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Mechanistic Study of Antiangiogenic Agents in Cancer Therapy

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ABSTRACT

Tumor growth and metastasis are dependent on angiogenesis that constantly alters the tumor microenvironment. Based on the antiangiogenic principle proposed more than 40 years ago by Dr. Judah Folkman, antiangiogenic drugs (ADs) are successfully developed and are routinely used in combination with chemotherapeutics for treatment of various cancers in human patients. However, nearly 10-year clinical experiences with these drugs have taught us some unexpected outcomes that are mechanistically challenging. These include: 1) Ineffective antiangiogenic monotherapy; 2) Modest therapeutic benefits in combination with chemotherapy; 3) Development of intrinsic and evasive drug resistance; 4) Broad adverse effects; 5) Timeline of treatment; 6) Searching for reliable and predictive biomarkers for patient selection and monitoring therapeutic efficacy. Currently, mechanisms underlying these important clinical issues remain poorly understood. In this thesis, we aimed to study the mechanisms that underlie clinical benefits of ADs, clinical adverse effects related to antiangiogenic therapy and interplay between different factors in the tumor microenvironment in modulation of tumor growth and drug responses. In the first published paper, we show that tumor-derived VEGF enters the circulation and causes a systemic effect by alteration of vessels numbers and structures in various tissues and organs. This systemic effect, which we term as cancer-associated systemic syndrome (CASS), is similar to paraneoplastic syndrome, often seen in cancer patients, manifesting anemia, endocrine dysfunction, and hepato-splenomegaly. Based on these findings, we hypothesized that treatment of high VEGF-expressing tumors with chemotherapeutics that often have hematopoietic suppressive effect would cause severe defects of bone marrow hematopoiesis. Indeed, treatment of high VEGF-expressing tumor-bearing mice results in severe anemia, leading to early demise of animals. Prior to chemotherapy, delivery of antiangiogenic agents markedly prevents chemotherapy-induced hematopoietic suppression. From this study we conclude: 1) Sequential delivery of ADs followed by chemotherapeutics is a preferred regimen; and 2) Reduction of chemotoxicity by ADs is, at least in part, a mechanism that underlies combination therapy. In the second paper, we show that PlGF, a member of the VEGF family, negatively regulates VEGF-induced tumor angiogenesis and tumor growth by the formation of PlGF-VEGF heterodimers. Notably, PlGF-overexpressing mouse and human tumors demonstrate superior sensitivity to ADs. Thus, PlGF may potentially be used as a surrogate marker to predict antiangiogenic therapy. In the third paper, we demonstrated that systemic delivery of ADs to mice causes a broad systemic effect on healthy vasculatures in various tissues and organs. These “off-tumor” effects in general correlated well with clinical adverse effects of these drugs in cancer patients. Thus these findings provide a new mechanistic insight of antiangiogenic therapy-related side effects. In the fourth paper, we show a new protocol of studying lymphangiogenesis, which is essential for lymphatic metastasis.

LIST OF PUBLICATIONS

I. Danfang Zhang, Eva-Maria E. Hedlund, Sharon Lim, Fang Chen, **Yin Zhang**, Baocun Sun and Yihai Cao. Antiangiogenic agents significantly improve survival in tumor bearing mice by increasing tolerance to chemotherapy-induced toxicity. Proc Natl Acad Sci U S A. 2011 March 8; 108 (10): 4117-4122.

II. Eva-Maria Eleonora Hedlund, Xiaojuan Yang, **Yin Zhang**, Yunlong Yang, Masabumi Shibuya, Weide Zhong, Baocun Sun, Yizhi Liu, Kayoko Hosaka, and Yihai Cao. Tumor cell-derived placental growth factor sensitizes antiangiogenic and antitumor effects of anti-VEGF drugs. Proc Natl Acad Sci U S A. 2013 Jan 8; 110 (2): 654-659.

III. Yunlong Yang[#], **Yin Zhang**[#], Ziquan Cao[#], Hong Ji, Eric Wahlberg, Toste Länne, Baocun Sun, Xuri Li, Yizhi Liu and Yihai Cao. Anti-VEGF- and anti-VEGFR- induced vascular alteration in mouse healthy tissues. *Submitted.* # Co-first author.

IV. Renhai Cao, Sharon Lim, Hong Ji, **Yin Zhang**, Yunlong Yang, Jennifer Honek, Eva Maria Hedlund & Yihai Cao**.** Mouse corneal lymphangiogenesis model. Nature Protocol. 2011 May 11; 6(6): 817-826.

RELATED PUBLICATIONS

V. Renhai Cao, Hong Ji, Ninghan Feng, **Yin Zhang**, Xiaojuan Yang, Patrik Andersson, Yuping Sun, Katerina Tritsaris, Anker Jon Hansen, Steen Dissing, and Yihai Cao. Collaborative interplay between FGF-2 and VEGF-C promotes lymphangiogenesis and metastasis. Proc Natl Acad Sci U S A. 2012 Sep 25; 109 (39): 15894-15899.

VI. Lasse Dahl Jensen, Ziquan Cao, Masaki Nakamura, Yunlong Yang, Lars Bräutigam, Patrik Andersson, **Yin Zhang**, Eric Wahlberg, Toste Länne, Kayoko Hosaka, and Yihai Cao. Opposing Effects of Circadian Clock Genes *Bmal1* and *Period2* in Regulation of VEGF-Dependent Angiogenesis in Developing Zebrafish. Cell Report. 2012 Aug 30; 2, 231-241.

VII. Rajesh K Nallapalli, Mohamed X Ibrahim, Alex X Zhou, Sashidar Bandaru, Sai Naresh Sunkara, Björn Redfors, David Pazooki, **Yin Zhang**, Jan Borén, Yihai Cao, Martin O Bergo and Levent M Akyürek. Targeting filamin A reduces K-RAS-induced lung adenocarcinomas and endothelial response to tumor growth in mice. Mol Cancer. 11: 50.

CONTENTS

LIST OF ABBREVIATIONS

Ang	Angiopoietin
AD	Antiangiogenic drug
AMD	Age-related macular degeneration
BM	Bone marrow
CASS	Cancer associated systemic syndrome
CTX	Cyclophosphamide
CD	Chemotherapeutic drug
DNA	Deoxyribonucleic acid
Epo	Erythropoietin
ELISA	Enzyme-linked immunosorbent assay
EC	Endothelial Cell
FGF	Fibroblast growth factor
HIF	Hypoxia induced factor
KO	Knock out
MMP	Matrix metalloproteinase
PDGF	Platelet-derived growth factor
PlGF	Placental growth factor
RB	Retinoblastoma protein
RBC	Red blood cell
TGF	Transforming growth factor
TK	Tyrosine kinase
TKI	Tyrosine kinase inhibitor
TSP	Thrombspondin
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cell
WBC	White blood cell

1 INTRODUCTION

1.1 CANCER

Cancer is a leading cause of death, it accounts for 7.6 million deaths in 2008 over the world, and the number of death estimation in 2030 would be 13.1 million according to the World Health Organization's report (Globocan 2008, IARC, 2010). Cancer is due to uncontrolled cell proliferation and abnormal cell differentiation. In most cases, cancer cells undergo rapid cell division resulting in lump formation, which is termed tumor. Tumor is the imbalanced result of constitutive cell proliferation versus cell death. Tumor is composed not only tumor cells, but also inflammatory cells, vascular cells, fibroblasts, and other supportive cells[1-3]. These non-tumor cells contribute to supporting tumor cell growth by creating structure foundation, supplying nutrients and promoting invasion and metastasis[4-7].

Cancer is classified into carcinoma and non-carcinoma[8]. Non-carcinoma includes sarcoma, melanoma, lymphoma and leukemia, germ cell tumor and blastoma[8]. Carcinoma is derived from epithelial cells in many organs, for example, lung, skin, breast and colon[8,9], whereas sarcoma originates from mesenchymal cells such as fibroblast, glial cell and bone cells[8,10]. Lymphoma and leukemia are from hematopoietic cells[8,11,12], whereas germ cell tumors arise from pluripotent cells[8,13]. Blastoma is from the precursor or embryonic cells[14], and melanoma is more specific from melanocytes[8].

