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REVIEW

Anti-angiogenic active immunotherapy: a new approach to cancer treatment

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Abstract Tumor angiogenesis plays an important role in tumor growth, aggression and metastasis. Many molecules have been demonstrated as positive regulators of angiogenesis, including vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), and others. In recent years, significant progress has been made in the research on anti-angiogenic strategies for tumor therapies. In this review, anti-angiogenic active immunotherapies for tumors based on vaccination with xenogeneic homologous molecules and non-xenogeneic homologous molecules are discussed.

Keywords Tumor · Angiogenesis · Active immunotherapy

Abbreviations

VEGF	Vascular endothelial growth factor
aFGF	Acidic fibroblast growth factor
bFGF	Basic fibroblast growth factor
EGF (R)	Epidermal growth factor (receptor)
TGF-α/β	Transforming growth factor-α/β
PlGF	Placental growth factor
ECs	Endothelial cells
EO-EPCs	Early-outgrowth of endothelial progenitor cells
MVEGF-P	Recombinant eukaryotic expression plasmid harboring VEGF-encoding gene of mice
XVEGF-P	Recombinant eukaryotic expression plasmid harboring VEGF-encoding gene of *Xenopus laevis*
FGFR-1	Fibroblast growth factor receptor-1
MMPs	Metalloproteinases
sVEGFR-2-IFN-γ	Soluble VEGFR-2 and IFN-γ fusion gene
TAMs	Tumor associated macrophages

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Introduction

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, 9, 10]. 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-α/β (TGF-α, TGF-β), 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 [11, 15, 47, 52]. 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 [6, 8], breast

cancer [44], bladder cancer [19], and renal cell cancer [43]. Importantly, it has been found that tumor growth can be attenuated via the suppression of angiogenesis [11].

Therapeutic strategies targeting tumor vascular endothelia rather than tumor cells have several merits in comparison to conventional anti-tumor therapies [22, 46]: (1) 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 [48]; (2) effector cells or antibodies can reach targeted endothelial cells more readily than they can reach tumor cells [46]; (3) treatment by targeting endothelial cells is not restricted to specific tumor entities [22, 42, 46]; (4) 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 [43]; (5) several specific anti-angiogenic agents, such as IFN-γ, have very low toxicity in some cases of drug combination-therapy regimens in both patients and animal models [22]. In recent years, the field of anti-angiogenic therapy for cancers has attracted much attention.

Active immunotherapy targeting tumor angiogenesis is thus a new modality for treatment of cancers which is based on several assumptions: (1) tumor-derived endothelial cells (ECs) possess characteristics distinct from those of normal tissue [48]; (2) specific immune responses against self-antigen can be elicited; (3) tumor growth can be attenuated via suppression of angiogenesis, as has been already shown [11].

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 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* [23]. In consequence, effective immune response to self-antigens associated with angiogenesis can thus be induced by vaccination with xenogeneic homologous molecules.

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 [48]. These proteins in murine vascular endothelial cells share homology to varying degree with counterparts of other species including human [48]. 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 [55]. 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 [55]. 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 [55]. Autoantibody sediments were detected on ECs within tumor tissues in the immunized mice by immunohistochemical analysis [55]. 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 and 130 kDa, had similar molecular sizes to those of ligand-binding sites of known VEGFR2 and αv integrin, respectively [55], although the authors did not provide direct evidence to demonstrate that the two positive bands aforementioned contained VEGFR2 and αv integrin, respectively. Immune cell subset depletion experiments showed that the production of autoantibodies against tumor vascular ECs and the anti-tumor effect was dependent on $CD4^+$ T lymphocytes [55].

