCR4<sup>+</sup>, VEGFR-1<sup>+</sup>, VEGFR-2<sup>-</sup>, CX3CR1<sup>+</sup>, and they are attracted by CXCL12 (also named stromal cell derived factor-1; SDF-1) (13). “Hemangiocytes” are BM-derived CXCR4<sup>+</sup> VEGFR-1<sup>+</sup> cells recruited by CXCL12 through MMP9, thrombopoietin (TPO) and soluble Kit-ligand (sKitL) (14). “Tie-2-expressing monocytes (TEMs)” are CD11b<sup>+</sup>, Tie2<sup>+</sup>, CD45<sup>+</sup>, c-Kit<sup>-</sup>, CD31<sup>low</sup>, VEGFR2<sup>-</sup>, and they represent 1-15 % of the total CD11b<sup>+</sup> myeloid hematopoietic cells (15). Other subgroups include VEGFR-2<sup>+</sup> CD14<sup>+</sup> monocytes (16), tumor-associated stromal cells (TASCs) (17), tumor-associated DCs (TADCs) (18) and monocyte-derived multi-potential cells (MOMCs) (19).

### 3.2. Tumor mosaic vessels and tumor vasculogenic mimicry

Tumors may sometimes generate unusual blood pathways that are not accompanied by ECs. In the region where endothelial lining is missing or attenuated, tumor cells may show ECs-like features. Luminal surface of tumor vessels may not always be covered by ECs. In some cases, tumor cells form a part of vessel wall, which is called “mosaic vessels” (20). At first, it was thought that tumor cells were exposed to blood channels in CD31<sup>-</sup> CD105<sup>-</sup> intimal regions. A later study (21) revealed that these luminal regions contained in part very thin, ultrastructurally identifiable CD31<sup>-</sup> CD105<sup>-</sup> ECs. The finding suggests that the tumor cells in mosaic vessels are closely juxtaposed to blood lumen with abnormally fragile ECs. Since these tumor cells do not express common endothelial markers, active transformation of tumor cells to endothelial phenotype may not be the case. Some researchers suggest spatiotemporal association between mosaic vessels and “tumor vasculogenic mimicry”, in which the transformation of tumor cells into ECs-like cells is taken place (22).

“Tumor vasculogenic mimicry” is the *de novo* formation of vascular channels by tumor cells (23). These specialized tumor cells are able to express some endothelial markers and embryonic vasculogenesis-related molecules such as VE-cadherin, CD34 and CD105 (23, 24). Such phenotypic alteration of tumor cells has been observed in several types of malignancies including aggressive melanoma, glioblastoma, and ovarian, prostatic, and breast cancers (23-29). A fraction of glioblastoma stem-like cells was shown to potentially trans-differentiate not only into ECs but also into another mesenchymal cell type with the property of vascular smooth muscle cells (VSMCs) (29). Collective data suggest that tumor cells may trans-differentiate into ECs- and VSMCs-like phenotypes during progression. The findings are supported by the studies that these aggressive tumor cells potentially express key angiogenic RTKs such as EphA2 (30) and VEGFR2 (31). These ECs-like tumor cells are reported to be associated with poor prognosis (32) and they may show resistant to antiangiogenic molecules *in vitro* (33).

Vascular remodeling by epithelial cells is not tumor specific phenomenon. It is also taken place under physiological control during gestation. The placenta is an invasive organ that remodels uterine endometrium. For

successful establishment of feto-placental circulation, certain phenotype of trophoblasts exerts vasculogenic property and replaces ECs of uterine spiral arteries. This process is called “pseudovasculogenesis” or “epithelial-endothelial transformation” (34). These transformed cells express common endothelial markers such as CD31, VE-cadherin, vascular cell adhesion molecule (VCAM)-1 and  $\alpha v \beta 3$  integrin (35, 36), and down-regulate the expression level of  $\alpha 6 \beta 4$  integrin (37).