However, tumor is different from cancer. Cancer is the more advanced stage or potent malignant type of tumor[8]. The difference between tumor and cancer are: first, tumor has a clear border which distinguishes itself from healthy tissue, whereas cancer is

invasive and metastatic; second, tumor can be benign or pre-malignant, and more likely to have better prognosis, whereas cancer on the other hand is malignant and more aggressive to spread[15].

Cancer is an outcome that induced by both genetic defects and epigenetic influence[16-19]. Genetic defects include the mutation in oncogenes and dysfunction of tumor suppressor genes, which have significant impact on regulation of cell cycle program[20]. The mutation in oncogenes such as mutations in Myc, Ras, and C-sis will over activate the signaling for cell proliferation[21-23], while mutation or loss of tumor suppressor genes such as mutations in Rb, p53 and p21 would result in dysfunction of inhibiting the abnormal cell proliferation[24,25]. Therefore, such genetic defects will increase the risk of developing cancer. On the other hand, epigenetic factors such as UV light, radiation, exposure to carcinogenic chemicals, and chronic infection by virus or bacteria, may cause DNA damage and disrupted DNA repair, which will lead to multiple gene mutations[8,17,26]. These mutations will ultimately lead to abnormal cell division.

1.1.1 Cancer is an age related disease

Cancer is an age related disease[27-30]. According to a clinical study, the incidence rate for cancer is closely correlated with age[31]. The underlying mechanisms are not well known, however, the possible mechanism could be that in the aged cells, cell machineries' function may not well maintained. Thus, the risk of the occurrence of an unrepaired DNA mutation is increased. Because the cell signaling system is poorly regulated in aged cells, it makes the DNA more vulnerable to be damaged by environmental factors, such as chemical or physical factors. Once the DNA damage occurs, it may not be properly repaired and thus cause mutations which can be accumulated and passed to next generation. Accordingly, mutation rate, genome

instability and heterogeneity increase. These changes will further disturb the cell cycle regulation and result in uncontrolled cell proliferation.

1.1.2 Cancer is a systemic disease

Rapid growth of tumor tissue could cause many problems, including local disease and systemic disease[32,33]. In the local environment, tumor expansion can impact organ function by physical affects, for example, colon tumor can lead to narrowing and blockage of the bowel and retinoblastoma may cause losing of vision. However, when cancer develops into malignant stage, it becomes a more complex disease which induces systemic dysfunction in multiple peripheral organs. This includes hematopoietic bone marrow (BM) defect, endocrine system chaos, ascites, gastrointestinal track disorders, muscular and adipose atrophy, and liver, spleen, kidney impairment[33,34]. In fact, cancer patients suffer more from the systemic syndrome rather than local effect[32,33].

1.1.3 Cancer is derived from healthy cells

Cancer cell is originated from healthy cell which has DNA mutations[8]. The outcome of the mutations usually cause constitutively uncontrolled cell cycle activity, resulting in over cell proliferation. After a certain period, accumulation of mutated cell population leads to a visible lump formation, also called neoplasm, which could be benign, pre-malignant or malignant. At the benign stage, the tumor lump is tiny and some tumors are covered by a membrane, which isolates the tumor lump from healthy tissue. Benign tumor could be small or big in size, growing slowly, and has limited local impact on the organ. When the tumor continues to progress, it enters a fast growing and

metastatic stage, releases multiple cytokines and induces cancer associated syndrome to many peripheral organs.

1.1.4 Cancer Therapy

Many strategies have been developed to fight cancer. The most ideal way to cure cancer is to remove tumor tissue completely by surgery. However, in clinical practice, it is very difficult for some invasive or non-solid cancers, especially when there is already microscopic metastasis in other organs[35,36]. Therefore, it is necessary to treat patients with drugs even after surgery to ensure that metastatic cells have been killed. Chemotherapeutic drugs (CDs), such as cyclophosphamide (CTX), Platins, Taxanes, and 5'-FU, are widely used as the second strategy in the clinic to kill cancer cells via blocking the cell cycle in rapid proliferating cancer cells[37-42]. However, these drugs also target the highly proliferating healthy cells, such as hematopoietic cells[43-46]. These drugs induce a broad spectrum of adverse effects. In clinical practice, patients severely suffer from CDs[45,46]. Some patients even die of the chemotherapy but not the cancer[34]. The third way, commonly seen in clinic to treat cancer is radiotherapy[47,48]. Radiotherapy applies ions, exited molecules and free radicals to target cancer cells. It induces chemical changes on both molecular composition and cell structure in cancer cells, resulting in DNA damage and further cell death. Radiotherapy can be applied externally, internally or systemically. It is widely used for treating prostate cancer, head and neck cancer, breast cancer, lung cancer, rectal cancer, gynecological cancer, Hodgkin lymphoma, soft tissue sarcoma, and some other types of cancer. Since we cannot avoid harming healthy tissues around tumor in radiotherapy treatment, the side effects include acute effects, like skin erosions, mucositis, epithelitis, diarrhea and hair loss[49]. The long term side effects include fibrosis, atrophy, bowel structures, myelitis of medulla spinalis, and even cancer[50].

1.2 ANGIOGENESIS

Angiogenesis is the growth of new capillaries from pre-existing blood vessels[3,51-53]. It differs from vasculogenesis which is the establishing of blood vessel stems during embryonic development. Since blood circulation transports oxygen and nutrients to the peripheral organs, and removes the metabolic waste and pathogens, the blood vessel system is the key network for supporting the whole body function[54]. Angiogenesis plays very important roles in physiology, such as embryonic development, wound healing and reproduction[55]. Under pathological conditions, excessive angiogenesis supports tumor growth and cause wet form of age related macular degeneration (AMD) and insufficient angiogenesis cause cardiovascular diseases[56].

Among these imbalanced angiogenesis related diseases, cancer is one of the main concerns. At the early stage of cancer of solid form, a lump which is called tumor can be detected. Like healthy tissues, the tumor also needs oxygen and nutrients to grow by the support of blood. However, tumor tissue does not have its own blood vessel network at the beginning. So it has to establish its own blood vessel system by stimulating the pre-existing vessels in the adjacent tissue to grow towards itself through releasing angiogenic factors. By secreting different angiogenic factors, such as vascular endothelial growth factor (VEGF)[57], platelet-derived growth factor (PDGF)[58], fibroblast growth factor (FGF)[59,60] etc, tumor tissue initiates angiogenesis from the nearby host blood vessels to grow towards tumor.

In such a context, Dr. Judah Folkman proposed that tumor growth depends on angiogenesis. Therefore, if we could inhibit angiogenesis, we may suppress tumor growth[61]. Today there are a lot of antiangiogenic drugs (ADs) developed by pharmaceutical companies widely used for cancer patients based on this concept, and

antiangiogenic therapy becomes a widely used therapy in combination with chemotherapy. Different ADs will be discussed in the later section.

1.2.1 Angiogenic stimulators

1.2.1.1 VEGF

One of the most well characterized angiogenic factor is VEGF. The VEGF family contains several proteins, VEGF-A, VEGF-B, VEGF-C, VEGF-D, and PlGF[62]. And two VEGF family members are found in virus (VEGF-E) and snake venom (VEGF-F) respectively[63,64]. VEGF-A is a 34-42 kDa homodimeric glycoprotein with a heparin binding region. The Human VEGF gene consists of 8 exons. As a result of alternative splicing, VEGF has several isoforms, such as $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$. VEGF family binds to three Vascular Endothelial Growth Factor Receptors (VEGFRs), VEGFR-1, VEGFR-2 and VEGFR-3. Among these receptors, VEGFR-2 is the key receptor to transduce positive signals to stimulate angiogenesis. VEGFR-2 can be activated by VEGF-A and VEGF-C and VEGF-D (also VEGF-E and VEGF-F). Binding of these proteins to VEGFR-2 induce auto-phosphorylation by VEGFR-2 receptor dimerization, resulting in activation of downstream signaling pathways and finally promoting endothelial cells (ECs) proliferation and migration and contribute to formation of the new blood vessels. Besides stimulating ECs, VEGF-A also increases the vascular permeability of capillaries resulting in vascular leakage[65,66].

VEGFR-1 is activated by VEGF-A, VEGF-B and PlGF and transduces signals to modulate angiogenesis and other non-vascular functions. Interestingly, VEGFR-1 has much higher binding affinity to VEGF than VEGFR-2, but the VEGFR-2 plays a key role to activate angiogenesis. VEGF and VEGFR-2 KO mice are lethal at embryonic

stage. VEGF-C and VEGF-D are mainly responsible for lymphangiogenesis, which is the formation of new lymphatic vessel network as shown in Figure 1[62].

