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# Anti-Angiogenic Active Immunotherapy for Cancers: Dawn of a New Era?

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Additional information is available at the end of the chapter

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## 1. Introduction

### 1.1. Angiogenesis in health and disease

The formation of blood vessels occurs by two mechanisms [1]: vasculogenesis and angiogenesis. Vasculogenesis is the process during which blood vessels are formed *de novo* by *in situ* differentiation of the primitive progenitor (i.e. angioblasts) into mature endothelial cells, which was thought to take place only during embryonic development. However, angiogenesis occurs both during embryonic development and postnatal life. Angiogenesis is defined as a process which gives rise to new blood vessels by proliferation and migration of preexisting differentiated endothelial cells. In embryonic life, angiogenesis is a critical process that leads to formation of stable vasculature comprised of endothelial cells, mural cells (pericytes) and basement membrane in the adult [2]. Vasculature in healthy adult is very stable with the exception of rare events such as cyclical growth of vessels in the ovarian corpus luteum or during pregnancy, angiogenesis activities are rare in adult individuals [2]. In addition to normal development, angiogenesis is known to be an important event in pathological conditions such as tissue repair during wound healing and in the growth of tumors [3].

Tumor angiogenesis, the formation of new blood vessels supplying the tumor mass, plays a critical role in tumor growth, progression, persistence and metastasis, because the proliferation and metastasis of malignant tumors are dependent on the sufficient nutrition supplied by the new vessels [4-6]. Many molecules have been demonstrated as positive regulators of angiogenesis, including vascular endothelial growth factor (VEGF), acidic or basic fibroblast growth factor (aFGF, bFGF), epidermal growth factor (EGF), transforming growth factor- $\alpha/\beta$  (TGF- $\alpha$ , TGF- $\beta$ ), placental growth factor (PlGF), angiopoietin, angiogenin, endoglin (CD105), prostate-specific membrane antigen (PSMA), the anthrax-toxin-receptor (ATR, TEM8), connective

tissue growth factor (CTGF, CCN2), urokinase plasminogen activator (uPA), and several others [7-10]. However, VEGF-mediated signaling through its receptor VEGFR-2 is the key rate-limiting step in tumor angiogenesis, and plays the most important role in neovascularization, development, and progression of various tumors including hematopoietic malignancies [11, 12], breast cancer [13], bladder cancer [14], and renal cell cancer [15]. Importantly, it has been found that tumor growth can be attenuated *via* the suppression of angiogenesis [7].

## 1.2. Anti-angiogenic strategies for cancer treatment

Given the role of angiogenesis in tumor growth and progression, therapeutic strategies targeting tumor vascular endothelia rather than tumor cells have several merits in comparison to conventional anti-tumor therapies [16, 17]: (a) Vascular endothelial cells have genetically stable MHC expression on the surface, which will not be down-regulated, in contrast to the surface of tumor cells [18]; (b) Effector cells or antibodies can reach targeted endothelial cells more readily than they can reach tumor cells [17]; (c) Treatment by targeting endothelial cells is not restricted to specific tumor entities [16, 17, 19]; (d) As each tumor vessel supplies hundreds of tumor cells, the inhibition or diminishment of a large amount of tumor cells could be achieved merely by the comparatively limited impairment of neovascularized endothelial cells; as a consequence, the efficiency of targeting tumor blood vessel endothelium should be higher than that of targeting tumor cells themselves [15]. (e) Several specific anti-angiogenic agents, such as IFN- $\gamma$ , have very low toxicity in some cases of drug combination-therapy regimens in both patients and animal models [16]. In recent years, the field of anti-angiogenic therapy for cancers has attracted much attention. In general, anti-angiogenic strategies can be divided into the following five categories:

1. **Passive immunotherapy:** The use of antibody to neutralize angiogenesis positive regulators such as VEGF. In 2003, the Food & Drug Administration (FDA) of the United States approved Bevacizumab (Avastin<sup>®</sup>; Genentech Inc.), a humanized variant of VEGF neutralizing monoclonal antibody, as the first anti-angiogenic agent for combinatorial treatment with standard of care in metastatic colorectal cancer [20] and subsequently for treatment of patients with non-small-cell lung cancer [21] or metastatic breast cancer [22]. The combinatorial treatment of Bevacizumab with conventional chemotherapy showed increased therapeutic efficacy, for example in patients with metastatic colorectal cancer, the median survival time was extended by 4.7 months [20]. Besides combining with conventional chemotherapy, bevacizumab could combine with radiation therapy safely and effectively [23].
2. **Therapy with VEGF inhibitors:** Since the approval of Bevacizumab in the clinical use by FDA, several VEGF inhibitors including small molecules targeting VEGF or its receptors came into different stages of clinical development. For example VEGF-Trap<sub>R1-R2</sub> (Aflibercept; Regeneron Inc.), a chimeric soluble receptor containing structural elements from VEGFR1 and VEGFR2, has the ability to bind to and neutralize the circulating VEGF [24]. VEGF-Trap<sub>R1-R2</sub> has shown potent anti-tumor activity in preclinical animal models and is currently in clinical trials [24]. In addition to VEGF inhibitors, several small molecule receptor tyrosine kinase inhibitors (RTKIs) target-

ing VEGF and other signaling pathways have been developed. Some of the most clinically relevant RTKIs are summarized in Table 1.

Compound	Company	Targets	Indications
Sunitinib (SU11248)	Pfizer	VEGFRs, PDGFRB, CSF1R, c-Kit	RCC, GIST
Pazopanib (Votrient)	GSK	VEGFRs, PDGFRs and c-kit	RCC
Sorafenib (Bay 43- 9006; Nexavar <sup>®</sup> )	Bayer	VEGFR-2 and -3, PDGFR-b, Flt-3, c-kit, Raf kinases	RCC, Inoperable HCC
Vendatanib (Caprelsa)	AstraZeneca	VEGFRs and EGFRs, RET- tyrosine kinases	Late-stage Medullary Thyroid Cancer
Cabozantinib (XL184)	Exelixis	VEGFRs Met	Prostate Cancer
Tivozanib (AV-951, KRN-951)	AVEO Pharmaceutical	VEGFRs	RCC
Axitinib (AG-013736)	Pfizer	VEGFRs	mRCC
Linifanib (ABT-869)	Abbott	VEGFRs, PDGFRB, CSF1R	RCC, NSCLC

**Table 1.** VEGF RTKIs and their indications in cancer patients (adopted from [2])

3. **Therapy with angiogenesis negative regulators:** Many negative regulators, such as angiostatin, endostatin, interferon- $\gamma$  etc., are involved in the angiogenesis [25]. Therefore the use of these agents to negatively regulate angiogenesis is another strategy for cancer treatment. For example, recombinant human endostatin has been approved in September 2005 by the State Food and Drug Administration (SFDA), P. R. China. The phase III clinical trial of endostatin in China showed promising effects. Combination of endostatin and NP regimen (vinorelbine and cisplatin) significantly improved the therapeutic efficacy in patients with advanced nonsmall-cell lung cancer and safely extended the median time of tumor progression [26].
4. **Therapy with vascular disrupting agents:** A strategy directly induce vascular collapse. ASA404, a flavonoid compound, is one of the vascular disrupting agents that induce apoptosis of tumor associated endothelial cells, resulting in the inhibition of blood flow, causing hypoxia and necrosis in tumor mass. ASA404 is currently in advanced stage of clinical trial in combination with standard of care in non-small-cell lung cancer [27].
5. **Active immunotherapy:** Active immunotherapy targeting tumor angiogenesis is a novel modality for treatment of cancers which is based on several assumptions: a) Tumor-derived endothelial cells (ECs) possess characteristics distinct from those of normal tissue [18]. b) Specific immune responses against self-antigen can be elicited. c) Tumor growth can be attenuated via suppression of angiogenesis [7]. The main aim of the active immunotherapy targeting tumor vessels is to break self immunological tolerance to the positive regulators of angiogenesis, hereby inhibiting tumor angiogenesis and thus leading to the inhibition of tumor growth and metastasis. Anti-angiogenic active immunotherapies can

be divided into two categories: one is based on the immunological cross-reactions mediated by vaccination with xenogeneic homologous molecules associated with angiogenesis, and the other targets non-xenogeneic homologous molecules. Therapeutic targets, vaccines and tumor models used in anti-angiogenic active immunotherapy for cancers are summarized in Table 2.