## 4. INHIBITORY EFFECTS OF VEGF AND RELATED SIGNALINGS

The molecules targeting VEGF signaling pathways are approved for several types of advanced tumors. One of the most popular anti-VEGF agents currently available is bevacizumab (Avastin), a humanized anti-VEGF monoclonal neutralizing antibody. In addition, some inhibitors targeting VEGFRs and PDGFRs such as sunitinib (Sutent) and sorafenib (Nexavar) are also under phase studies. Clinical studies have revealed that these targeting agents somewhat improve overall survival, although statistically non-significant results are included. It should be noted, however, that significant percentage of patients have to discontinue anti-VEGF therapies due to adverse events. Although severe toxicities have not been explored in detail in the studies of rodent tumor models, these drugs very likely disturb physiological VEGF-related homeostasis in some degree, and unfavorable events may be even life-threatening. Some characteristic adverse events such as hypertension and proteinuria are reminiscent the symptoms of preeclampsia (38-40). We discuss general features of VEGF and PlGF, and systemic effects of their inhibitors.

### 4.1. VEGF and PlGF

VEGF functions as a main ligand for VEGFR1 (Flt1) and VEGFR2 (Flk1/ KDR) (3). The kinase activity of VEGFR2 is about ten-fold higher than that of VEGFR1 (41). Therefore, angiogenic VEGF signaling is thought to be mediated mainly by VEGFR2 (42, 43). VEGF is required in many non-malignant events including wound healing and ovulation, whereas physiological VEGF suppression is also necessary for certain tissue(s). It is known that sVEGFR1 (sFlt1) is essential for physiological avascularity in the cornea (44). Neovascular eye diseases such as diabetic retinopathy are attributed to VEGF, and bevacizumab has been successfully used in the treatment of these eye diseases. sVEGFR1 is a natural soluble factor, and is the truncated version of VEGFR1 that lacks transmembrane and intracellular signaling domains (38). sVEGFR1 is also produced in some types of tumor tissues such as colorectal and breast cancers (45, 46). In clinical studies on these tumors, the expression level of sVEGFR1 was correlated with favorable prognosis, probably owing to its antiangiogenic property. sVEGFR1 generally inhibits the signaling pathways of VEGF and PlGF by binding to free forms of VEGF and PlGF (38, 47).

Heterozygous VEGF<sup>+/-</sup> embryos die due to vascular defects (48), whereas PlGF deficient mice are fertile with normal looking (49). Therefore, the roles of

PlGF are not well understood as contrasted with those of VEGF. The importance of PlGF in neovascularization, however, has been suggested in various pathological conditions. PlGF binds to VEGFR1 but not to VEGFR2 (50, 51). The finding suggests that enriched PlGF may displace VEGF from VEGFR1 and direct VEGF towards VEGFR2 (50). Since the kinase activity of VEGFR2 is much higher than that of VEGFR1 (41), PlGF-directed VEGF-VEGFR2 interaction seems to accelerate angiogenesis. On the other hand, some groups argue angiogenic property of PlGF. Antiangiogenic effect of PlGF has been reported in autochthonous and orthotopic tumor model mice in which PlGF/VEGF heterodimers suppressed VEGF homodimers-induced angiogenic signaling (52-54). Free VEGF and PlGF levels increase during gestation in maternal circulation in order to increase placental perfusion. In preeclamptic women, however, the levels of these molecules are suppressed. Instead, circulatory sVEGFR1 increases abnormally in the patients (55-57). Predominance of sVEGFR1 frequently causes poorly developed placental vasculature and circulatory disorders such as hypertension and renal dysfunction (38, 58-60). The findings support the notion that disturbance of homeostatic activities of VEGF and PlGF leads to systemic endothelial dysfunction.