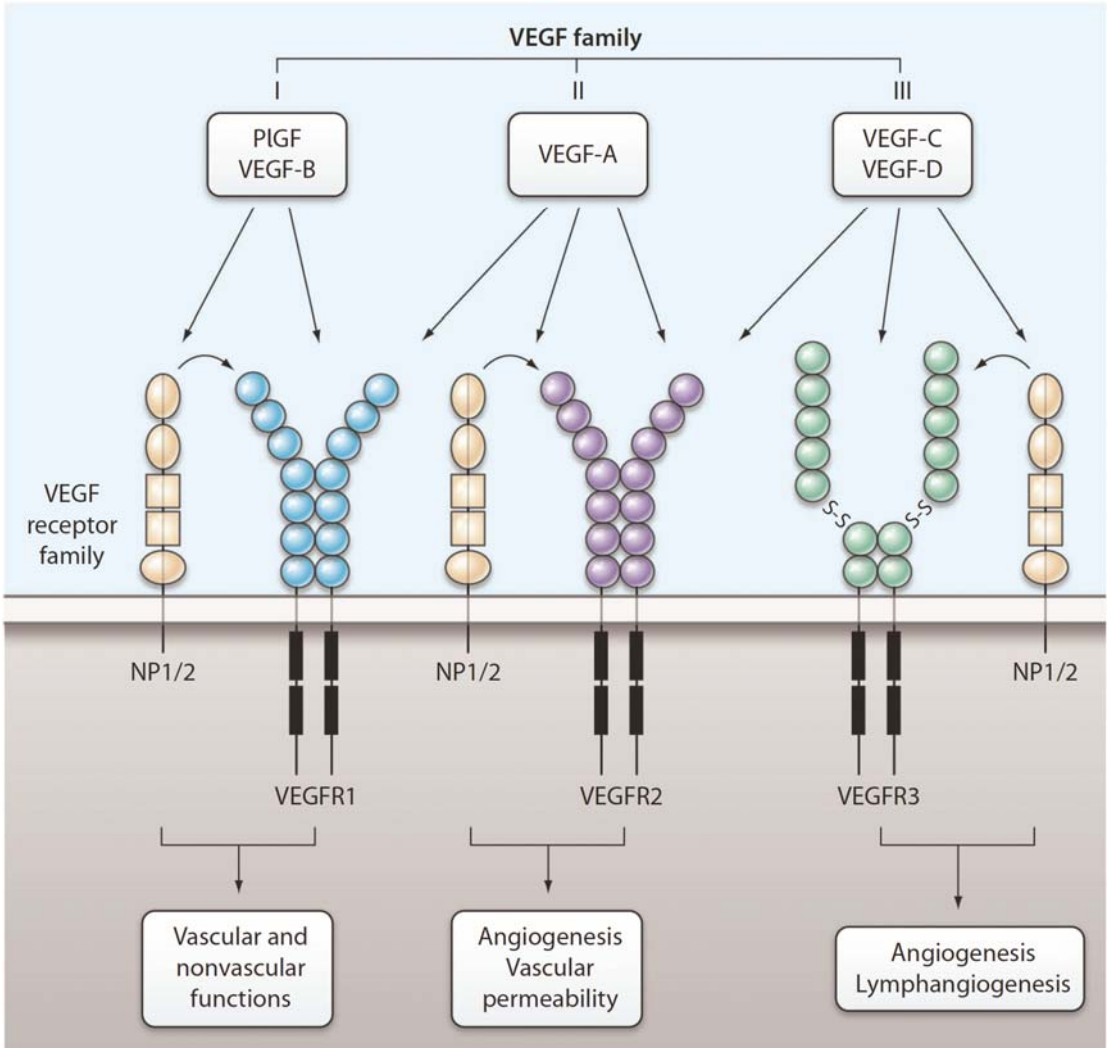

Figure 1. VEGF family and VEGF receptors. (adapted from Y Cao, Science signalling, 2009).

1.2.1.2 PDGF

The PDGF is another mitogenic growth factor family. PDGF family has four members, PDGF-A, PDGF-B, PDGF-C and PDGF-D. They bind to two receptors, platelet-derived growth gactor receptor-alpha (PDGFR-α) and platelet-derived growth factor receptor-beta (PDGFR-β). PDGF proteins usually form homodimers such as PDGF-AA, PDGF-BB. They bind to PDGFR-α, but PDGF-BB has higher binding affinity to

PDGFR-β. PDGF-A and PDGF-B also form PDGF-AB heterodimer, which binds to both α and β receptors[67,68].

PDGF promotes angiogenesis through β receptor and increases ECs migration and proliferation through PI3K/Akt pathway. PDGFR-β is expressed on endothelial, stromal and mural cells, including myofibroblasts, fibroblasts, pericytes and Vascular Smooth Muscle Cells (VSMCs)[69,70]. Thus, PDGF-BB modulates angiogenesis by either targeting endothelial cells or perivascular cells such as pericyte and vascular smooth muscle cells. PDGF-BB stimulates vascular ECs proliferation and migration, which contribute to angiogenesis. On the other hand, PDGF-BB has marked effects on vascular remodelling, maturation and stability by targeting pericyte and VSMCs[71,72]. genetic knock out (KO) of PDGF-BB and PDGFR-β is embryonic lethal due to depletion of pericytes and increasing of vascular leakage. A recent study shows that PDGF-BB can also induce erythropoietin (EPO), which is a potent angiogenic protein[73].

1.2.1.3 FGF

FGF family is a big family and contains 22 members[60]. Among these members, FGF-1 and FGF-2 have been shown to have more potent ability in promoting angiogenesis[68,74]. FGF-1 and FGF-2 lack a signal leading sequence for being transported out to the extracellular matrix, whereas most other members of the FGF family are secreted. FGFs have multiple functions induced by binding to four FGF receptors (FGFR-1, FGFR-2, FGFR-3 and FGFR-4), which are expressed on a wide range of different cell types, including ECs. FGF-1 and FGF-2 promote EC proliferation, migration, differentiation, protease production, integrin and cadherin receptor expression and intercellular gap junctions[60,74].

1.2.1.4 Angiopoietin

The Angiopoietin (Ang) family contains Angiopoietin-1, -2, -3 and -4. Angs bind to tyrosine kinase with immunoglobulin-like and EGF-like domains-1 and -2 (Tie-1 and Tie-2) Tie-1 and Tie-2 receptors. Ang1 and Ang2 are required for maturation of blood vessels shown in a mouse model. Ang-2 binds to Tie-2 receptor competitively with Ang-1. The roles of Ang-1 and Ang-2 remain controversial. Tie-2 can be activated by Ang-1, but Ang-2 can either activate or inhibit Tie-2 depending on cell types[75].

1.2.1.5 TGF-β

Transforming growth factor-beta (TGF-β) is a protein that has multiple functions on various cell types. TGF-β induces apoptosis, controls cell cycle, and induces p15 and p21 expression, which block the cyclin/CDK complex responsible for Retinoblastoma protein (Rb) phosphorylation[76,77]. Thus, TGF-β blocks G1 phase. TGF-β plays key roles in immunity, cancer, heart disease, diabetes and AIDS. TGF-β is a secreted protein that has three isoforms named TGF-β1, TGF-β2 and TGF-β3[78,79].

1.2.1.6 Matrix metalloproteinase

Matrix metalloproteinase (MM) regulates angiogenesis by indirect releasing angiogenic related proteins. It is a proteinase that degrades the cell matrix, including heparin, thus releasing heparin bound VEGF, PDGF and other angiogenic cytokines[80,81]. These cytokines may promote tumor angiogenesis.

1.2.2 Angiogenic inhibitors

1.2.2.1 Angiostatin

Angiostatin is a 38 kDa fragment of plasmin formed by autoproteolytic cleaveage of plasminogen. Angiostatin can be cleaved by different MMPs. Angiostatin binds ATP

(Adenosine-5'-triphosphate) synthase on ECs and inhibits endothelial cell proliferation, migration and induce apoptosis[82,83].