Strategies	Therapeutic targets	Vaccines	Tumor models	References	
Vaccination based on xenogeneic homologous molecules	Murine vascular endothelial cells	Human umbilical vein endothelial cells, human dermal microvascular endothelial cells, and bovine glomerular endothelial cells	Meth A, H22, MA782/5S, FM3A, and Lewis Lung carcinoma	29	
	Murine VEGF	Recombinant plasmid encoding VEGF of <i>xenopus laevis</i>	Meth A, H22 and MA782/5S	32	
		pMAE5 $\Delta$ 5 vectors harboring human VEGF 121 gene and mutated human VEGF 121 gene	EL-4, B16, and TC-1	33	
	Canine VEGF	Liposome-DNA adjuvant	Soft tissue sarcoma	34	
	Murine VEGFR-2	Plasmid DNA encoding quail VEGFR-2		Meth A, EL-4 and MOPC-315,	36
			Plasmid DNA encoding the C terminal 37 amino acids of hCG $\beta$ , 5 different CTL epitopes from human surviving and the 3 <sup>rd</sup> and 4 <sup>th</sup> extracellular domains of VEGFR-2	LL/2 lung carcinoma	37
		<i>Bifidobacterium infantis</i> expressing human sKDR	Lewis lung cancer	38	
	Murine FGFR-1	cDNA encoding <i>Xenopus</i> FGFR-1	Meth A, H22, and MA782/5S	41	
	Murine $\alpha$ v $\beta$ 3	Plasmid DNA encoding the ligand-binding domain of chicken integrin $\beta$ 3	Meth A, H22, and MA782/5S	43	
	Murine MMP-2	Plasmid DNA encoding chicken MMP-2	LL/2, Meth A, H22	48	
	Murine RHAMM	Plasmid encoding <i>Xenopus</i> RHAMM	B16	49	
	Murine DLL4	Plasmid encoding human DLL4	D2F2/E2	55	
	Murine Angiomotin	Plasmid encoding human angiomotin	TUBO breast cancer	57, 61	

Strategies	Therapeutic targets	Vaccines	Tumor models	References
Vaccination based on non-xenogeneic homologous molecules	Murine VEGFR-2	flk-1 protein pulsed DC	B16, 3LL	62
		flk1 mRNA-transfected DC	B16, MBT-2	64
		Murine sVEGFR-2 and IFN- $\gamma$ fusion gene-transfected DC	B16, 3LL	68
		Recombinant plasmid encoding flk-1	B16, MC38, D121	63
		H-2D <sup>b</sup> -restricted KDR2 or KDR3 peptide	MC38	69
		<i>L. monocytogene</i> -based Flk-1	4T1-Luc	71, 72
		pSG.SS.FlK-1 <sub>ECD</sub> .C3d3	BTT739	73
VEGFR-2 in transgenic mice expressing HLA-A*0201	HLA-A*0201-restricted VEGFR-2 epitope peptide	MC38, B16	70	
Human VEGFR-2	HLA-A*2402-restricted VEGFR2-169	Patients with advanced pancreatic cancer	74	
Murine FGF-2	FGF-2 heparin-binding structural domain peptide	B16BL6, LLC-LM	39	
Murine EGFR	Recombinant mouse EGFR ectodomain pulsed DC	Lewis lung carcinoma and mammary cancer	80	
Legumain	Minigene plasmid DNA encoding murine MHC class I antigen epitopes of Legumain	D2F2	85	
Endoglin	Plasmid DNA encoding murine endoglin	D2F2	86	
		Listeria-based vaccines encoding murine endoglin	4T1-Luc and NT-2 breast cancer	87
Endothelium	Endothelium lysates pulsed DC	Colon-26 colon cancer	89	
		Glutaraldehyde-fixed HUVECs	Patients with recurrent malignant brain tumour or metastatic colorectal cancer	90

Notes: B16: B16 melanoma; B16BL6: B16BL6 tumor cells; D121: D121 lung carcinoma; D2F2: breast carcinoma cells; D2F2/E2: mammary tumor cells derived from BALB/c mouse and were transfected with the cDNA encoding ERBB2; EL-4: EL-4 lymphoma; FM3A: mammary cancer; H22: H22 hepatoma; LL/2: LL/2 Lewis lung carcinoma; LLC-LM: LLC-LM tumor cells; MA782/5S: MA782/5S mammary cancer; Meth A: Meth A fibrosarcoma; MBT-2: MBT-2 bladder tumor; MC38: MC38 murine colon cancer; MOPC-315: MOPC-315 plasmacytoma; TC-1: TC-1 carcinoma; 3LL: 3LL Lewis lung carcinoma; HUVECs: human umbilical vein endothelial cells.

**Table 2.** Anti-angiogenic active immunotherapy for cancers

## 2. Anti-angiogenic active immunotherapy

### 2.1. Anti-angiogenic active immunotherapy based on xenogeneic homologous molecules

Homologous molecules in different species are formed as the result of evolution. Molecules with essential functions keep the stability of their molecular sequences, although some moderate degree of evolution is essential for adaptation to different environments and physiological requirements in different species. Many genes in the human and mouse genome are similar (but not identical) to the corresponding genome sequences of the fruit fly *Drosophila melanogaster* and other non-vertebrates such as *Xenopus laevis* [28]. In consequence, effective immune response to self antigens associated with angiogenesis can thus be induced by vaccination with xenogeneic homologous molecules.

#### 2.1.1. Cell vaccine

Neovascular endothelial cells in tumor tissues express proteins not present or not detectable in normal vascular endothelial cells, such as  $\alpha v \beta 3$  integrin and receptors for certain angiogenic growth factors [18]. These proteins in murine vascular endothelial cells share homology to varying degree with counterparts of other species including human [18]. Vaccination of mice with paraformaldehyde-fixed xenogeneic human and bovine proliferative vascular endothelial cells, such as human umbilical vein endothelial cells, human dermal microvascular endothelial cells, and bovine glomerular endothelial cells, resulted in successful breaking of the immunological tolerance to autogeneic vascular endothelial cells in several murine tumor models, such as Meth A fibrosarcoma, MA782/5S and FM3A mammary cancer, H22 hepatoma, and Lewis lung carcinoma, generating a protective and therapeutic anti-tumor immunological reaction [29]. Antibodies against the receptors associated with tumor angiogenesis generated in mice immunized with the xenogeneic homologous proliferative vascular endothelial cell vaccines might inhibit the proliferation of endothelial cells *in vivo*, leading to the regression of established tumor, and the prolonged survival of tumor-bearing mice [29]. Tumor angiogenesis could be suppressed by the adoptive transfer of autoreactive immunoglobulins purified from the immunized mouse, resulting in inhibition of tumor growth in mice [29]. Autoantibody sediments were detected on ECs within tumor tissues in the immunized mice by immunohistochemical analysis [29]. Furthermore, Western blot analysis showed that reactions between the extract from murine ECs and the serum from the immunized mice resulted in several positive bands, at least two of which, with the molecular weight of 220 kDa and 130 kDa, had similar molecular sizes to those of ligand-binding sites of known VEGFR2 and  $\alpha v$  integrin, respectively [29], although the authors did not provide direct evidence to demonstrate that the two positive bands aforementioned contained VEGFR2 and  $\alpha v$  integrin respectively. Immune cell subset depletion experiments showed that the production of autoantibodies against tumor vascular ECs and the anti-tumor effect were dependent on CD4<sup>+</sup> T lymphocytes [29].