Table 1 Anti-angiogenic active immunotherapy for tumors

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, MA782/5S, FM3A, H22, and Lewis lung carcinoma	[55]
	Murine VEGF	Recombinant plasmid encoding VEGF of *Xenopus laevis*	Meth A, MA782/5S and H22	[54]
		pMAE5Δ5 vectors harboring human VEGF 121 gene and mutated human VEGF 121 gene	EL-4, B16, and TC-1	[3]
	Canine VEGF	Liposome-DNA adjuvant	Soft tissue sarcoma	[18]
	Murine VEGFR-2	Plasmid DNA encoding quail VEGFR-2	Meth A, EL-4 and MOPC-315,	[27]
	Murine FGFR-1	cDNA encoding *Xenopus* homologous FGFR-1	Meth A, H22, and MA782/5S	[14]
	Murine $\alpha v\beta 3$	Plasmid DNA encoding the ligand-binding domain of chicken integrin $\beta 3$	Meth A, H22, and MA782/5S	[31]
	Murine MMP-2	Plasmid DNA encoding chicken homologous MMP-2	LL/2, Meth A , H22	[49]
Vaccination based on non-xenogeneic homologous molecules	Murine VEGFR-2	flk-1 protein pulsed DC	B16, 3LL	[26]
		flk1 mRNA-transfected DC	B16, MBT-2	[33]
		Murine sVEGFR-2 and IFN-γ fusion gene- transfected DC	B16, 3LL	[36]
		Recombinant plasmid encoding flk-1	B16, MC38, D121	[34]
		H-2D^b-restricted KDR2 or KDR3 peptide	MC38	[7]
	VEGFR-2 in transgenic mice expressing HLA-A*0201	HLA-A*0201-restricted VEGFR-2 epitope peptide	MC38, B16	[53]
	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	[16]
	Legumain	Minigene plasmid DNA encoding murine MHC class I antigen epitopes of Legumain	D2F2	[25]
	Endoglin	Plasmid DNA encoding murine endoglin	D2F2	[24]

B16 B16 melanoma; *B16BL6* B16BL6 tumor cells; *D121* D121 lung carcinoma; *D2F2* breast carcinoma cells; *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

Recently, early-outgrowth of endothelial progenitor 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) [2]. EO-EPCs also incorporated DiLDL and bound UEA-I, which are endothelial features, and additionally, they formed vascular-like structures on Matrigel [2]. 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 or syngeneic proliferative endothelial cells to break self-tolerance, thereby inducing profound anti-angiogenic effects in vivo.

Non-cell vaccines

VEGF/VEGFR2

The VEGF is a potent and crucial vasculogenic and angiogenic factor, which can induce endothelial cell proliferation, promote cell migration, and inhibit endothelial cell apoptosis [9, 10]. 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 [21]. The amino acid sequence of VEGF in *X. laevis* shares 75 and 73% homology with that of VEGF164 in mice and that of VEGF165 in humans, respectively [54]. Recombinant eukaryotic expression plasmids harboring VEGF-encoding gene of mice and *X. 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 [54]. Anti-VEGF specific autoantibody was detected in serum of mice vaccinated with XVEGF-P by Western blot and ELISA [54]. The VEGF levels in the tumor-bearing mice immunized with XVEGF-P was lower than that in the control groups [54]. 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 [54]. 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 [54]. Anti-$CD4^+$ 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 $CD4^+$ T lymphocytes were responsible for XVEGF-P-induced anti-tumor effects [54]. 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-α, IFN-β, TNF-α, or β-chemokine in sera was found in immunized mice [54]. Recently, it was reported that when immunized with human VEGF isoform 121 gene (hVEGF121) inserted into pMAE5Δ5 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 [3]. 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 [3]. 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 pMAE5Δ5 ''empty'' vector [3]. IFN-γ ELISPOT assay revealed a significant increase in the number of spots in spleen cells from mice immunized with pM-VEGF [3]. Vaccination with a mutated hVEGF121 gene inserted into the pMAE5Δ5 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 [3]. Serum VEGF levels decreased 8-fold in mice vaccinated with pM-VEGF or pM-VEGFmut as compared with those in pMAE5Δ5 treated mice [3]. A significant correlation was also found between the elevation of serum VEGF level and the increase of the tumor dimensions [3]. 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 [3]. 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 [3].

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 [18]. Recently, 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 [18]. 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 [18]. 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 [18]. 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 [18].

The VEGF receptor-2 [VEGFR-2, also known as fetal liver kinse-1 (flk-1) in mouse and kinase-containing domain receptor (KDR) in human] is the main receptor responsible for the VEGF-mediated angiogenic activity [10]. 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 [45]. Overexpression of KDR was found on activated endothelial cells of newly formed vessels [10]. 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 [27]. 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 [27]. 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 [27]. In contrast, the sera isolated from controls showed negative staining [27]. 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 [27]. Sera from qVEGFR-2-immunized mice also recognized recombinant protein qVEGFR-2 and mVEGFR-2 in ELISA [27]. Detectable IgG1 and IgG2b with significantly elevated concentration in sera were found to be responsible for the immunoglobulin response to VEGFR-2 [27]. 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 [27]. Deposition of immunoglobulins on endothelial cells was found within tumors from qVEGFR-2-immunized mice, but not from controls [27]. 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 [27]. Angiogenesis was markedly suppressed within the tumors, and the vascularization of alginate beads was also diminished [27]. Depletion of $CD4^+$ T lymphocyte could abrogate the anti-tumor activity and the production of autoantibodies against flk-1 [27].