PlGF is expressed in several types of cells including ECs, myelomonocytic cells, hematopoietic stem cells (61), and some tumor cells (62). The effects of sVEGFR1 on PlGF-VEGFR1 axis seem to be complex depending on cell types and pathological milieu. In tumor progression, VEGFR1<sup>+</sup> cells are thought to be important for pre-metastatic niche formation (63, 64). Tumor metastasis studies using VEGFR1 tyrosine kinase-deficient homozygous mice and VEGFR1-antibody administrated mice strongly suggest that VEGFR1<sup>+</sup> immature hematopoietic cells are mobilized from the BM to a distant organ of tumor-bearing mice in pre-metastatic phase, and that they activate vascular bed to attract circulating tumor cells for metastasizing (63, 64). Tissue-resident VEGFR1<sup>+</sup> cells such as alveolar macrophages in the lung may also modulate tumor progression in metastatic sites (65). Several lines of investigations showed that the blockade of VEGFR1 led to antiangiogenesis and tumor suppression (66-68). A study showed that anti-PlGF antibody treatment suppressed tumor progression in part by preventing macrophage trafficking to tumor lesion (69), suggesting that the tumor associated macrophages (TAMs) enhance angiogenic cascades and that anti-PlGF antibody negatively mediates the property of TAMs in proinflammatory milieu. Considering angiogenic and antiangiogenic properties shown in different tumor models, PlGF seems to exert different effects depending on targeting cells and primary/distant lesions, and the actual effects of the blockade of PlGF-VEGFR1 axis require further studies. At least, a recent study on more than one orthotopic tumor models, neither anti-PlGF antibody nor VEGFR1 signaling blockade exhibited inhibitory effects on primary tumor lesions (70).

### 4.2. Other related molecules

There are several angiogenic receptors other

than VEGFRs that participate in angiogenesis. For example, CD105 is an auxiliary receptor for transforming growth factor (TGF)- $\beta$ 1 and TGF- $\beta$ 3, and is expressed in ECs, VSMCs, hematopoietic cells and so on (40, 71, 72). CD105 is thought to support the properties of TGF- $\beta$  for proliferation and migration of ECs (73). CD105 deficient mice embryos die at mid gestation due to poor vascular smooth muscle development (74). In cardiovascular system, CD105 interacts with endothelial nitric oxide synthase (eNOS), and mediates eNOS-dependent vascular tone (75, 76). Like sVEGFR1 that antagonize VEGF/PlGF cascades, sCD105 inhibits CD105-mediated angiogenesis. A recent study on colorectal cancers demonstrated that plasma sCD105 levels in cancer patients were lower than those in healthy controls. The study suggests that antiangiogenic potential of sCD105 is attenuated in cancer patients group (77). To support the notion, sCD105 concentration is significantly elevated in preeclamptic patients in antiangiogenic condition, especially in severe cases named HELLP syndrome (Hemolysis, Elevated Liver enzyme, Low Platelets syndrome) (40, 56).

In addition to sVEGFR1 and sCD105, some other soluble forms of decoy receptors such as, sVEGFR2, sNotch-1 and sTie-2 (78-80) are thought to negatively control angiogenesis by antagonizing membrane-bound counterparts. A study demonstrated that sVEGFR2 level was inversely correlated with VEGF level in tumor bearing mice *in vivo* (78). The amounts and the effects of these soluble molecules may be altered in response to antiangiogenic therapies. In breast cancer patients who were treated with bevacizumab, plasma levels of sVCAM-1 (sCD106) were reported to be elevated (81), which might reflect endothelial damage due to targeting therapy. Very limited information is available about whether endogenous antiangiogenic systems are disturbed and whether tumor immune responses are attenuated in response to bevacizumab and other antiangiogenic drugs. Further studies are required to improve targeting therapies with minimal adverse effects.

## 5. G PROTEIN-COUPLED RECEPTORS (GPCRs) SIGNALINGS

Seven-transmembrane receptors, named G protein-coupled receptors (GPCRs), represent the targets of 50-60% of all current therapeutic agents (82), and they include vasoactive receptors that become potential antiangiogenic targets. Several GPCR ligands are crucially involved in embryonic vascular development and tumor neovascularization. Angiotensin II, endothelin-1, Bv8 and CC- and CXC- chemokines are among them. They play important roles not only in angiogenesis but sometimes also in tumor immunology. A study demonstrated that endothelin B receptor (ET<sub>B</sub>R) gene is much higher in the ECs from tumor infiltrating lymphocytes (TILs)-rich tumors than those in TILs lacking tumors (83). It is suggested that ET<sub>B</sub>R controls ICAM-1 (CD54) expression and TILs binding ability to ECs (83). We highlight renin-angiotensin system (RAS) and some chemokine axes in this review.