In 2006, early-outgrowth progenitor endothelial cells (EO-EPCs) have been characterized on the basis of their dendritic-like phenotypes (such as expression of HLA-DR, CD40, CD54, CD80, and CD86), phagocytotic and antigen-presenting functions, and endothelial markers

(such as VEGFR2, von Willebrand factor, CD105) [30]. EO-EPCs also incorporated DiLDL and bound UEA-I, which are endothelial features, and additionally, they formed vascular-like structures on Matrigel [30]. Thus, it might be a promising strategy toward anti-angiogenic cancer treatment to use EO-EPCs as cell vaccine to inhibit tumor angiogenesis, since such cells might function both as dendritic-like cells to augment anti-tumor immunity and as xenogeneic proliferative endothelial cells to break self-tolerance, thereby inducing profound anti-angiogenic effects *in vivo*.

### 2.1.2. Non-cell vaccines

#### 2.1.2.1. VEGF/VEGFR2

VEGF is a potent and crucial vasculogenic and angiogenic factor, which can induce endothelial cell proliferation, promote cell migration, and inhibit endothelial cell apoptosis [5, 6]. In most types of cancers, VEGF is often present at elevated levels, and strategies aimed at blocking its activity usually lead to suppression of tumor angiogenesis and consequently tumor growth inhibition [31]. The amino acid sequence of VEGF in *Xenopus laevis* shares 75 % and 73 % homology with that of VEGF164 in mice and that of VEGF165 in humans, respectively [32]. Recombinant eukaryotic expression plasmids harboring VEGF-encoding gene of mice and *Xenopus laevis*, respectively, designated as MVEGF-P and XVEGF-P, have been constructed. Immunization of mice with XVEGF-P provoked protective and therapeutic anti-tumor immunological effects in mouse tumor models with Meth A fibrosarcoma, MA782/5S mammary cancer and H22 hepatoma [32]. Anti-VEGF specific autoantibody was detected in serum of mice vaccinated with XVEGF-P by Western blot and ELISA [32]. The VEGF levels in the tumor-bearing mice immunized with XVEGF-P was lower than that in the control groups [32]. Furthermore, the frequency of anti-VEGF antibody-producing B cells in the spleen of mice immunized with XVEGF-P was remarkably higher than that in the spleen of control groups where such B cells were undetectable [32]. VEGF-mediated proliferation of ECs could be inhibited *in vitro* by purified immunoglobulins from XVEGF-P-immunized mice. Adoptive transfer of the purified immunoglobulins into non-immunized tumor-bearing mice could also inhibit tumor angiogenesis *in vivo* and generate anti-tumor effects [32]. Anti-CD4<sup>+</sup> monoclonal antibody could obstruct the escalation of concentration of immunoglobulin IgG1 and IgG2 in serum and also block the anti-tumor effects of XVEGF-P DNA vaccines, indicating that CD4<sup>+</sup> T lymphocytes were responsible for XVEGF-P-induced anti-tumor effects [32]. The possibility that the anti-tumor activity may result from nonspecifically augmented immune response could be ruled out by the findings that no increase in NK activity of spleen cells or in the level of cytokines such as IFN- $\alpha$ , IFN- $\beta$ , TNF- $\alpha$ , or  $\beta$ -chemokine in sera was found in immunized mice [32]. Recently, it was reported that when immunized with human VEGF isoform 121 gene (hVEGF121) inserted into pMAE5 $\Delta$ 5 vector (pM-VEGF) and later challenged with melanoma or lung carcinoma tumor cells, a reduction of tumor growth and an increased survival of tumor-bearing C57BL/6 mice were observed because the hVEGF121 gene is highly homologous to its murine counterpart [33]. A decrease in tumor cell density around vessels and in mitotic figures, as well as an increase in apoptotic tumor cells were manifested by histopathological analyses of tumors from C57BL/6 mice immunized with hVEGF121 [33]. Spleen cells

from mice immunized with pM-VEGF showed a significant enhanced cytotoxic activity against VEGF-secreting tumor cells, including EL-4 lymphoma, B16-F10 melanoma, and TC-1 carcinoma, as compared with those obtained from the mice immunized with the pMAE5 $\Delta$ 5 “empty” vector [33]. IFN- $\gamma$  ELISPOT assay revealed a significant increase in the number of spots in spleen cells from mice immunized with pM-VEGF [33]. Vaccination with a mutated hVEGF121 gene inserted into the pMAE5 $\Delta$ 5 vector (pM-VEGFmut) produced similar *in vitro* and *in vivo* results, and remarkably reduced the number of spontaneous metastases in a murine model with Lewis lung carcinoma [33]. Serum VEGF levels decreased 8-fold in mice vaccinated with pM-VEGF or pM-VEGFmut as compared with those in pMAE5 $\Delta$ 5 treated mice [33]. A significant correlation was also found between the elevation of serum VEGF level and the increase of the tumor dimensions [33]. However, antibody responses against the GSThVEGF121 fusion protein or GST alone used as capture antigens in ELISA were undetectable in animals vaccinated with pM-VEGF or pM-VEGFmut [33]. These findings indicate that human VEGF-harboring DNA vaccine can be employed for anti-angiogenic active immunotherapy for cancers in mice and direct cell cytotoxicity contributes to the overall anti-tumor effects observed in immunized mice [33].

Previous studies in rodent tumor models have indicated that immunization against xenogeneic growth factors is more likely to induce effective anti-tumor responses than immunization against the syngeneic growth factor [34]. In 2007, an investigation was conducted to assess the safety and anti-tumor and anti-angiogenic effects of a xenogeneic VEGF vaccine in pet dogs with spontaneous cancer. Nine dogs with soft tissue sarcoma were immunized with a recombinant human VEGF vaccine over a 16-week period [34]. The xenogeneic VEGF vaccine was well-tolerated by all dogs and resulted in induction of humoral responses against both human and canine VEGF in animals that remained in the study long enough to receive multiple immunizations [34]. Three of five multiply immunized dogs also experienced sustained decreases in circulating plasma VEGF concentrations and two dogs had a significant decrease in tumor microvessel density [34]. The overall tumor response (>50% decrease in tumor volume) rate was 30% for all treated dogs in the study. Thus, it was concluded that a xenogeneic VEGF vaccine may be a safe and effective alternative means of controlling tumor growth and angiogenesis [34].