FGFR-1

Fibroblast growth factor receptor-1 (FGFR-1) is expressed on endothelial cells and many types of tumors [39, 51]. 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 [14]. 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 [14]. 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 [14]. However, the immunoglobulins had no direct inhibitory effect on the proliferation of above three tumor cell lines [14]. 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 [14]. Autoantibodies deposited on the endothelial cells within tumor tissues and significantly suppressed intratumoral angiogenesis were found in pxFR1-immunized mice by histological examination [14]. Furthermore, this anti-tumor activity and production of FGFR-1-specific autoantibodies were abrogated by depletion of $CD4^+$ T lymphocytes, again pointing to their essential helper function for antibody production [14].

Integrins

Integrins are heterodimeric transmembrane proteins consisting of α and β 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 [12]. $\alpha v\beta 3$ 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 [12]. This distributive characteristic also makes $\alpha v\beta 3$ an attractive target for tumor therapy [12]. A plasmid DNA encoding the ligand-binding domain of chicken integrin $\beta 3$ was constructed to test this assumption. Immunization with chicken homologous integrin $\beta 3$-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 [31]. Autoantibodies against integrin $\beta 3$ in sera of the immunized mice were found by Western blot analysis and ELISA [31]. 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 [31]. The anti-tumor activity and the production of integrin $\beta 3$-specific autoantibodies were again $CD4^+$ T lymphocyte-dependent [31].

MMP

Angiogenesis is an invasive process, requiring proteolysis of the extracellular matrix [56]. Inappropriate destruction of extracellular matrix components is involved in certain pathological conditions, including arteriosclerosis, rheumatoid arthritis, and tumor aggression and metastasis [56]. The matrix metalloproteinases (MMPs), a family of extracellular endopeptidases, can selectively degrade components of the extracellular matrix [56]. In vivo, elevated stromal MMP-2 and MMP-9 activity is highly correlated with increased metastatic potential in most malignant tumors [29]. 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 [1]. Moreover, numerous pathological and clinical studies demonstrated that the MMPs were frequently overexpressed in various solid tumor cells and peritumoral stromal cells [1]. 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 [17]. Furthermore, the suppression of tumor-induced angiogenesis and of invasion and metastasis of tumor cells could be observed in MMP-2-deficient mice [17]. 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 [49]. 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 [49]. The elevation of MMP-2 in the sera of tumor-bearing mice was abrogated with the vaccination of c-MMP-2 [49]. 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 [49]. 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 [49]. However, the vaccination did not inhibit the gelatinase activity of MMP-9 [49]. 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 $CD4^+$ T lymphocytes [49].

The above observations indicated 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.

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.

VEGFR2

As has been discussed above, VEGF-mediated signaling pathway through VEGFR2 is a rate-limiting step during tumor angiogenesis. Thus, VEGF/VEGFR2 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 [26]. Significant inhibition of tumor growth and metastasis was observed in both melanoma and

Lewis lung carcinoma metastasis murine models [26]. Oral administration of mice with DNA vaccines encoding murine VEGFR2 carried by attenuated *Salmonella typhimurium* could break the immunotolerance 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 [34]. 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 [33]. We studied the regulatory effects of interferon-γ on the differentiation and development of DC and found that IFN-γ is an autocrine mediator for DC maturation [37]. IFN-γ gene transfection could promote differentiation, development, and functional maturation of DC [38]. IFN-γ gene-modified DC had increased capacity to induce Th1 type immune response, and intratumoral injection of IFN-γ gene-modified DC in a murine model with pre-established B16 melanoma resulted in the potentiation of the anti-tumor effect of DC [38]. On the other hand, it was demonstrated that IFN-γ itself is also a negative regulator of neoangiogenesis [41]. 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-γ fusion protein, pcDNA3.1/sVEGFR-2-IFN-γ, and found that the fusion protein expressed by recombinant plasmid shared biological activities of both sVEGFR-2 and IFN-γ [36]. Immunization of mice with murine sVEGFR-2-IFN-γ fusion gene-transfected DC could significantly augment the CTL response to murine VEGFR-2 and pronouncedly inhibit tumor cell-induced angiogenensis and tumor metastasis in comparison with murine sVEGFR2 gene-transfected DC [36].