### 5.1. RAS signaling

AT1 is a principle GPCR for angiotensin II, and AT1 signaling leads to strong vascular contraction (84, 85). RAS participates in a wide variety of biological activities including vascular remodeling, inflammation, (84, 86-88) and tumor progression (89). RAS-mediated signaling contributes to tumor angiogenesis in part by directly activating vascular cells. Therefore, some RAS inhibitors used for hypertension therapies become under clinical trials as potential tumor inhibitors, especially in respect of antiangiogenic approaches. The mechanisms generally involve AT1, but some tumors may take advantage of RAS in AT1-independent manner (90). Some types of tumor cells express AT1 on the plasma membrane, and angiotensin II potentially activates tumor cells in direct manner and induces tumor proliferation. Cell cycle activities are significantly suppressed by AT1 antagonist in pancreatic and prostate cancer cells (91, 92). The results suggest that RAS inhibitors may suppress tumor cells activities efficiently. With regard to angiogenesis, administration of angiotensin II was shown to increase the expression levels of VEGF and other angiogenic cytokines such as TGF- $\beta$ , tumor necrosis factor (TNF)- $\alpha$  and CC- and CXC-chemokines (86, 89) in several types of cells including VSMCs, mesangial cells and cancer cells *in vitro* (93-97). Since angiogenic ECs potentially express AT1 (96), RAS inhibitors are expected to work for antiangiogenesis by both direct blockade of AT1 signaling on ECs and indirect suppression of angiogenic microenvironment. A study on tumor bearing model demonstrated that tumor angiogenic activities were significantly impaired in AT1a-deficient mice. The mechanism of AT1-induced angiogenesis of this model was attributed to AT1<sup>+</sup> TAMs that were attracted from circulation to the tumor lesion and produced VEGF (98). Collectively, RAS may accelerate tumor angiogenic activities by utilizing local angiogenic signaling as well as by stimulating circulating hematopoietic lineages.

With respect to pathological net balance of angiogenesis under hypoxic microenvironment, however, actual mechanism of RAS-mediated signaling *in vivo* is not fully understood. As mentioned above, cumulative studies suggest that RAS contributes to tumor angiogenic milieu by increasing vascular permeability, recruiting inflammatory cells, and up-regulating VEGF and other pro-angiogenic factors. On the other hand, accelerated AT1 signaling in preeclampsia induces antiangiogenic events such as increases of sVEGFR1 and sCD105. In a hind limb ischemic models, suppression of RAS through angiotensin converting enzyme (ACE) inhibitor improved revascularization (99, 100), suggesting that accelerated RAS signaling in ischemic organs/tissues works for antiangiogenesis. These contradictory effects of RAS depending on pathological conditions *in vivo* have raised arguments about the safety of RAS inhibitors for tumor patients (101, 102). RAS inhibitors may be of great advantage in both tumor suppression and cardiovascular diseases. On the other hand, if ACE inhibitors and AT1 inhibitors work for revascularization in hypoxic tumor areas as it does in ischemic tissues, such therapeutic approach might induce unfavorable effects on tumor therapy. The application of RAS inhibitors in progressive

tumor patients, therefore, should be carefully monitored.