VEGF receptor-2 (VEGFR-2, also known as fetal liver kinase-1 (flk-1) in mouse and kinase-containing domain receptor (KDR) in human) is the main receptor responsible for the VEGF-mediated angiogenic activity [6]. The impairment of vasculogenesis and death of embryo at day 8.5 were observed as the result of the targeted inactivation of flk-1 gene in mice [35]. Overexpression of KDR was found on activated endothelial cells of newly formed vessels [6]. It was discovered that the primary sequence of quail VEGFR-2 (qVEGFR-2) was 67% and 70% identical at the amino acid level with mouse and human homologues (flk-1 and KDR), respectively [36]. Immunotherapy with a vaccine based on quail homologous VEGFR-2 elicited protective and therapeutic anti-tumor immunity in both solid and hematopoietic tumor models in mice, such as LL/2 Lewis lung carcinoma, CT26 colon carcinoma, Meth A fibrosarcoma, MOPC-315 plasmacytoma, and EL-4 lymphoma [36]. Autoantibodies against flk-1 in the immunized mice were identified. Sera from qVEGFR-2-

immunized mice recognized not only recombinant qVEGFR-2, but also recombinant mouse VEGFR-2 (mVEGFR) in Western blot analysis [36]. In contrast, the sera isolated from controls showed negative staining [36]. Sera from mice immunized with qVEGFR-2 recognized a single band in flk-1-positive mouse SVEC4-10 endothelial cells and KDR-positive human umbilical vein endothelial cells, with the same size as recognized by commercially available flk-1 or KDR antibodies [36]. Sera from qVEGFR-2-immunized mice also recognized recombinant protein qVEGFR-2 and mVEGFR-2 in ELISA [36]. Detectable IgG1 and IgG2b with significantly elevated concentration in sera were found to be responsible for the immunoglobulin response to VEGFR-2 [36]. Anti-VEGFR-2 specific antibody-producing B cells were detected by ELISPOT. The number of anti-VEGFR-2 antibody-producing B cells was elevated in the spleens of mice immunized with qVEGFR-2, compared with that in controls [36]. Deposition of immunoglobulins on endothelial cells was found within tumors from qVEGFR-2-immunized mice, but not from controls [36]. Adoptive transfer of the purified immunoglobulins from qVEGFR-2-immunized mice resulted in inhibition of VEGF-mediated endothelial cell proliferation and effective protection against tumor growth [36]. Angiogenesis was markedly suppressed within the tumors, and the vascularization of alginate beads was also diminished [36]. Depletion of CD4<sup>+</sup> T lymphocyte could abrogate the anti-tumor activity and the production of autoantibodies against flk-1 [36].

Very recently, a DNA vaccine designed by synergizing different tumor antigens with VEGFR2 was constructed. A DNA fragment (HSV) encoding the C terminal 37 amino acids of human chorionic gonadotropin  $\beta$  chain (hCG $\beta$ ), 5 different HLA-restricted cytotoxic T lymphocyte epitopes from human survivin and the third and fourth extracellular domains of VEGFR2 was inserted into the sequence between the luminal and transmembrane domain of human lysosome-associated membrane protein-1 cDNA for the construction of a novel DNA vaccine (p-L/HSV) [37]. Vaccination of the mice with p-L/HSV elicited potent and long-lasting cellular and humoral immune responses to the specific antigens and showed a prominent anti-tumor effect on the LL/2 lung carcinoma model in syngeneic C57BL/6 mice. In addition, the tumor vasculature was abrogated as observed by immunohistochemistry in p-L/HSV immunized mice [37]. These data indicates that the strategies of combining anti-tumor with anti-angiogenesis cooperate well. Such a study may shed new light on the designing of vaccine for cancer in the future.

Again in 2012, a *Bifidobacterium infantis*-based vaccine that express human extracellular domain of VEGFR2 (sKDR) was established [38]. Immunization of the mice with the *Bifidobacterium infantis*-based vaccine through caudal vein could significantly suppress the tumor growth and prolong the survival of the tumor-bearing mice. On the other hand, this immunization strategy could significantly increase the tumor necrosis, and obviously decrease microvessel density and the blood flow signals in tumor [38].

#### 2.1.2.2. FGFR-1

Fibroblast growth factor receptor-1 (FGFR-1) is expressed on endothelial cells and many types of tumors [39, 40]. The Xenopus homologue of FGFR-1 is 80% and 74% identical at the amino

acid level with mouse FGFR-1 and human FGFR-1, respectively [41]. Therefore, FGFR-1 may be used as another ideal target for anti-angiogenesis therapy. Vaccination with Xenopus FGFR-1 (pxFR1) provoked protective and therapeutic effects in three murine tumor models, including Meth A fibrosarcoma cells, H22 hepatoma cells, and MA782/5S mammary carcinoma [41]. FGFR-1-specific autoantibodies were detected in sera of pxFR1-immunized mice by Western blot analysis, and the purified immunoglobulins effectively inhibited endothelial cell proliferation *in vitro* [41]. However, the immunoglobulins had no direct inhibitory effect on the proliferation of above three tumor cell lines [41]. Adoptive transfer of sera or purified immunoglobulin isolated from pxFR1-immunized mice into unimmunized mice provided effective protection against tumor growth, while adsorption of sera or immunoglobulin with FGFR-1-positive endothelial cells before adoptive transfer could abrogate its anti-tumor activity [41]. Autoantibodies deposited on the endothelial cells within tumor tissues and significantly suppressed intratumoral angiogenesis were found in pxFR1-immunized mice by histological examination [41]. Furthermore, this anti-tumor activity and production of FGFR-1-specific autoantibodies were abrogated by depletion of CD4<sup>+</sup> T lymphocytes, again pointing to their essential helper function for antibody production [41].

#### 2.1.2.3. Integrins

Integrins are heterodimeric transmembrane proteins consisting of  $\alpha$  and  $\beta$  subunits with large extracellular domain and short cytoplasmic tail. They play very crucial roles in angiogenesis as the migration of endothelial cells is dependent on their adhesion to extracellular matrix proteins such as vitronectin [42].  $\alpha\beta3$  is not generally found on blood vessels in normal tissues, but its expression is enhanced on newly developing blood vessels in human wound tissue, tumors, diabetic retinopathy, macular degeneration and rheumatoid arthritis, which implies that this integrin may play an important role in angiogenesis and development of neovascularization [42]. This distributive characteristic also makes  $\alpha\beta3$  an attractive target for tumor therapy [42]. A plasmid DNA encoding the ligand-binding domain of chicken integrin  $\beta3$  was constructed to test this assumption. Immunization with chicken homologous integrin  $\beta3$ -based vaccine could elicit both protective and therapeutic anti-tumor immunity in murine tumor models with Meth A fibrosarcoma, H22 hepatoma, or MA782/5S mammary carcinoma [43]. Autoantibodies against integrin  $\beta3$  in sera of the immunized mice were found by Western blot analysis and ELISA [43]. The purified immunoglobulins could effectively inhibit endothelial cell proliferation *in vitro*, and adoptive transfer of the purified immunoglobulins into non-immunized mice could provide effective protection against tumor growth and markedly inhibit tumor angiogenesis [43]. The anti-tumor activity and the production of integrin  $\beta3$ -specific autoantibodies were CD4<sup>+</sup> T lymphocyte-dependent [43].

