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Review

Antibody-based antiangiogenic and antilymphangiogenic therapies to prevent tumor growth and progression*

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Blood and lymphatic vessel formation is an indispensable factor for cancer progression and metastasis. Therefore, various strategies designed to block angiogenesis and lymphangiogenesis are being investigated in the hope to arrest and reverse tumor development. Monoclonal antibodies, owing to their unequalled diversity and specificity, might be applied to selectively inhibit the pathways that cancer cells utilize to build up a network of blood vessels and lymphatics. Among the possible targets of antibody-based therapies are proangiogenic and prolymphangiogenic growth factors from the VEGF family and the receptors to which they bind (VEGFRs). Here, we present molecular mechanisms of angiogenesis and lymphangiogenesis exploited by tumors to progress and metastasise, with examples of antibody-based therapeutic agents directed at interfering with these processes. The expanding knowledge of vascular biology helps to explain some of the problems encountered in such therapies, that arise due to the redundancy in signaling networks controlling the formation of blood and lymphatic vessels, and lead to tumor drug resistance. Nonetheless, combined treatments and treatments focused on newly discovered proangiogenic and prolymphangiogenic factors give hope that more prominent therapeutic effects might be achieved in the future.

Key words: monoclonal antibodies, antiangiogenic therapy, antilymphangiogenic therapy, VEGF- A, VEGF-C, VEGF-D

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INTRODUCTION

Cancer is the leading cause of death in developed countries. Although localized tumors are successfully treated by surgery, radiotherapy and other more selective methods, medicine is often helpless in the face of metastatic disease — the most lethal form of cancer. Therefore, enormous efforts are being made to create specific agents with a potential to suppress tumor growth and prevent the processes that cause benign tumors to gain metastatic competence and begin to spread to distant organs. Progression from benign to malignant tumor involves an increase in tumor cell proliferation rate and in the ability to migrate and invade other tissues. Metastatic tumor cells utilize blood and lymph vessels as routes for dissemination. Tumor-derived factors stimulate formation of new blood vessels (angiogenesis) and lymph vessels (lymphangiogenesis), which actively support tumor growth and spreading. Different approaches are applied to inhibit tumor progression. This review will particularly focus on antibody-based therapies inhibiting proangiogenic and prolymphangiogenic signals mediated by endothelial cell growth factors and their receptors. An overview of potential targets for therapeutic monoclonal antibodies in these processes is shown in Table 1. In the future, strategies utilizing such antibodies, alone or combined with other antitumor therapies, may become a method of choice in the treatment of metastatic cancers.

ROLE OF ANGIOGENESIS IN CANCER PROGRESSION

Angiogenesis is a process involving formation of new blood vessels from preexisting ones. Over forty years ago, Judah Folkman hypothesized that the process of angiogenesis is critical for tumor growth: solid tumors need to develop new blood vasculature to obtain nutrients and oxygen they need to survive and proliferate (Folkman, 1971). Angiogenesis is also an essential component of the metastatic process. Tumor vasculature is a route by which malignant cells exit the primary site to enter general circulation and establish new foci in distant organs. The newly formed blood vessels are immature and leaky, and may not form continuous layer that would prevent intravasation of cancer cells.

Blood vessels are also a dynamic structure connecting tumor environment with the host immune system. Tumor endothelial cells (TECs) interact with the extracellular matrix and stromal tumor cells as well as with

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Abbreviations: ACT, adoptive cell transfer; ADCC, antibody-dependent cell-mediated cytotoxicity; ANG-2, angiopoietin 2; CCL, chemokine (C-C motif) ligand; CCR, C-C chemokine receptor; CSCs, cancer stem cells; CXCL, chemokine (C-X-C motif) ligand; dLN, draining lymph node; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ErbB, v-erb-a erythroblastic leukemia viral oncogene homolog; Fc, crystallizable fragment; FDA, Food and Drug Administration; FGF, fibroblast growth factor; HB-EGF, heparin-binding EGF-like growth factor; HER2, human epidermal growth factor receptor 2; HGF, hepatocyte growth factor; IFP, interstitial fluid pressure; IL, interleukin; IR, ionizing radiation; KRAS, v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; LECs, lymphatic endothelial cells; Lyve-1, lymphatic vessel endothelial hyaluronan receptor-1; mAb, monoclonal antibody; mCRC, metastatic colorectal cancer; NCAM, neural cell adhesion molecule; NF-κB, nuclear factor κ-light-chain-enhancer of activated B cells; Nrp, neuropilin; NSCLC, non-small cell lung carcinoma; PDGF, platelet- derived growth factor; PlGF, placental growth factor; Prox1, Prospero homeobox protein 1; scFv, single-chain variable fragment; TECs, tumor endothelial cells; TGF, transforming growth factor; TNF, tumor necrosis factor; Tregs, regulatory T cells; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