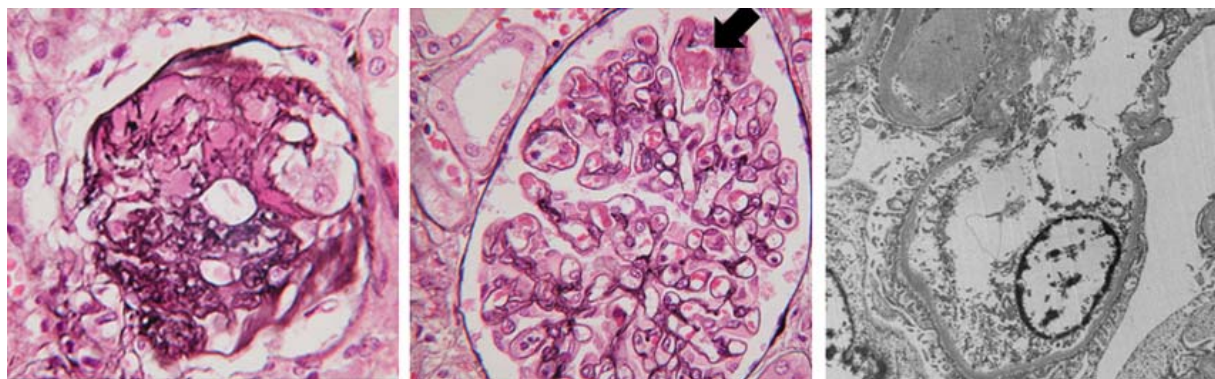
### 5.2. Chemokine signaling

As mentioned above, tumor cells secrete chemoattractants to recruit various types of hematopoietic cells from the BM and blood/lymphatic vessels, and tumor cells themselves express chemoreceptors for invasion and metastasis. Chemokines are a family of low molecular weight cytokines, and they exert the activities by binding to corresponding GPCRs. Chemokines are subgrouped into CC, CXC, C and CX3C chemokines depending on the spacing or the presence of four N-terminal cysteine residues, and most members are classified into CXC and CC chemokines. The ligands-receptors interactions of these chemokines seem to be rather promiscuous than one-to-one correspondence (103-105).

CXC chemokines with Glu-Leu-Arg (ELR) motif (ELR<sup>+</sup>), such as CXCL1 (GRO- $\alpha$ ), CXCL2 (GRO- $\beta$ ), CXCL3 (GRO- $\gamma$ ), CXCL5 (ENA-78), CXCL6 (GCP-2), CXCL7 (NAP-2) and CXCL8 (IL-8) induce ECs migration and proliferation. CXCR2 is regarded as a primary functional receptor for ELR<sup>+</sup> chemokines. IFN- $\gamma$ -inducible CXC chemokines without ELR motif (ELR<sup>-</sup>), such as CXCL9 (Mig), CXCL10 (IP-10) and CXCL11 (I-TAC) share a common receptor CXCR3 and the axes are thought to inhibit endothelial migration and proliferation. Although these ELR<sup>-</sup> chemokines generally function as antiangiogenic and anti-tumorigenic factors, CXCR3 is also expressed in some tumor cell types and the role is not fully understood (105, 106).

Another ELR<sup>-</sup> CXC chemokine CXCL12 binds to CXCR4. Contrary to other ELR<sup>-</sup> CXC chemokines, CXCL12 promotes angiogenesis and CXCR4 is essential for embryonic blood vessel formation (107). There are at least three important mechanisms in CXCL12-CXCR4 axis that contribute to tumor progression. (I) Tumor metastasis; CXCR4<sup>+</sup> tumor cells preferentially metastasize to the organs enriched in CXCL12 (108). (II) Tumor survival; CXCL12 is highly expressed in cancer-associated fibroblasts (CAFs), and CAFs-derived CXCL12 supports the survival and growth of CXCR4<sup>+</sup> tumor cells in a paracrine fashion (109). (III) Angiogenesis; CXCL12-enriched tumor stroma accelerate tumor angiogenesis by stimulating pre-existing vascular ECs as well as by attracting circulating CXCR4<sup>+</sup> EPCs and other vascular progenitor cells in an endocrine fashion (109). It is expected that ECs in hypoxic tumor areas are CXCR4<sup>high</sup>, because hypoxia-inducible factor-1(HIF-1) up-regulates CXCR4 in various cell types. In addition, CXCL12-CXCR4 axis is also involved in pre-metastatic niche formation. A subset of circulating CXCR4<sup>+</sup> precursor cells may be attracted to a distant organ and activate vascular beds there before tumor cells establish the secondary lesion (64).