#### 2.1.2.4. MMP

Angiogenesis is an invasive process, requiring proteolysis of the extracellular matrix [44]. Inappropriate destruction of extracellular matrix components is involved in certain pathological conditions, including arteriosclerosis, rheumatoid arthritis, and tumor aggression and metastasis [44]. The matrix metalloproteinases (MMPs), a family of extracellular endopepti-

dases, can selectively degrade components of the extracellular matrix [44]. *In vivo*, elevated stromal MMP-2 and MMP-9 activity is highly correlated with increased metastatic potential in most malignant tumors [45]. Increased activity of MMPs appears to permit the tumor to remodel its surrounding microenvironment, to grow in a permissive space, and to promote the development of supporting stroma, including angiogenesis [46]. Moreover, numerous pathological and clinical studies demonstrated that the MMPs were frequently overexpressed in various solid tumor cells and peritumoral stromal cells [46]. It was reported that the abrogation of MMP-2 alone resulted in the inhibition of the transition from the prevascular to the vascular stage during tumor development and then of tumor growth [47]. Furthermore, the suppression of tumor-induced angiogenesis and of invasion and metastasis of tumor cells could be observed in MMP-2-deficient mice [47]. These findings indicated that MMP-2 alone played an important role in angiogenesis and tumor growth. Sequence comparison analysis showed that the primary sequence of mouse MMP-2 at the amino acid level was 82% and 91% identical with chicken and human homologues, respectively [48]. It was reported that the plasmid DNA vaccination with chicken homologous MMP-2 (c-MMP-2)-based model antigen could induce both protective and therapeutic anti-tumor immunity in murine tumor models with LL/2 Lewis lung carcinoma, Meth A fibrosarcoma, and H22 hepatoma [48]. The elevation of MMP-2 in the sera of tumor-bearing mice was abrogated with the vaccination of c-MMP-2 [48]. The autoimmune response against MMP-2 may be provoked in a cross-reaction by the immunization with c-MMP-2, and the autoantibody targeting to MMP-2 was elevated and probably responsible for the anti-tumor activity [48]. Moreover, gelatinase activity of MMP-2, including both latent MMP-2 and active MMP-2, derived from the above mentioned three murine tumor models was apparently inhibited by the vaccination with c-MMP-2 [48]. However, the vaccination did not inhibit the gelatinase activity of MMP-9 [48]. These findings indicate that the activity of MMP-2 is impaired by immunization with c-MMP-2 in mice. Angiogenesis was apparently inhibited within tumors in immunized mice. The anti-tumor activity and production of auto-antibodies against MMP-2 were abrogated by depletion of CD4<sup>+</sup> T lymphocytes [48].

#### 2.1.2.5. *xRHAMM*

In 2010, Yang et al. [49] used a cross-reactive serological expression cloning (SEREX) strategy (CR-SEREX) to identify novel xenogenic angiogenesis- and tumor-associated antigens in oocytes of *Xenopus laevis* and found that *Xenopus* receptor for hyaluronic-acid-mediated motility (xRHAMM) was the most frequently clone among 78 CR-SEREX positive clones, suggesting that xRHAMM has the strongest immunogenic potential for xenogenic immunotherapy. It was demonstrated that expression of RHAMM is restricted to the testis, thymus, placenta, vascular endothelial cells, and various cancer cells, and RHAMM functions in vascular endothelial cell migration, angiogenesis, and in hyaluronic-acid-induced cell mobility [50]. In order to examine the anti-angiogenic effects, a DNA vaccine based on xRHAMM (pcDNA3.1-xRHAMM) was constructed [49]. Intramuscular vaccination of the cationic liposome encapsulated pcDNA3.1-xRHAMM DNA effectively induced a protective anti-tumor immunity against local tumor and lung metastasis in B16 melanoma mouse models. Angiogenesis was inhibited and cell apoptosis was increased within tumors. Anti-tumor

activity of xRHAMM was mediated by both the antigen-specific cellular and humoral responses against RHAMM, as confirmed by the depletion of immune cell subsets *in vivo*. Furthermore, the anti-angiogenic and anti-tumor effects induced by vaccination of pcDNA3.1-xRHAMM were significantly stronger than that induced by vaccination of the corresponding autologous counterpart pcDNA3.1-mRHAMM [49].

#### 2.1.2.6. *DLL4*

Notch signaling has recently emerged as a critical regulator of developmental and tumor angiogenesis. Notch signaling in both endothelial and smooth muscle cells appears to provide critical regulatory information to these cells downstream of the initiating signal induced by VEGF [51, 52]. Studies in humans and mice have demonstrated that Notch ligand delta-like 4 (DLL4) is strongly expressed by the tumor vasculature and generally not by the tumor cells themselves. In various mouse models, strong DLL4 expression was observed in the majority of tumor vessels, contrasting with significantly lower vascular expression in adjacent normal tissues [51]. In humans, DLL4 expression was analyzed in tumors from kidney, bladder, colon, brain and breast [53, 54]. Robust DLL4 expression was observed specifically in the tumor vasculature in all of these tumor types, whereas DLL4 expression was low to undetectable in the vasculature of adjacent normal tissue. Furthermore, at least in the case of breast cancer, the degree of DLL4 expression correlated with outcome: tumors with high DLL4 in the vasculature progressed more rapidly [54]. These findings suggest that DLL4 is an attractive new therapeutic anti-angiogenesis target. To generate the DLL4 plasmid vaccine, the cDNA encoding human DLL4 was cloned into the pVAX1 expression vector (DLL4 vaccine), which is specifically designed for the development of DNA vaccines and approved for use in humans. Immunization of Balb/c mice with DLL4 vaccine could bring about a break in tolerance against the self-antigen, DLL4. Readily detectable titers of serum antibodies against DLL4 were induced. Moreover, immunization with DLL4-encoding plasmid DNA severely retarded the growth of orthotopically implanted D2F2/E2 mammary carcinomas in mice by induction of a non-productive angiogenic response. In addition to the promising therapeutic effects, no evidence for a delayed wound healing response, or for toxicity associated with pharmacological blockade of DLL4 signaling, was observed in mice immunized with the DLL4 vaccine [55].

#### 2.1.2.7. *Angiomotin*

Angiomotin (Amot), one of angiostatin receptors [56], is a membrane-associated protein present on the endothelial cell surface of angiogenic tissues [57] characterized by conserved coiledcoil and carboxy termini-PDZ domains [58]. A shorter Amot isoform (p80) confers a hyper-migratory and invasive phenotype in transfected cells [59] and induces endothelial cell migration during angiogenesis [60]. The longer (p130) isoform localizes to tight junctions, regulates cell shape and appears to play a role in the later phase of angiogenesis [60]. It was demonstrated that increased Amot expression on tumor endothelia concomitant with the progression from pre-neoplastic lesions to full-fledged carcinoma, therefore, plasmid vaccine encoding human p80 Amot (pAmot) was constructed [57]. Immunization of mice with pAmot can overcome immune tolerance and induce a significant antibody response that mimic the

effect of angiostatin. These antibodies inhibit endothelial cell migration, block tumor cell- and bFGF-induced angiogenesis in the matrigel plug assay and prevent growth of transplanted tumors without impairing normal stromal or retina vessels [57]. Very recently, Arigoni et al. further showed that the pAmot-induced antibodies alter tumor vessel permeability and structure. These combined effects of vaccine-induced anti-Amot antibodies lead to inhibition of established clinically evident mammary tumors, massive tumor perivascular necrosis, and an effective tumor antigen presentation in a form of epitope spreading that induces an immune response against other oncoantigens overexpressed by tumor cells [61]. Greater tumor vessel permeability also markedly boosts the local accumulation of doxorubicin and enhances the anti-tumor effect of the drugs [61]. These data provide a rationale for the development of fresh anticancer treatments based on anti-Amot vaccination in conjunction with chemotherapy regimens.