1.2.2.2 Endostatin

Endostatin is a 20 kDa fragment derived from type XVIII collagen. It has a high affinity for heparin binding. Endostatin is produced by proteolytic cleavage of collagen XVIII. It has a broad spectrum of antiangiogenic effect and interacts with FGF-2 and VEGF[84]. Endostatin inhibits angiogenesis by down-regulating many signaling pathways, including TNF-α, NFkB, ephrin, and adhesion cascades, which decrease viability and migration of endothelial cells[85]. Endostatin blocks pro-angiogenic gene expression controlled by c-Jun N-terminal kinase through interfering with TNF-α activation. It reduces the growth of cells by inhibiting cyclin D1, and thus induces apoptosis. Endostatin also regulates FGF signaling transduction and further inhibits migration of ECs[86]s. Endostatin may prevent the activity of certain MMPs.

1.2.2.3 Thrombospondin

Thrombospondins (TSP) are secreted endogenous proteins that inhibit angiogenesis. TSP was discovered by Nancy L. Baenziger[87-90]. This family has five members (TSP1-5). TSP1 is a well studied protein. It is encoded by THBS1, first isolated from platelets that stimulated with thrombin. TSP1 has been found in multiple biological processes, including angiogenesis, activation of TGF-β and immune regulation. TSP1 binds to several receptors. It inhibits proliferation and migration of ECs by activation of apoptosis[91].

1.2.3 Tumor angiogenesis

Tumor growth is depending on several biological processes. From a single tumor cell to a lump, it goes through environmental changes and genome instability[8]. During the highly dynamic progression, energy and materials undoubtedly are very much demanded by the tumor tissue for supporting the growth and metabolism. However, perfusion of oxygen and exchange of materials by cell contacts are not sufficient for tumor tissue to grow when the tumor lump grows beyond 2 mm^3. In this situation, tumor tissue will become more and more hypoxic, and hypoxia will up-regulate gene expression of many cytokine including VEGF, FGF and PDGF by the transcription factor Hypoxia Induced Factor 1-alpha (HIF1-α)[92]. The up-regulation of these pro-angiogenic factors induce vascular endothelial cells on the nearby blood vessels to proliferate and migrate towards to the hypoxic region and finally form new blood vessels that connected with pre-existing vessels. Through this biological process, tumor tissue obtains much more energy supply by creating its own blood vessel network through manipulating the host system.

However, newly formed blood vessels in tumor tissue are not well functioning. Most of the vessels are less perfused, leakier and disorganized compare to functional vessels in healthy tissue[93]. These vessels lack of the support by the mural cells such as pericytes and smooth muscle cells[94].

Tumor tissue is not composed only of cancer cells, but also stromal cells such as fibroblast, vascular cells, adipocytes, and inflammatory cells such as macrophages, lymphocytes and neutrophils[2]. All these cells contribute to tumor progression and tumor angiogenesis by producing angiogenic factors, such as VEGF family[95].

As proposed by Dr. Judah Folkman, tumor growth is angiogenesis dependent. Therefore targeting tumor vessels by antiangiogenic drugs could be an effective therapy to combat cancer.

1.3 ANTIANGIOGENIC AGENTS

1.3.1.1 Bevacizumab

Nowadays there are many ADs widely used for the patients. Basically, there are two types of ADs. One type is antibody. In clinic, one of the widely used antibodies is bevacizumab, a humanized monoclonal antibody against VEGF-A, named AVASTIN in the market[96]. By targeting VEGF-A, bevacizumab can inhibit the VEGF/VEGFRs pathway signaling resulting in suppression of angiogenesis. Bevacizumab was first approved in 2004 by the Food and Drug Administration (FDA) for combination use together with chemotherapy for colorectal cancer[97]. Now it has been approved for various cancers such as non-small cell lung cancer, metastatic renal cell cancer (RCC), recurrent glioblastoma multiform, AMD and diabetic retinopathy[98] a. It was approved for metastatic breast cancer in 2010 December. However, later clinical trials showed that bevacizumab did not prolong the overall survival or slower disease progression either in breast cancer. In October 11, 2011, FDA withdrew the approval for bevacizumab from breast cancer treatment.

Bevacizumab also has adverse effects, since it inhibits blood vessels in all tissues due to the systemic administration of this drug. The main side effects are hypertension, bleeding, intestinal perforation and thrombotic microangiopathy[99]. Beside, the cost is another big concern regarding its limited benefits for prolonging overall survival. Dr. Robert J. Mayer published in the New England Journal of Medicine that AVASTIN

extended life by 4.7 months of colorectal cancer at a cost of 42,800-55,000 US dollars[100]. Such high cost of bevacizumab raises needs for seeking cheaper and better alternatives. Small molecules that inhibit VEGF/VEGFR signaling, such as sunitinib, sorafenib, pazopanib and imatinib have been developed as ADs.

1.3.1.2 Sunitinib

Sunitinib, commercial name is Sutent, was approved by the FDA in 2006 for RCC, imatinib-resistant gastrointestinal stromal tumors and other tumors[101-104]. Sunitinib is one of the tyrosine kinase inhibitors (TKIs). It targets the intracellular domain of several tyrosine kinases, including VEGFRs, PDGFRs, c-Kit, Ret, CSF-1R and Flt-3[105-107]. By blocking the signaling pathway of VEGF and PDGF, tumor blood vessels are reduced significantly according to several pre-clinical and clinical studies[33,57,73]. However, multiple inhibitions in sunitinib treatment caused several side effects such as asthenia, fatigue, hair depigmentation, cardiotoxicity, hypothyroidism, hypertension, hematologic and gastrointestinal toxicities, and dermatologic adverse events[108-111]. Sunitinib has been approved for treating RCC, Imatinib resistant Gastrointestinal Stromal Tumor (GIST) and pancreatic neuroendocrine tumors.

1.3.1.3 Sorafenib

Another small molecule is sorafenib. Similar to sunitinib, sorafenib targets VEGFR-2, VEGFR-3, PDGFRs, Raf kinase (B-Raf and c-Raf), and c-kit. Sorafenib has been approved for treatment of advanced RCC and hepatocellular carcinoma[112-115]. And there are studies ongoing for this drug to treat liver cancer, lung cancer thyroid cancer and recurrent glioblastoma[116,117]. Sorafenib also has adverse effects similar with sunitinib, such as skin rash, hand-foot skin reactions, diarrhea and hypertension[118,119].

1.3.1.4 Pazopanib

Pazopanib (Votrient) blocks VEGFRs, PDGFRs and c-kit. It has been approved for RCC and soft tissue sarcomas[120-123].

1.4 ANTIANGIOGENIC THERAPY

1.4.1 Principle of antiangiogenic therapy

The principle of antiangiogenic therapy is based on the hypothesis that tumor growth is dependent on angiogenesis, and inhibiting angiogenesis might block tumor growth by cutting off the nutrient supply for tumor tissue. Many ADs have been developed based on this theory, such as Avastin, Sutent, Nexavar[98,124,125] and so on. These drugs target vascular ECs and suppress tumor growth in the following way. Firstly, by inhibiting the ECs proliferation and migration, the formation of new blood vessels is blocked. This results in oxygen, growth factors, substrate supply decrease and metabolites increase due to the limited material exchange with the circulation system. Consequently, the tumor grows slower because of a lack of blood supply. Secondly, usually in the tumor tissue, blood vessels are extremely disorganized. After antiangiogenic treatment, these vessels may become normalized, and regain their function, which benefits the drug delivery of CDs. Thereby increase the efficiency of CDs is enhanced. Thirdly, ADs will also target circulating endothelial progenitor cells, which contribute to angiogenesis. Taken together, antiangiogenic therapy inhibits tumor growth by directly targeting blood vessels, blocking progenitor cells and assisting the delivery of CDs.

1.4.2 Clinical practice and perspective

In the pre-clinical study, ADs showed very significant outcome in inhibiting tumor growth and prolonging the overall survival in the animal models[33,34]. However, in clinical settings, ADs are always administered in combination with chemotherapy for cancer patients. There is no antiangiogenic monotherapy that has been approved so far for the ADs[126]. And even in combination therapy, ADs only give modest benefits, but never cure cancer patients completely[127-129]. Weighing the high cost and the limited benefits, we should reconsider about the low efficiency of ADs regarding the price and adverse effects.