CC chemokines contribute to neovascularization generally in indirect manners, although direct role of certain CC chemokines has also been reported. Several CC chemokine members, including CCL2 (MCP-1), CCL5 (RANTES), CCL7 (MCP-3), CCL8 (MCP-2), CCL17 (TARC) CCL 20 (LARC/MIP-3 $\alpha$ ), CCL22 (MDC) and



**Figure 1.** Microvascular changes of the kidney in a sunitinib-treated patient: Left: Glomerular capillary structure is damaged severely, and exudate is noted (Mehtenamine-silver, x 400). Center: Segmental thrombotic occlusion of the glomerular capillary are observed (arrow) (Mehtenamine-silver, x 400). Right: Electron microscopy reveals widening of subendothelial space of the glomerular capillary (x 2,500).

CCL23 (MPIF-1/MIP-3), are produced mainly by TILs, and some of them also by tumor cells. They attract circulating mesenchymal cells and tumor cells expressing corresponding receptors, and the axes accelerate tumor angiogenesis and metastasis. In colon cancer model mice, BM-derived CCR1<sup>+</sup> immature myeloid cells migrate into tumor invasion front and interact with CCL9<sup>+</sup> (putative human homolog of CCL23) cancer cells (110). CCL2 highly produced by TAMs induce monocyte recruitment and activate progression cascades in solid tumors (111, 112).

### 6. SYMPTOMS ASSOCIATED WITH ANTIANGIOGENIC MOLECULES

In tumor antiangiogenic therapies, unfavorable effects are the consequence of direct suppression of VEGF and related RTK signaling cascades. Disturbed VEGF/PlGF signalings in preeclampsia lead to host renal dysfunction and hypertension. Therefore, such symptoms provide us with important information about adverse effects of VEGF suppression on homeostatic regulation of circulatory system under antiangiogenic milieu.

#### 6.1. Renal dysfunction

Under physiological condition, permeability of capillaries varies among organs and tissues. For example, cerebral capillary ECs are particularly impermeable. On the other hand, capillary ECs of renal glomeruli are characterized by fenestrate for fine traffic control of fluid and molecules. In the rodent model's kidney, depletion of VEGF from podocytes using Tet-On system under podocyte-specific promoter resulted in proteinuria and hypertension in four-five weeks after doxycycline treatment (113). In this model, renal glomeruli were damaged by fibrin deposit and endotheliosis, suggesting that local effusion of physiological VEGF from podocytes toward ECs is indispensable for maintaining fenestrated structure of glomerular vasculature (113). Glomerular damages are also detectable in the kidneys of preeclamptic patients. Histological studies on rodent models revealed that the pregnant rats administrated adenovirus sVEGFR1 presented with glomerular endotheliosis (40). These studies

suggest that the disturbance of either local VEGF production or circulatory VEGF level potentially causes glomerular dysfunction.

We reviewed the autopsy cases of three renal cell carcinomas (RCCs) and a gastrointestinal stromal tumor (GIST) that had been administrated sunitinib, a multi-targeted RTK inhibitor that suppresses VEGFRs. None of the patients had a history of renal dysfunction or proteinuria before sunitinib administration. Among them, a RCC case showed the progression of renal dysfunction and sodium-potassium imbalance starting a month after sunitinib administration. The increase of serum creatinin became overt in a month after sunitinib administration (1.7 mg/dL) and was elevated for twelve months (2.1 mg/dL) until the drug was changed to everolimus, an inhibitor of mammalian target of rapamycin (mTOR). The patient died of the disease ten months after. Pathologically, endotheliosis and luminal narrowing were observed in several glomerular capillaries (Figure 1, left). Another RCC case showed mild increases of blood urea nitrogen (BUN) (48 mg/dL) and serum creatinin (1.5 mg/dL) in a month after sunitinib administration, and the patient died of the disease soon. In the autopsy specimen, thrombotic glomerular capillary with mild endotheliosis was occasionally noted (Figure 1, center and right). The other two cases did not show overt renal dysfunction, however, mild increase of BUN (44 mg/dL) was observed in a GIST patient one month after sunitinib administration. Since these patients had received other drugs such as interferon in RCCs and imatinib in GIST, glomerular changes may not be attributable thoroughly to the VEGFRs inhibitor. At least, clinical data and pathological examination supported the notion that the incidence of renal dysfunction and elevated BUN/creatinin were triggered by sunitinib administration in these cases. Careful monitoring for potential damage of physiological VEGF-related homeostasis is required in the patients especially with limited functional capacity of the kidney.