Taken together, it is obvious that vaccination with xenogeneic homologous molecules associated with angiogenesis, such as pro-angiogenic factors, integrins, MMP, could induce anti-tumor immunity and thus might be a feasible strategy for cancer therapy with potential clinical applications.

## **2.2. Anti-angiogenic active immunotherapies based on non-xenogeneic homologous molecules**

Given that vaccination with xenogeneic homologous molecules associated with tumor angiogenesis could effectively induce anti-tumor immunity, it can be assumed that vaccines based on non-xenogeneic homologous molecules, such as allogeneic homologues of some pro-angiogenic factors or other important molecules associated with angiogenesis, could also successfully induce specific and potent anti-tumor immunity. To date, several vaccines based on non-xenogeneic homologous molecules were used in anti-angiogenic active immunotherapy for tumors.

### *2.2.1. VEGFR-2*

As has been discussed above, VEGF-mediated signaling pathway through VEGFR-2 is a rate-limiting step during tumor angiogenesis. Thus, VEGF/VEGFR-2 is still an ideal target in the non-xenogeneic homologous molecules-based anti-angiogenic strategy. Immunization of mice with VEGF receptor-2 (flk-1)-pulsed dendritic cells (DC) can break self-tolerance to VEGFR-2, induce CTL and antibody responses to VEGFR-2 [62]. Significant inhibition of tumor growth and metastasis was observed in both melanoma and Lewis lung carcinoma metastasis murine models [62]. Oral administration of mice with DNA vaccines encoding murine VEGFR-2 carried by attenuated *Salmonella typhimurium* could break the immune tolerance to VEGFR-2, induce CTL response to VEGFR-2, inhibit tumor cell-induced neoangiogenesis, and suppress the formation of spontaneous and experimental pulmonary metastases, with slight impact on wounds healing and no influence on hematopoiesis and pregnancy [63]. Immunization of mice with flk1-encoding mRNA-transfected DC could induce specific CTL response to VEGFR-2, partially inhibit the tumor cell-induced neoangiogenesis, and suppress tumor growth and metastasis in murine B16/F10.9 melanoma and MBT-2 bladder tumor models [64]. We studied

the regulatory effects of IFN- $\gamma$  on the differentiation and development of DC and found that IFN- $\gamma$  is an autocrine mediator for DC maturation [65]. IFN- $\gamma$  gene transfection could promote differentiation, development, and functional maturation of DC [66]. IFN- $\gamma$  gene-modified DC had increased capacity to induce Th1 type immune response, and intratumoral injection of IFN- $\gamma$  gene-modified DC in a murine model with pre-established B16 melanoma resulted in the potentiation of the anti-tumor effect of DC [66]. On the other hand, it was demonstrated that IFN- $\gamma$  itself is also a negative regulator of neoangiogenesis [67]. In order to combine the anti-angiogenic immunotherapy with the cytokine immunotherapy, we constructed recombinant plasmid expressing murine VEGFR-2 extracellular domain (sVEGFR-2) and IFN- $\gamma$  fusion protein, pcDNA3.1/sVEGFR-2-IFN- $\gamma$ , and found that the fusion protein expressed by recombinant plasmid shared biological activities of both sVEGFR-2 and IFN- $\gamma$  [68]. Immunization of mice with murine sVEGFR-2-IFN- $\gamma$  fusion gene-transfected DC could significantly augment the CTL response to murine VEGFR-2 and pronouncedly inhibit tumor cell-induced angiogenesis and tumor metastasis in comparison with murine sVEGFR2 gene-transfected DC [68].

In 2006, three CTL epitope candidates, designated as KDR1, KDR2 and KDR3, respectively, from VEGFR-2 with high binding affinity to the H-2D<sup>b</sup> molecule were predicted by two computer programs: Bimas and SYFPEITH [69]. Two of them, KDR2 and KDR3, were from the extracellular domain; KDR1 was from the intracellular part of the receptor [69]. Immunization of mice with KDR2 or KDR3 peptide in combination with murine GM-CSF and agonist anti-mouse CD40 antibodies as adjuvant could break self-tolerance and induce specific immune responses in C57BL/6 mice [69]. Furthermore, immunization of mice with these two peptide epitopes elicited pronounced specific CTL responses to murine VEGFR-2, effectively inhibited VEGF-induced angiogenesis, and suppressed tumor growth in MC38 murine colon cancer model [69]. Similarly, the epitope peptides of human VEGFR-2 restricted by HLA-A\*0201 and HLA-A\*2402 were also identified by analyzing the binding affinities to the corresponding HLA molecules [70]. Antigen based on the epitope peptide with high binding affinity to human HLA-A\*0201 could successfully induce specific CTL response *in vitro* [70]. Furthermore, transgenic mice expressing HLA-A\*0201, A2/Kb, were generated, and the vascular endothelial cells in that mice could not only express human VEGFR-2 (KDR), but also express human MHC class I molecules [70]. After inoculation of A2/Kb with HLA-A\*0201 restricted VEGFR-2 epitope peptide, specific IFN- $\gamma$ -expressed CTL was induced [70]. Immunization of tumor-bearing A2/Kb transgenic mice with VEGFR-2 epitope peptide could markedly inhibit tumor-induced angiogenesis, hereby inhibiting tumor growth in MC38 colon cancer and B16 melanoma models, and prolong survival of the tumor-bearing animals without fatal adverse effects [70]. To further study whether specific CTL response to KDR can be elicited in human or not, KDR epitope peptide vaccines were used to stimulate peripheral blood mononuclear cells derived from 6 cancer patients *in vitro*, and CTLs specific for the peptide epitope were successfully induced in all patients [70].

In comparison with the full-length protein, peptide vaccines like the aforementioned KDR epitope peptides can be easily synthesized in high purity and are less expensive. Moreover, immunization with such vaccines could avoid the potential dangers involving induction of an infection by recombinant viruses or exposure to a latently allergenic exogenous protein.

In 2009, Seavey, et al developed *Listeria monocytogens* based VEGFR-2 vaccines that encode the peptide of VEGFR-2 extracellular domain fused to the first 441 residues of the microbial adjuvant listeriolysin O (*Lm*-LLO-Flk-E1 and *Lm*-LLO-Flk-E2) and the peptide of VEGFR-2 intracellular domain that also fused to LLO (*Lm*-LLO-Flk-I1), respectively [71, 72]. Immunization of the mice with the *Listeria*-based Flk1 vaccines elicited potent antitumor CTL responses. *Lm*-LLO-Flk-1 was able to eradicate some established Her-2/neu<sup>+</sup> breast tumors, reduce microvascular density in the remaining tumors, protect against tumor rechallenge and experimental metastases, and induce epitope spreading to various regions of the tumor-associated antigen Her-2/neu. Tumor eradication was found to be dependent on epitope spreading to Her-2/neu and was not solely due to the reduction of tumor vasculature [71]. In an autochthonous model for Her-2/neu<sup>+</sup> breast cancer, these vaccines could significantly delay tumor onset, while tumors that grew out overtime accumulated mutations in the Her-2/neu molecule near or within CTL epitopes [72]. Moreover, vaccine efficacy did not affect normal wound healing nor have toxic side effects on pregnancy [71]. These data suggest that an anti-angiogenesis vaccine can overcome tolerance to the host vasculature driving epitope spreading to an endogenous tumor protein and drive active tumor regression.