However, ADs are still popular in the research field, and pharmaceutical companies continue to invest money in the development of this type of drugs, which implies that the antiangiogenesis theory proposed by Dr. Judah Folkman has been widely accepted and it is very promising to become a way to cure cancer. Thus, we have to overcome many issues regarding practical difficulties[55,65,66,93,130-132]. First, a finely established model to mimic the clinical situation is needed. At present, animal tumor models are used which are associated with some disadvantages in several aspects. In the xenograft model, it is easy to standardize the experimental procedures, but it is quite different from the clinical reality. The orthotopic model is more clinically relevant compare with the xenograft model. But in this model several parameters such as tumor growth and tumor weight are difficult to evaluate. Moreover, orthotopic model also requires sophisticated skills to manipulate which makes the results fluctuate from different people. The spontaneous model mimics cancer patients the best among all models, but the result can varies a lot. The second issue is that the animals used for research are syngeneic, i.e. they have the same genetic background. However, in human patients, the genomic background is rather individually different. Consequently, pre-clinical

results often cannot simply be applied to human patients. Ideally, personalized therapy based on the individual's genetic constitutions would prefect a very pronouncing approach. However, this strategy requires very advanced technology. Third, cancer itself is a very complex process. There are many RNA and DNA regulations, proteins and pathways cross talks during the tumor progression. In such complex networks, it could be difficult to figure out the detailed lines among all the participants. But as long as we can identify the central players in regulating tumor growth, invasiveness, metastasis, and drug resistance, we might be able to develop promising treatment for cancer patients.

2 AIMS

The overall aim is to elucidate the mechanism of multiple antiangiogenic agents in inhibiting tumor growth by blocking blood vessels in cancer therapy.

More specifically, the aims are:

1) To study the beneficial effect of antiangiogenic agents in improving survival by combination with chemotherapeutic drugs
2) To study the mechanism of PlGF on antiangiogenic outcome
3) To study the side effects of antiangiogenic therapy with different agents in healthy tissues
4) To study angiogenesis and lymphangiogenesis in a mouse cornea model

3 METHODS

In vitro methods:
Histology and Immunohistochemistry
Imaging of immunostaining with various microscopes
Enzyme linked immunosorbent assay
Electron Microscope
Western blot
RNA Interference
Blood chemistry

In vivo models:
Mouse tumor model
Mouse cornea model
Vascular permeability and perfusion assay

Histology and Immunohistochemistry

Histology is widely used method to detect the morphological changes of tissues. In this thesis, Hematoxylin and Eosin (H&E) staining was applied to stain the cell nuclei and cytoplasm in different tissues. To study angiogenesis, specific cell surface markers for blood vessels are needed. Here, CD31, a pan endothelial cell marker was used for immunohistochemistry[133]. Endomucin is another good marker for detecting ECs with both fresh tissue staining and paraffin embedded samples. Quantification of the $CD31^+$ and $endomucin^+$ signals can give information of blood vessels, including vessel density, vessel diameter, and branches. Together with a pericyte maker NG2 staining[134], the coverage of blood vessels can be determined by this approach to compare the changes among different groups. Since immunofluorescence gives clear signals when staining with multiple markers, we also applied the fluorescent conjugated antibody in this method, and thus generated color labeled cells. By fluorescent microscopy, colorful images can be produced, that provide more beautiful and clear pictures.

Imaging of samples with various microscopes

Optical microscope, fluorescence microscope and confocal fluorescence microscope were applied in this thesis. For H&E staining and cell morphology observation, regular optical microscope is sufficient to distinguish tissue structures and pathology changes. However, when it comes to detecting the fluorescence labeled blood vessels or cell sub types, fluorescence microscope can easily work with immunohistochemistry samples. Fluorescence microscopy was commonly used on paraffin-embedded samples with color-labeled staining, such as endomucin staining, DAPI staining, α-SMA staining[135], caspase-3 staining and hypoxic probe staining. To get a 3D structure of blood vessels, confocal microscope is the key tool to collect and combine such information. With this advanced microscope, tumor cells and other matrix structure, such as blood vessels, and their association between perivascular cells and vascular cells become easily to obtain.

ELISA

To study angiogenesis, it is also interesting to measure the changes of angiogenic factors' level in cells, local tissue and circulation. Therefore, ELISA is a necessary mean to achieve the goal. In this thesis, all the ELISA was based on sandwich method.

Electron Microscopy

Tiny capillaries in some organs such as thyroid and glomerulus have a finely regulated structure called fenestration, which is the window of the endothelial wall. This particular structure is very important for material exchange between perivascular cells and blood system. To investigate the subtle changes on this fenestration, EM is the only approach to detect such tiny structures of a size less than one μm.

Western blot

Western blot is widely used to detect protein level changes under different condition. Here western blot was applied for detecting the hypoxic indicated protein levels.

Transduction of specific plasmid and shRNA interferance

To study protein functions, usually gain and loss of function approaches are necessary to provide compelling evidence. In this thesis, multiple angiogenic protein over-expression cell lines were established by transducing retro- and lenti- virus vector, while RNA interference[136,137] was done with several commercially available plasmids to knock down the expression level in some wild type cell lines.

Blood chemistry

Tumors often induce systemic syndromes in the peripheral organs and bone marrow and blood circulation is one of the targets. Therefore, it is necessary to measure different parameters of blood cell populations. The typical parameters tested in our study were RBC, WBC, HGB and HCT. These data supported our observation that CDs induce bone marrow suppression.

Mouse cornea model

The Mouse cornea is a non-vascular tissue under physiological conditions. Thus, it is a great model to study angiogenic and lymphangiogenic factors in a pure environment. By implanting small pellets into the cornea, visible angiogenesis can be induced by VEGF, FGF and PDGF. Combination with immunostaining, blood vessels and lymphatic vessels are easily detected under a confocal microscope. This model allows us to study the potential candidates that induce angiogenic response and inhibitors without any disturbance by a pre-existing vessels system[138].

Mouse tumor model

In this thesis, a mouse xenograft tumor model was used for most of the experiment[34,139,140]. In this model, tumor cells were re-suspended in PBS and inoculated subcutaneously on the dorsal back of C57 or SCID mice. After a few days, visible tumor tissue will grow. It usually takes around 1 month for a tumor to reach the size of $1cm^3$-$1.5cm^3$ depending on different tumor cell lines and mouse strain. Compared with other models, such as spontaneous model or orthotopical model, the xenograft model has several advantages: first, it is very easy to perform and it is easy to be standardized

and highly reproducible. The Spontaneous model usually needs long term stimulation with chemicals or other factors to induce tumors. And the variations of tumor size and growth rate are hardly to control. The orthotopical model usually requires very sophisticated surgical techniques and sometimes it is very difficult to perform on very tiny tissues in mice, such as thyroid, adrenal gland or brain. Second, the result from xenograft model is easy to repeat because the model is well established. Third, usually in this simple model, there are fewer factors which have impacts on tumor development. The spontaneous model often has multiple gene mutations which may contribute to tumor progression, and orthotopic model may also have difficulties on visualize the tumor size and condition.

Vascular permeability and perfusion

One of the characteristics of tumor blood vessels is high permeability and poor perfusion, which lead to dysfunction of intra-tumor vessels. Thus, to determine the permeability and perfusion is very important to evaluate the tumor vessels function. Here we use a fluorescent labeled molecule as a probe. This molecule is injected I.V. into the circulation system and can be detected by the fluorescence under microscope. Therefore, it allows to easily detection of potential leakage and perfusion of the tumor vessels[140].

4 RESULTS AND DISCUSSIONS

4.1 ANTIANGIOGENIC AGENTS IMPROVE SURVIVAL BY ATTENUATING THE CHEMO TOXICITY (PAPER I)

Cancer patients usually suffer from cancer associated systemic syndrome (CASS), such as impairment of live function, severe anemia, and bone marrow defect. CDs, such as cyclophosphamide (CTX), platins, and taxanes usually have a broad toxicity in several tissues, often leading to immune suppression, anemia, liver dysfunction, hair loss, gastrointestinal disorders and kidney damage. Tumors that express high levels of VEGF also have severe impact on tissue functions. Since both tumor-derived VEGF and CDs impair normal functions of multiple tissues and organs, we hypothesize that blocking VEGF with antiangiogenic drugs (ADs) might improve the tissue functions by the mechanism of reducing VEGF induced organ function impairment and increasing the tissue tolerance to CDs' toxicity.

ADs are widely used in clinical in combination with CDs. The principle is that using CDs to kill tumor cells directly while cut off the nutrient supply by blocking blood vessels growth in tumors. However, modest survival benefits have been achieved by combination therapy. And the underlying mechanism is not well known.