#### 6.2. Hypertension

Hypertension is also a common adverse effect of anti-VEGF therapies. The majority of cancer patients are

middle-aged and older adults who have increased risks of cardiovascular diseases. Therefore, it may be difficult to estimate precisely the risk of drug-induced hypertension. Nevertheless, the incidence of hypertension associated with antiangiogenic therapies is statistically significant by the analyses in several types of solid malignancies (114-116).

Postulated mechanisms of antiangiogenic drugs-induced hypertension include suppressed production of nitric oxide (NO)(2) and prostacyclin. Since NO works as a potent vasodilator, VEGF inhibitors probably decrease NO production in ECs and increase systemic blood pressure (117). The production of NO and prostacyclin is also disturbed in preeclampsia (118). The mechanism is explained in part by the inhibitory condition of TGF- $\beta$ 1-mediated eNOS activation (76). In preeclampsia, the symptom of hypertension is generally limited to gestational period and disappears after delivery. It is widely recognized, however, that pregnancy-induced hypertension increases the risk of cardiovascular diseases in later life (119). Currently, very limited information is available about long term effects of antiangiogenic drugs on circulatory system in advanced tumor patients. Some of these drugs will be approved for first-line, thus, the detail of physiological recovery from drug-induced endothelial dysfunction is a subject for future study.

### 6.3. Reversible posterior leukoencephalopathy syndrome

A reversible posterior leukoencephalopathy syndrome (RPLS, also named posterior reversible encephalopathy syndrome) is another possible disorder in the patients under antiangiogenic therapies. Clinical and radiological features of RPLS were reported by Hinchey *et al.* in 1996 (120). The most frequent cause that led to brain edema was disease-associated hypertension in each case. RPLS is currently accepted as a syndrome associated with endothelial dysfunction triggered by idiopathic hypertension, preeclampsia, systemic lupus erythematosus (SLE), and so on. With regard to malignancies, bevacizumab and sorafenib have been reported to cause RPLS (121). All these conditions potentially perturb blood-brain-barrier and lead to brain edema. Common manifestations of RPLS include headache, visual disturbance and seizure (122). RPLS is essentially curable without post-complications if treated properly based on anti-convulsion drugs and blood pressure control. Precise diagnosis of RPLS and appropriate management are necessary for clinicians to minimize physical damage of the patients.

## 7. PERSPECTIVE

We have discussed general view of tumor neovascularization and representative angiogenic signaling pathways that become effective therapeutic targets. Tumor angiogenesis and proinflammatory microenvironment depend on tumor types, progression stages, host conditions, and so on. BM-derived cells recruited by special chemoattractants may disturb TILs property and accelerate distant metastasis. These tumors frequently obtain

resistance to chemotherapeutic agents during cyclic administration, thus combined chemotherapies with antiangiogenic drugs are expected to improve overall prognosis. Clinical data of phase studies on combined therapies are encouraging, but tumor vessels potentially reconstruct alternative signaling pathways in response to VEGF-targeting therapies. Unfavorable effects of antiangiogenic therapies include systemic vascular resistance and local endotheliosis in some critical organs. Current clinical information about several antibodies against VEGF-mediated pathways is obtained from the patients in advanced stages in most cases. Therefore, further information is necessary about the safety and effects of these targeting therapies in long term prognosis. A better understanding of the molecular and cellular cross-talks of antiangiogenic molecules on local lesion as well as on pre-metastatic organs will contribute to the improvement in therapies for tumor patients and for those suffering from vasculature diseases.

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