More recently, three CTL epitope candidates, designated as KDR1, KDR2 and KDR3, respectively, from VEGFR-2 with high binding affinity to the H-2D^b molecule were predicted by two computer programs: Bimas and SYFPEITH [7]. Two of them, KDR2 and KDR3, were from the extracellular domain; KDR1 was from the intracellular part of the receptor [7]. 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 [7]. 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 [7]. 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 [53]. Antigen based on the epitope peptide with high binding affinity to human HLA-A*0201 could successfully induce specific CTL response in vitro [53]. 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 [53]. After inoculation of A2/Kb with HLA-A*0201 restricted VEGFR-2 epitope peptide, specific IFN-γ-expressed CTL was induced [53]. 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 [53]. 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 six cancer patients in vitro, and CTLs specific for the peptide epitope were successfully induced in all patients [53].

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.

bFGF

Basic fibroblast growth factor (bFGF/FGF2) is an important proangiogenic factor, which is secreted by tumor cells and macrophages or released by extracelluar matrix, and functions in the autocrine or paracrine manner. FGF2 can upregulate the expression of several dominant pro-angiogenic factors, such as VEGF [50], and activator of plasminogen [5], and inhibit apoptosis of endothelial cells by bcl-2 pathway [20]. bFGF exerts its biological activities through its binding to high affinitive 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 galatin 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 [40]. 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 [40]. Furthermore, histological analyses did not reveal any alterations in organogenesis in these offsprings [40]. 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.

EGFR

Epidermal growth factor receptor (EGFR), a membrane surface sensor with tyrosine kinase activity, is widely distributed on the membrane of mammalian cells [13]. 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 [13]. 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 [13]. 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 [16]. Spleen cells isolated from DC-edMER-immunized mice showed a high frequency of EGFR-specific antibody-producing cells [16]. Anti-EGFR specific antibody was markedly elevated in sera of immunized mice and was shown to be effective against tumor growth by adoptive transfer [16]. Immunization with DC-edMER vaccine also elicited CTL responses [16]. Depletion of $CD4^+$ T lymphocytes could completely abrogate the anti-tumor activity and generation of EGFR-specific antibody responses, whereas depletion of $CD8^+$ T lymphocytes showed partial abrogation of the anti-tumor activity but antibody was still detected [16]. Furthermore, tumor-induced angiogenesis was suppressed in DC-edMER-immunized mice or mice treated with antibody adoptive transfer [16]. 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 [16].

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 released by TAMs such as VEGF, TGF-β, TNF-α and MMP-9 [30]. 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 [28, 30, 32, 35]. 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 [25]. Expression vectors encoding these epitopes were designated as pLegu-H-$2D^d$ and pLegu-H-$2K^d$, respectively [25]. Oral administration of those vaccines by transforming them into attenuated *Salmonella typhimurium* (Dam^-, $AroA^-$) resulted in significant suppression of angiogenesis in tumor tissues of D2F2 breast carcinoma in syngeneic BALB/c mice [25]. 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 [25]. 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 [25].

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-β superfamily and modulates TGF-β signaling [15]. Expression of CD105 is correlated with vascular density and poor prognosis [15]. Endoglin is over-expressed on proliferating endothelial cells in the breast tumor neovasculature and thus offers an attractive target for anti-angiogenic therapy [15]. It was reported that an oral murine endoglin-encoding DNA vaccine carried by double attenuated *Salmonella typhimurium* (dam^-, $AroA^-$) to a secondary lymphoid organ, i.e., Peyer's patches, resulted in activation of antigen-presenting dendritic cells, induction of immune responses mediated by $CD8^+$ 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 [24].

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 almost all of the outcomes reported to date were based on pre-clinical animal experiments. Because 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 [10], 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., we could also anticipate a promising clinical application of anti-angiogenic active immunotherapy alone or in combination with other anti-tumor strategies. However, there exist as well *caveats* and deficiencies in this strategy. First, 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 break self-tolerance and to induce effective immune response. With these issues being solved continuously, anti-angiogenic active immunotherapy for cancers will become more applicable and effective.

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