Recently, a DNA vaccine (pSG.SS.Fl<sub>1</sub><sub>ECD</sub>.C3d3) encoding Flk-1 extracellular domain and the complement fragment C3d fusion protein was constructed [73]. Vaccination of mice with pSG.SS.Fl<sub>1</sub><sub>ECD</sub>.C3d3 could also elicit Flk-1 specific antibody response, leading to suppression of angiogenesis and tumor growth in bladder translational cell carcinoma mouse model, suggesting that C3d can be used as an adjuvant to enhance the immune response [73].

In 2010, Miyazawa, et al. [74] reported the results of phase I clinical trial combining of epitope peptide for VEGFR-2 (VEGFR2-169) with gemcitabine for patients with advanced pancreatic cancer. 18 HLA-A\*2402-positive patients with metastatic and unresectable pancreatic cancer were enrolled in the trial. Gemcitabine was administered at a dose of 1000 mg/m<sup>2</sup> on days 1, 8, and 15 in a 28-day cycle. The VEGFR2-169 peptide was subcutaneously injected weekly in a dose-escalation manner (doses of 0.5, 1, and 2 mg/body, six patients/one cohort). Safety and immunological parameters were assessed. No severe adverse effect of grade 4 or higher was observed. Of the 18 patients who completed at least one course of the treatment, 15 (83%) developed immunological reactions at the injection sites. VEGFR2-169 specific CTLs were induced in 11 (61%) of the 18 patients. The disease control rate was 67%, and the median overall survival time was 8.7 months. This combination therapy for pancreatic cancer patients was tolerable at all doses. Peptide-specific CTL could be induced by the VEGFR2-169 peptide vaccine at a high rate, even in combination with gemcitabine. Therefore, they suggested that the optimal dose for further clinical trials might be 2 mg/body or higher.

### 2.2.2. bFGF

Basic fibroblast growth factor (bFGF/FGF2) is an important proangiogenic factor, which is secreted by tumor cells and macrophages or released by extracellular matrix, and functions in the autocrine or paracrine manner. FGF2 can upregulate the expression of several dominant pro-angiogenic factors, such as VEGF [75], and activator of plasminogen [76], and inhibit apoptosis of endothelial cells by bcl-2 pathway [77]. bFGF exerts its biological activities

through its binding to high affinity receptor, fibroblast growth factor receptor-1 (FGFR1). It was found that both peptide segments of synthetic human FGF2 heparin-binding structural domain and receptor-binding structural domain could inhibit the *in vitro* proliferation of human umbilical vein endothelial cells [39]. Immunization of mice with vaccine based on heparin-binding structural domain peptide could induce production of anti-FGF2 specific antibody, which could hamper the binding of FGF2 to heparin sulphate, and inhibit tumor-induced angiogenesis in a gelatin sponge model and tumor growth in a tumor metastatic model [39]. Surprisingly, despite an immune response toward FGF2, this modality of treatment did not affect wound healing as shown by the fact that the treatment did not alter the mean time of wound healing [78]. It also did not affect fertility, because the vaccinated females were not impaired in their ability to become pregnant, to support the growth and development of their embryos, and to deliver viable offspring when compared with control animals [78]. Furthermore, histological analyses did not reveal any alterations in organogenesis in these offsprings [78]. Therefore, the authors concluded that although vaccination against FGF2 induced a specific FGF2 antibody response and inhibited angiogenesis and tumor development in a pathological setting, it did not adversely alter normal physiological events dependent on FGF2.

### 2.2.3. EGFR

Epidermal growth factor receptor (EGFR), a membrane surface sensor with tyrosine kinase activity, is widely distributed on the membrane of mammalian cells [79]. In the physiological condition, EGFR exerts, through binding to ligands (epidermal growth factor, EGF), its physiological activities in regulation of cell division, proliferation and differentiation [79]. Results from clinical studies show that high expression level of EGFR is frequently observed in non-small cell lung cancer, and has been implicated in aggressive biological behavior of tumor cells and poor prognosis of tumor patients [79]. Therefore, immunotherapy targeting EGFR should be another attractive approach to the treatment of EGFR-positive tumors. In murine tumor models with Lewis lung carcinoma and mammary cancer, immunization of mice with DC pulsed with recombinant ectodomain of mouse EGFR (DC-edMER) inhibited tumor angiogenesis, reduced tumor growth, and prolonged the survival of tumor-bearing mice [80]. Spleen cells isolated from DC-edMER-immunized mice showed a high frequency of EGFR-specific antibody-producing cells [80]. Anti-EGFR specific antibody was markedly elevated in sera of immunized mice and was shown to be effective against tumor growth by adoptive transfer [80]. Immunization with DC-edMER vaccine also elicited CTL responses [80]. Depletion of CD4<sup>+</sup> T lymphocytes could completely abrogate the anti-tumor activity and generation of EGFR-specific antibody responses, whereas depletion of CD8<sup>+</sup> T lymphocytes showed partial abrogation of the anti-tumor activity but antibody was still detected [80]. Furthermore, tumor-induced angiogenesis was suppressed in DC-edMER-immunized mice or mice treated with antibody adoptive transfer [80]. These findings indicate that vaccination with DC-edMER can induce both humoral and cellular anti-tumor immunity, and may suggest novel strategies for the treatment of EGFR-positive tumors through the induction of active immunity against EGFR [80].

#### 2.2.4. Legumain

Tumor associated macrophages (TAMs) are well known to play a very important role in tumor angiogenesis and metastasis, as the abrogation of TAMs in tumor tissues effectively reduced several pro-tumor growth and angiogenesis factors, such as VEGF, TGF- $\beta$ , TNF- $\alpha$  and MMP-9 [81]. Thus, the suppression of TAMs in the tumor-microenvironment provides a novel strategy to inhibit tumor growth and dissemination by remodeling the tumor's stroma. Legumain is an asparaginyl endopeptidase and a member of the C13 family of cystine proteases which was found to be highly upregulated in many murine and human tumor tissues and, furthermore, also overexpressed on TAMs in the murine tumor stroma, but absent or present at only very low levels in all normal tissues from which such tumors arose [81-84]. Recently, several oral minigene vaccines against murine MHC class I antigen epitopes of Legumain were constructed based on the binding predictions for these MHC class I molecules by the HLA peptide binding predictions program [85]. Expression vectors encoding these epitopes were designated as pLegu-H-2D<sup>d</sup> and pLegu-H-2K<sup>d</sup> respectively [85]. Oral administration of those vaccines by transforming them into attenuated *Salmonella typhimurium* (Dam<sup>-</sup>, AroA<sup>-</sup>) resulted in significant suppression of angiogenesis in tumor tissues of D2F2 breast carcinoma in syngeneic BALB/c mice [85]. The possible mechanism of angiogenic inhibition involved the induction of a specific CTL response capable of killing Legumain positive cells, especially TAMs, which is likely to be responsible for anti-tumor angiogenesis [85]. Generally, the anti-angiogenic effect aided in the protection of BALB/c mice from lethal challenges with D2F2 breast tumor cells in a prophylactic setting [85].