In our study, we first established a mouse tumor model with treatment of combination therapy of a representative AD, sunitinib and a CD, carboplatin at clinically relevant dose to mimic the clinical therapy. In this model we successfully reproduced the benefits of prolonged survival of tumor bearing mice treated with combination therapy compare with monotherapy of carboplatin alone. Interestingly, we also found that when administering sunitinib and carboplatin individually, carboplatin significantly decrease

the survival by inducing nearly 80% animal death within ten days of treatment. On the contrary, in the sunitinib treated group, all animals were still alive during the experimental schedule. This data indicated that antiangiogenic drugs can significantly improve survival of tumor bearing mice.

Since CDs produce side effects on multiple organs, we compared BM in carboplatin and sunitinib treated group. Indeed, as we expected, BM in carboplatin treated group showed a dramatic decrease of hematopoietic niches. In the blood count, the numbers of red blood cells (RBCs) decreased more than half compared with the buffer group. However, sunitinib treated group show no significant impact on either BM cells or red blood cells decrease and RBC even increased slightly, which further demonstrates the protective effect of antiangiogenic drug on bone marrow.

Using a $VEGF_{165}$ overexpression tumor cell line in the tumor model showed significant BM suppression compared with the vector tumor group. Accordingly, survival of VEGF tumor bearing mice was shortened. However, when sunitinib was given to the VEGF tumor mice, bone marrow was restored back to the vector tumor mice level and survival was greatly increased. To further study the additive effect of CDs on VEGF tumor bearing mice, we administrated VEGF tumor mice with carboplatin. As expected, Carboplatin further suppressed BM of VEGF tumor bearing mice and the RBC level decreased to a quarter of the level in vector tumor mice.

To generalize the findings, we also used cyclophosphamide (CTX) in the same settings. As expected, CTX decreased survival and BM and also suppressed hematopoiesis. Together with VEGF, CTX manifests the impairment of BM and survival on VEGF tumor bearing mice. Based on these findings, we conclude that both VEGF and CDs

have a similar impact on suppressing BM cells, and sunitinib has a protective effect induced by both VEGF and CDs on BM cells. Thus, we hypothesize that AD could improve BM cells and survival to CDs treated mice by a sequential delivery regimen.

To test our hypothesis, we gave VEGF tumor bearing mice sunitinib as a neoadjuvant therapy before administrating CDs. Surprisingly, we found that antiangiogenic therapy improves survival significantly compared with combination therapy alone from the very beginning. The histology results also showed that sequential delivery of ADs followed by CDs restored BM cells and showed even better effect than combination therapy.

These data demonstrate that sunitinib can protect BM against CDs induced toxicity and improve survival when using sequential delivery in combination therapy. These data also explained the mechanism of combination therapy. In conventional therapy, ADs and CDs are administrated simultaneously, however, CDs and VEGF produced systemic toxicity is attenuated by ADs. Restored BM function will result in more tolerance to the CDs and VEGF induced suppression.

In clinical settings, tumor tissue is always the central concern of the therapy. However, the survival benefit of all the CDs may not necessarily correlate with tumor suppression. Hereby, to improve the off-tumor organ function is also an effective way to prolong survival and also the quality of life for the cancer patients. In our study, we proposed a modified regimen in pre-clinical settings indicating that by improving the off-tumor target organ function may increase the survival of cancer patients without dramatically inhibiting tumor volume. This approach of taking into account the off-tumor targets might open new possibilities to improve anti-cancer therapy.

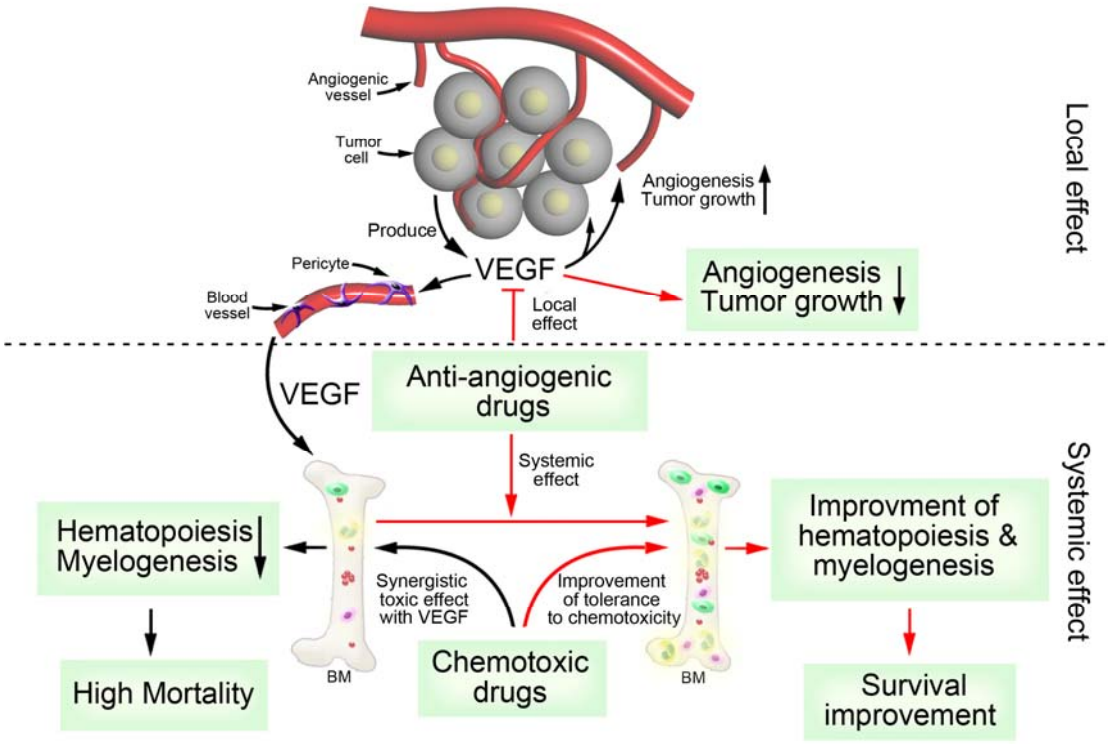

Fig 2. Cartoon shows the mechanisms of how antiangiogenic drugs contribute to prolong the survival and increase the tolerance to chemotoxicity (adapted from Danfang Zhang *et al,* PNAS, 2010).

In Figure 2, we summarize the mechanism that how antiangiogenic drugs protect BM and improve survival. Tumor derived VEGF targets on local endothelial cells and promotes tumor angiogenesis, supporting tumor growth. On the other hand, VEGF also enters the circulation and targets peripheral organs such as BM. VEGF induces BM suppression and results in hematopoiesis and myelogenesis defects. Thus, in VEGF high expression tumor patients, when administration of CDs, which also suppress BM function, will lead to further damage of the BM and a dramatic mortality increase due to additive dual toxicity. However, when treating with ADs before chemotherapy, not only tumor vessels and tumor growth would be inhibited locally, but the VEGF induced BM defect would be blocked as well. Therefore, the tolerance of BM for taking CDs induced toxicity will be increased and the survival would be improved.

4.2 PLACENTAL GROWTH FACTOR IS A NEGATIVE REGULATER OF ANTIOGENESIS (PAPER II)

PlGF is a member of the VEGF family. Unlike other VEGF family members, PlGF specifically binds to VEGFR-1. The study of PlGF has raised a lot of controversial results. Some researchers reported that PlGF is an angiogenic factor that promotes angiogenesis and tumor growth[141-143]. However, data from different labs showed opposite results[94,140,144,145]. This discrepancy requires more extensive studies on the mechanisms of PlGF function under different context. In our study, we found that PlGF is a negative regulator of angiogenesis and PlGF over expression could normalize tumor blood vessels and moreover, sensitize the anti-VEGF treatment. Thus, it can be used as a surrogate marker for cancer patients who would respond effectively to antiangiogenic therapy.

We first analyzed a series of human tumor cell lines to detect PlGF expression level. In seven tumor cell lines, including human breast cancer, squamous cell carcinoma, melanoma, pancreatic carcinoma, hepatocellular carcinoma, ovarian carcinoma, and choriocarcinoma. A choriocarcinoma JE-3 expressed the highest level of PlGF around 46.58 ± 2.26 ng/ml compared to the second highest one 1.01± 0.17 ng/ml. In our previous study, we showed that PlGF normalized blood vessels in tumor tissue[94]. To investigate the anti-VEGF drug effect on PlGF expressing tumors, we injected VEGF neutralizing antibody to the JE-3 tumor bearing mice. Interestingly, we found that anti-VEGF treatment had a marked effect on PlGF tumors. At day 22 after treatment, the tumor remained dormant and some tumors even showed 93% reduction. Immunofluorescence staining showed a marked decrease of blood vessels in the tumor tissue. Besides, vascular branch points, pericyte coverage and vessel diameters were all decreased in the anti-VEGF group compare with the buffer treated group. These data

demonstrated that PlGF expression in tumor cells does not confer anti-VEGF resistance. On the contrary, PlGF expressing tumor is sensitive to anti-VEGF treatment.

PlGF binds to VEGFR-1 specifically, but not VEGFR-2. Thus, to elucidate the role of VEGFR-1 and VEGFR-2 in this PlGF effect, JE-3 tumor bearing mice were treated with VEGFR-1 neutralizing antibody (MF1) and VEGFR-2 neutralizing antibody (DC101) respectively. Surprisingly, VEGFR-1 blockade did not significantly affect the tumor growth compare with the control group. However, VEGFR-2 blockade dramatically inhibited JE-3 tumor growth, and at day 19 after treatment, two of six DC101 treated tumors remained as tiny nodules where MF1 treated tumors reached a size of 1.2 cm^3. Consistent with tumor growth rate, VEGFR-2 blockade virtually completely depleted tumor microvessels, resulting in a large avascular area in the tumors. Only few scattered tumor microvessels were detected in one of six tumors. In contrast, VEGFR-1 blockade significantly increased blood vessel density in tumors. These data indicated that the activation of VEGFR-1 by PlGF has a direct effect on sensitizing the tumor tissue to anti-VEGF treatment.

To further validate that PlGF expressing tumors are highly sensitive to VEGFR-2 blockade, we silenced the *plgf* gene with shRNA that targeting the *plgf* transcripts. First we checked the VEGF level after *plgf* shRNA. VEGF expression was not changed compared with scrambled shRNA transduced cells. After shRNA transduction, a six-fold decrease of PlGF was detected in the tumor. Plgf shRNA transduced tumors exhibited a marked increase of vascular density compared with control tumor. Additionally, microvessels in *plgf* shRNA transduced JE-3 tumors appeared to have more disorganized and premature vascular networks, finally resulting in accelerated tumor growth rate.

These data again demonstrated that PlGF negatively regulates angiogenesis. We next treated JE-3 tumor with *plgf* shRNA transduced by VEGFR-2 blockade. Interestingly, silencing *plgf* led to a significant increase of resistance to VEGFR-2 blockade. Our results show that JE-3 tumor cell derived PlGF is responsible for the drug hypersensitivity of antiangiogenic agents that target the VEGFR-2 signaling pathway.

To elucidate our findings, we established a mouse fibrosarcoma cell line that over expresses human plgf-cDNA (T241-PlGF) as previously described[94]. As we expected, the T241-PlGF tumor grow significantly slower relative to vector T241 tumors. To exclude the possibility that PlGF may have direct effect on tumor cells, we measured mRNA expression of *vegfr-1* and *vegfr-2* in T241 tumor cells. The data showed that T241 tumor cells lack a detectable level of *vegfr-1* and *vegfr-2* expression compared with a mouse endothelial cell line. And PlGF or VEGF protein did not stimulate tumor cell proliferation. These findings demonstrate that extrogenous or endogenous VEGF and PlGF do not affect tumor cell growth. Markedly, VEGF blockade increased the antitumor effect on PlGF expressing tumor. Similar to VEGF blockade, VEGFR-2 blockade resulted in a suppressive effect of tumor volume compare with vector tumor. In contrast to VEGFR-2 blockade, VEGFR-1 blockade did not show anti-tumor effect in neither PlGF nor vector tumor.

To address the underlying mechanism, we measured both VEGF and PlGF homodimer respectively, and also VEGF/PlGF heterodimer by ELISA. As we previously showed, only the homodimer form of VEGF ligand can bind the VEGFR-2 receptor and activate the downstream pathways. VEGF/PlGF heterodimer can only bind to VEGFR-1, which will transduce negative signals to remodel angiogenesis. We found that in the PlGF over-expressing cell line, VEGF was competitively bound to PlGF, only very low

levels of VEGF homodimers were detected in JE-3 tumor. The explanation could be that the VEGF homodimers in the tumor environment is essential for maintaining vessel survival. Delivery of anti-VEGF drugs to PlGF-expressing tumors may result in functional depletion of VEGF homodimers, leading to vascular regression. In support of this notion, treatment of anti-VEGF with anti-VEGFR-2 leads to dormancy of a small number of avascular tumors that lack detectable blood vessels. The other possibility is that PlGF can normalize the tumor vessels thus increase the function of perfusion. This leads to an increase of anti-VEGF drug delivery efficiency in the tumor environment.

4.3 SIDE EFFECTS PRODUCED BY ANTIANGIOGENIC DURGS ON OFF TUMOR TARGETS. (PAPER III)

Bevacizumab is an antibody that neutralizes human VEGF, which is now widely used in clinical settings for treating various cancers, including colorectal cancer, non-small cell lung cancer, glioblastoma, kidney and ovarian cancer. However, this antibody also has broad side effects on several off tumor tissues[146-148]. But in clinical settings, bevacizumab is usually used together with chemotherapy, therefore the side effects induced by VEGF blockade alone have not been well evaluated due to the clinical practice. In this paper, we used a rabbit anti-mouse neutralizing monoclonal antibody and rat anti-mouse VEGFR-1/-2 neutralizing antibodies to investigate the impacts of VEGF and VEGFRs blockades on multiple tissues with the mouse model.

In this study, we first examined blood vessels in multiple tissues. We found that anti-VEGF decreases blood vessel density in various tissues. For example, in the endocrine organs, such as thyroid, adrenal gland and pancreatic islets, blood vessel level was reduced to more than 50%, and also in GI tract, liver, kidney and reproductive organs,

blood vessel density was decreased significantly. Since this antibody only binds VEGF, our data shows that VEGF plays a key role in maintaining the homeostasis of blood vessel renewal. Further studies on retina, muscles, BM and different brain sections indicated that there were no changes of blood vessels in these tissues. This data demonstrated that different tissues have different response to VEGF blockade, although VEGF is the main driving force for angiogenesis. This might due to the difference of tissue structure, local environment, and antibody efficiency.

To address the mechanism of the blood vessel change, we used two specific antibodies to block VEGFRs. VEGF has three receptors, VEGFR-1, VEGFR-2 and VEGFR-3. It is widely accepted that VEGFR-1 and VEGFR-2 are involved in angiogenesis while VEGFR-3 is responsible for lymphangiogenesis. By targeting VEGFR-1 and VEGFR-2 respectively with MF1 and DC101, we found that VEGFR-2 blockade had the same impact on reducing blood vessels, which is consistent with VEGF blockade. However, anti-VEGFR-1 did now show any inhibition on blood vessels. These data revealed that VEGF/VEGFR-2 is the main signaling pathway in maintaining blood vessels.

The quantity changes of blood vessel led us to consider the consequence of structure changes as well. Electron microscopy on thyroid vessels showed decreased fenestrations in anti-VEGF treated vessels, but increased the caveolae numbers in the endothelial layer. Fenestration is the '*small window*' in the vessel wall. Its function is to exchange materials between cells and circulation system. Once the '*windows*' are closed, the balance of matters and energy will be broken, leading to dysfunction of several organs. However, we also found that the numbers of caveolae increased after anti-VEGF treatment. Caveolae are the small invaginations of the plasma membrane in endothelial cells and adipocytes. These structures are rich in proteins and lipids, which

are involved in signaling transduction, exo- or endocytosis, and uptake of pathogenic bacteria or certain viruses. Thus, this means the increased caveolae actually is a substitute approach to compensate the fenestration reduction induced functional defects.

To test our hypothesis that anti-VEGF could cause tissue function changes, we measured the thyroid hormones, free Triiodothyronine (fT3) and free Thyroxine (fT4). Interestingly, in a short inhibition of VEGF in two weeks, fT3 and fT4 did not change. However, when we prolonged the treatment to four weeks, fT3 significantly has reduced, which is a sign of thyroid function impairment.