#### 2.2.5. Endoglin (CD105)

Endoglin, a 95 kDa cell surface protein expressed as a homodimer, functions as an accessory protein for kinase receptor complexes of the TGF- $\beta$  superfamily and modulates TGF- $\beta$  signaling [8]. Expression of CD105 is correlated with vascular density and poor prognosis [8]. Endoglin is over-expressed on proliferating endothelial cells in the breast tumor neovasculature and thus offers a target for anti-angiogenic therapy [8]. It was reported that an oral murine endoglin-encoding DNA vaccine carried by double attenuated *Salmonella typhimurium* (dam<sup>-</sup>, AroA<sup>-</sup>) to a secondary lymphoid organ, i.e., Peyer's patches, resulted in activation of antigen-presenting dendritic cells, induction of immune responses mediated by CD8<sup>+</sup> T cells against endoglin-positive target cells, and suppression of angiogenesis and dissemination of pulmonary metastases of D2F2 breast carcinoma cells presumably by eliminating proliferating endothelial cells in the tumor vasculature, thus providing an promising strategy to therapies for breast cancer [86]. More recently, Wood et al. [87] developed *Listeria*-based vaccines directed against CD105, Lm-LLO-CD105A and Lm-LLO-CD105B. The region of CD105 in Lm-LLO-CD105A vaccine contains at least three predicted H-2K<sup>d</sup> epitopes, while the region of CD105 in Lm-LLO-CD105B contains at least two predicted H-2K<sup>d</sup> epitopes. Immunization of the *Listeria*-based vaccines led to therapeutic responses against primary and metastatic tumors in the 4T1-Luc and NT-2 mouse models of breast cancer. In a mouse model for autochthonous Her-2/neu-driven breast cancer, Lm-LLO-CD105A vaccination prevented tumor incidence in 20% of mice by week 58 after birth while all control mice developed tumors by week 40. In

comparison with previous *Listeria*-based vaccines (Lm-LLO-HMWMAA-C [88] and Lm-LLO-FLK-I1 and Lm-LLO-FLK-E2 [71] ) targeting tumor vasculature, Lm-LLO-CD105A and Lm-LLO-CD105B demonstrated equivalent or superior efficacy against two transplantable mouse models of breast cancer. Mechanism analysis revealed that the anti-tumor therapeutic efficacy of *Listeria*-based CD105 vaccines was mediated by epitope spreading to endogenous tumor antigens and reduction in tumor vascularity [87]. These data suggest that CD105 therapeutic vaccines are highly effective in stimulating anti-angiogenesis and anti-tumor immune responses leading to therapeutic efficacy against primary and metastatic breast cancer.

#### 2.2.6. Endothelial cell lysates-pulsed dendritic cells

Dendritic cells (DCs) are the most potent professional antigen-presenting cells, they play crucial roles in the initiation of an immune response. DCs prepared from BALB/c mouse were pulsed with lysates of autologous or xenogeneic endothelium, and their anti-tumor effects were tested in two syngeneic models of colon cancer [89]. Immunization of endothelium lysates pulsed DCs could induce a break in self tolerance against endothelial cells and mount both the endothelium-specific CTL response and antibody response, leading to significant inhibition of tumor angiogenesis and the growth of subcutaneous tumors as well as pulmonary metastases in mice. Furthermore, the decrease in the mean vascular density of tumors correlates well with the extent of tumor inhibition [89]. Therefore, immunization of endothelium lysates pulsed DCs is also an effective modality of anti-angiogenic active immunotherapy for cancers, and should have important clinical implications for adjuvant cancer therapy.

#### 2.2.7. Endothelial cell vaccine

In 2008, Okaji Y, et al. [90] reported a pilot phase I clinical study in which glutaraldehyde-fixed human umbilical vein endothelial cells (HUVECs) were used as the vaccine. Six patients with recurrent malignant brain tumour and three patients with metastatic colorectal cancer were given intradermal injections of  $5 \times 10^7$  HUVECs/dose, first month weekly, and then every 2 weeks (in total 230 vaccinations). ELISA and flow cytometry revealed immunoglobulin response against HUVECs' membrane antigens. ELISPOT and  $^{51}\text{Cr}$ -release cytotoxicity assay revealed a specific cellular immune response against HUVECs, which were lysed in an effectors:targets ratio-dependent manner. Gadolinium-contrasted MRI showed partial or complete tumour responses in three malignant brain tumour patients. Except for a DTH-like skin reaction at the injection site, no adverse effect of vaccination was observed. These results suggested that the endothelial vaccine can overcome peripheral tolerance of self-angiogenic antigens in clinical settings, and therefore could be useful for adjuvant immunotherapy of cancer.

### 3. Concluding remarks

Recent research achievements have disclosed inspiring pragmatic perspectives of anti-angiogenic active immunotherapy for cancers. In comparison with application of angiogenic

inhibitors and angiogenic antibodies, anti-angiogenic active immunotherapy has its obvious merits. Provided that a break of immunological tolerance to positive regulators of angiogenesis is successfully induced, the long-lasting immune response to angiogenesis-related molecule will be present in the body, hereby providing long-lasting inhibitory effects on angiogenesis. Therefore, it is expected to be the more cost-effective strategy than angiogenic inhibitor or anti-angiogenic antibody therapy where continuous use of the drugs is needed.

Here we divided anti-angiogenic active immunotherapy into two categories: therapies based on vaccination with xenogeneic homologous molecules and with non-xenogeneic homologous molecules related to angiogenesis. Presently, it is difficult to point out which one is better for clinical application because most of the outcomes reported to date were based on pre-clinical animal experiments. As VEGF-mediated signaling through its receptor VEGFR-2 is the key rate-limiting step in tumor angiogenesis, and plays the most important role in neovascularization, development, and progression of various tumors [6], as well as human VEGFR2-169 peptide vaccination could effectively break peripheral self tolerance against VEGFR-2 in patients with metastatic and unresectable pancreatic cancer [74], anti-angiogenic active immunotherapy targeting VEGF or VEGFR-2 might be the most effective strategy among all these therapies. Moreover, considering the potential clinical application of anti-angiogenic immunotherapy based on the specific antibodies raised against a variety of angiogenesis-associated molecules in different tumor entities like glioma, renal cell cancer, and breast cancer, etc, a promising clinical application of anti-angiogenic active immunotherapy alone or in combination with other anti-tumor strategies could be expected. However, there exist as well *caveats* and deficiencies in this strategy. Firstly, in the early phase of tumor growth when the tumor diameter is less than 2-3 mm, tumor cells simply depend on passive diffusion rather than blood perfusion to acquire enough oxygen and nutrition indispensable for growth. Therefore, anti-angiogenic therapy against tumor in this early stage might be ineffective when applied alone. Secondly, although current anti-angiogenic active immunotherapy is focused on specific targets, potential adverse effects might include impairment of wound healing and menstrual cycle. Furthermore, this approach has also limited application perspectives in children with cancers. Therefore, along with recent developments in molecular biology and immunology, future studies will focus on multiple approaches, such as series analysis of gene expression to analyze the gene expression in normal endothelial cells and in proliferative endothelial cells, phage display technology to search for new endothelial cell receptors, and proteomics to discover peptide segments or proteins regulating endothelial cell growth. These approaches are expected to discover more tumor-specific endothelial cell markers for the purpose of selecting specific targets for anti-angiogenic active immunotherapy. In addition, further studies are also required to optimize protocols how to construct vaccines to effectively break self-tolerance and to induce efficient immune response. With these issues being solved continuously, anti-angiogenic active immunotherapy for cancers will become more applicable and effective.

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