To further address the mechanisms, we co-stained apoptosis and vascular ECs markers on thyroid tissue. We found that compare with buffer treated group, anti-VEGF increased apoptosis in ECs resulting in cell death. Moreover, western blot showed significant elevated hypoxic proteins in thyroid.

Very interestingly, when we withdraw the inhibition, blood vessels grew back to normal level in two weeks in thyroid and other tissues. This data indicated that the anti-VEGF induced blood vessel degeneration is reversible. Which means the balance of angiogenesis is finely regulated by VEGF under physiological condition.

Taken together, VEGF plays a key role in keeping homeostasis of blood vessel renewal in multiple organs, whereas some tissues do not respond to VEGF sufficiently. Further, anti-VEGF induced side effects are due to the reduction of blood vessels, resulting in both vessel and organ function impairment. However, stopping the treatment can reverse the side effect by restoring the blood vessels.

4.4 MOUSE CORNEA MODEL TO STUDY LYMPHANGIOGENESIS AND ANGIOGENESIS. (PAPER IV)

Lymphangiogenesis is the formation of lymphatic vessels that involves lymphatic endothelial cells proliferation, migration and remodeling. This process is highly regulated by lymphangiogenic factors and inhibitors. Lymphangiogenesis has been associated with cancer metastasis, and inhibition of such process would offer a new opportunity for the treatment of cancer metastasis[7,74,149-151].

There are several in vitro and in vivo assays to study lymphangiogenesis, but these assays do not usually describe the lymphangiogenic process in vivo in a functional lymphatic network. In vitro assays do not answer the questions of lymphatic vessel remodeling.

Lymphatic vessels are not perfused with blood cells and remain invisible in the tissues. Detection of lymphatic vessels is usually achieved by injection of dyes such as Evans blue. Alternatively, immunostaining of lymphatic marker such as lymphatic vessel endothelial hyaluronan receptor-1, podoplanin or vascular endothelial growth factor receptor 3 (VEGFR3) provides a way to study the structure of lymphatic microvessels.

However, these methods generally do not allow us to distinguish the pre-existing lymphatic vessels and the newly formed lymphatic vessels. In this paper, we developed a quantitative lymphangiogenesis model in the cornea, which is similar to a corneal angiogenesis model.

As the cornea is an avascular tissue without blood vessel and lymphatic vessels, all lymphatic vessels are newly formed in the assay. Thus, the corneal model provides a

great advantage to study lymphatic vascular formation, structure and remodeling. And it is also convenient to quantify the lymphatic vessels.

Similar to this corneal model, there is an inflammation induced lymphangiogenesis in the mouse cornea. In this model, a thread is embedded into the epithelial layer in the cornea and induces a robust inflammatory response. Although this inflammation-induced corneal lymphangiogenesis model allows detection of lymphatic vessel growth, this method uses different principles. First, the corneal suturing assay is not a reliable quantitative assay because the size of the suture thread is not easy to be uniformed. Second, inflammation induced lymphangiogenesis can be mediated by a various cytokines and growth factors, which can be present in different amounts and ratios. These variations make it also difficult to test the pure effect of single lymphangiogenic factor. Third, in the inflammation-induced corneal model, it is difficult to study the synergistic lymphangiogenic activity between two or more lymphangiogenic factors.

Different from the inflammation induced corneal model, implantation of a polymer based micropellet containing a single factor or combination of two or more factors would not induce inflammatory responses. In addition, the present protocol describes a method in which the amount of lymphangiogenic factor, size of the micropellet, the implantation procedure and the time point of responses are well defined. Thus, allowing investigating quantitatively the lymphangiogenic activity of any given factor in this model. Another advantage is that our protocol enables to study the inhibition of specific growth factor induced lymphangiogenesis by any proteins or compounds. This assay can be used for assessment of potential therapeutic drugs.

5 CONCLUSIONS

Cancer is an age related systemic disease. People who are diagnosed with cancer usually do not die directly due to the tumor itself, but because of systemic organ dysfunction and regression[33]. Thus, targeting tumor or peripheral organs has becoming a debate for treating cancer patients. Some researchers support the dogma that inhibiting tumor growth or even diminishing the tumor nodule is the key to fight against cancer. With this notion, clinical doctors are always trying to concentrate on how to kill the tumor cells: surgery, radiotherapy and all kind of toxic chemo compounds. However, each of them has side effects that may even, in turn, cause a lot of problems. For some tumors, surgery is not performed due to practical issue. And sometimes, surgery cannot resect the tumor completely, which will cause tumor recurrence and metastasis. Radiotherapy also causes unavoidable DNA damage to healthy cells due to affecting the adjacent healthy tissue around tumor region in the therapy procedure. Accordingly, radiotherapy has the potential to induce cancer as well. Chemotherapeutic drugs target both cancer cells and healthy cells. Chemotherapy induces a broad toxicity such as heart failure, bone marrow suppression, hair loss, immunosuppression, anemia, liver dysfunction, and gastrointestinal disorders. Due to these adverse effects, many patients actually suffer much more than the tumor tissue induced syndrome. According to the clinical experience, 20% patients die of chemotherapy but not cancer[33,34]. Under such context, we have to reconsider how to achieve the balance of treating cancer versus improving quality of life by alternative thinking.

On the other hand, treating the patients by targeting off-tumor organs is also becoming an alternative way to improve the life quality for cancer patients. Off-tumor target

means that treating the patients aiming to reduce the systemic syndromes rather than killing tumor cells. Tumor tissue usually produces a lot of cytokines, which enters the circulation, and reach peripheral organs can further cause imbalanced signaling pathway activations, leading to regulation chaos of the cell machineries and causing functional impairment. If any drugs can neutralize the tumor induced paraneoplastic syndrome, the life quality of the patients will be significantly improved although the tumor is still live with them. Like other chronic diseases, such as cardiovascular disease, obesity, diabetics, immune system diseases and chronic liver infection, patients have to live with the disease for a life-long time with daily control by medicines. So does the cancer. If patients can live with the tumor by controlling the progression, we do not need to kill the tumor as long as it does not turn into an aggressive syndrome. Accordingly, off-tumor target is really a feasible idea for clinical practice.

Antiangiogenic therapy targets on the tumor microenvironment- the stromal cells to be more specific. It shows very promising results in the preclinical research. However, in the clinical patients, antiangiogenic therapy showed modest benefits in combination with chemotherapy. Among these patients, only a few patients respond to the antiangiogenic therapy. Thus, a surrogate and reliable marker to predict the prognosis is needed. PlGF is a member of VEGF family, and it is highly expressed in choriocarcinoma. By heterodimerizing with VEGF and normalizing the blood vessel, PlGF sensitize the tumor vessels for anti-VEGF treatment. Therefore, PlGF might be a good marker for clinical prediction for the efficiency of antiangiogenic therapy.

In this thesis, we also discussed the mechanisms of adverse effects caused by anti-angiogenic therapy. Since all the tissues are depending on blood vessels to maintain normal function, keeping the balanced turnover of blood vessels is very important. Here

we showed that VEGF plays a key role in maintaining the homeostasis of blood vessels. Considering the different tissue function and metabolism rate, blood vessels density and structures also differ, which induced the different sensitivity of blood vessels to anti-VEGF treatment. In the endocrine organs, micro vessels are surprisingly high compared with other organs such as retina or bone marrow. Thus VEGF blockade reduces the blood vessels by inhibition of angiogenesis. And dramatic decrease was demonstrated in the paper that blood vessels in endocrine organs are very sensitive to anti-VEGF treatment. Alterations in tissue functions were detected by measuring free T4 levels in serum after a relative long period treatment. These data showed that anti-VEGF treatment cause vessel reduction and further influences the tissue function. However, when ending treatment, vessels will grow back to a normal level, which means, the vessel reduction is reversible. These findings provided mechanisms of anti-VEGF adverse effect and may help to optimize the clinical regimen for cancer patients.

For better understanding the mechanisms of angiogenesis and lymphantiogenesis in tumor progression and metastasis, a reliable and relevant model is needed. In this thesis, we developed a mouse cornea model to study multiple angiogenic and lymphangiogenic factors in vivo. This model is also a significant powerful approach for testing any potential antiangiogenic compounds.

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