Table 1. Monoclonal antibodies as inhibitors of lymph- and angiogenesis

Target	Name	Type	Description	Research phase
Antiangiogenic action				
VEGF-A	Bevacizumab	humanized IgG1 [1]	binds to VEGF-A and inhibits its biological activity [1]	approved for colorectal cancer, NSCLC*, renal cell carcinoma, glioblastoma [1]
VEGFR-2	Ramucirumab	human IgG1 [2]	blocks VEGF ligand (VEGF-A, VEGF-C and VEGF-D) binding to VEGFR-2 [2]	Phase 3 breast cancer, gastric cancer, hepatocellular carcinoma, NSCLC,gastric cancer, colorectal cancer [3]
VEGFR-2	Tanibirumab	human IgG1 [4]	blocks VEGF ligand (VEGF-A, VEGF-C and VEGF-D) binding to VEGFR-2 [4]	preclinical studies, animal models[4]
Dll-4 (Notch Delta--like ligand 4)	YW152F	humanized Ig [5]	neutralizes Dll-4, induces endothelial cell hyperproliferation and formation of defective vessels [5] when Dll4-Notch signaling is blocked, there is a decreased tumor growth and a reduction in: bone marrow-derived pericytes, vascular smooth muscle cell formation, vessel functionality, lumen-bearing vessels [6]	preclinical studies, animal models [5,6]
HK (high molecular weightkininogen)	C11C1	murine IgG1 [7]	inhibits angiogenesis by reducing tumor microvascular density andblocks binding of HK to endothelial cells [8]	preclinical, animal models [7,8]
EphB4 (Ephrin type--Breceptor 4)	hAb47 and hAb131	humanized IgG1 [9]	hAb131 induces degradation of human EphB4, inhibits human endothelial tube formation *in vitro* and growth of human tumors expressing EphB4 *in vivo* hAb47 targets human and murine EphB4, inhibits angiogenesis and growth of both EphB4-positive and EphB4-negative tumors in amouse model [9]	preclinical, animal models [9]
globotriaosylceramide Gb3	3E2	murine IgM [10]	inhibits: endothelial cell proliferation *in vitro*, angiogenesis *ex vivo*, neuroblastoma development and liver metastase spreading in murine models [10]	preclinical, animal models [10]
YKL-40secreted glycoprotein	mAY	murine Ig [11]	inhibits tube formation of microvascular endothelial cells *in vitro*,prevents YKL-40-induced activation of VEGFR-2, restrains tumor growth and angiogenesis in animal models [11]	preclinical, animal models [11]
Ang-2 (angiopoietin-2)	LC06	human IgG1 [12]	prevents binding of Ang-2 to its receptor Tie2, inhibits tumor growth, reduces: intratumoral microvessel density, tumor vessel branching and dissemination of tumor cells to the lungs, increases pericyte coverage [12] coverage [12]	preclinical studies, animal models [12]
Ang-2 (angiopoietin-2)	MEDI3617	human Ig [13]	prevents Ang-2 bindisng to the Tie2 receptor *in vitro*, inhibits angiogenesis and tumor growth *in vivo* [13]	preclinical studies, animal models [13, 14]
HB-EGF(heparin--binding EGF-like growth factor)	Y-142	murine Ig [15]	inhibits sHB-EGF-induced cancer cell proliferation and sHB-EGF- induced angiogenesis [15]	*in vitro* studies [15]
Antiangiogenic and antitumor action				
EGFR	Cetuximab	chimeric human/ mouse IgG1[16]	EGFR antagonist [16], may reduce pro-angiogenic factor secretion by cancer cells [17] cancer cells [17]	approved for head and neck cancerand colorectal cancer [16]

EGFR	Panitumumab	human IgG2 [18]	human IgG2 competitively inhibits binding of ligands (eg. EGF, TGFα) to EGFR [18]	approved for metastatic colorectal-cancer [18] Phase 3 (head and neck cancer) Phase 2 (pancreatic cancer) [19]
HER2	Trastuzumab	humanized IgG1 [20]	mediator of ADCC [20]	approved for metastatic breast and gastric cancers [20]
HER2	Pertuzumab	humanized IgG1 [21]	blocks ligand-dependent dimerization of target receptor, mediator of ADCC [21]	approved for breast cancer [22]
VEGFR-1	Icrucumab	human IgG1 [23]	blocks VEGF-A, VEGF-B and PlGF binding to VEGFR-1; inhibits VEGFR-1 activation in both cancer and non malignant supporting cells that contribute to tumor progression [23]	Phase 2, colorectal cancer, breast cancer, bladder, urethra, ureter, and renal pelvis carcinoma [24]
PDGFRα	Olaratumab, IMC--3G3	human IgG1 [25]	targets PDGFRα present on both tumor and stromal cells, inhibits tumor and vasculature formation [25, 26]	Phase 2NSCLC, soft tissue sarcoma, prostate cancer [27]
integrin α5β1	Volociximab	chimeric human/mouse IgG4 [28]	human/mouse chimeric antibody, inhibits endothelial cell proliferation and formation of capillary like structures by endothelial cells, inhibits tumor growth *in vivo* [28]	Phase 1b/2NSCLC [29]
Notch1	anti-NRR1	human IgG1 [30]	specific toward negative regulatory region (NRR) of Notch1; stabilizes NRR quiescence, inhibits cancer cell growth and disrupts tumor angiogenesis, blocks both ligand-dependent and -independent Notch activation [30]	preclinical studies, animal models [30]
HER2	chA21	single-chain chimeric antibody [31]	it does not directly disrupt dimerization of target receptor but leads to its internalization and down-regulation [31] inhibits VEGF secretion and down-regulates migration and proliferation of endothelial cells [32]	preclinical, animal models [32]
Antilymphangiogenesis				
VEGFR-3	IMC-3C5	human IgG1 [33]	blocks VEGF-C and VEGF-D binding to VEGFR-3; inhibits mitogenic response to mature VEGF-C [33]	Phase 1 [34]
VEGF-C	VGX-100	human Ig [35]	human Ig, blocks VEGF-C binding to VEGFR-2 and VEGFR-3 [35]	Phase 1 [36]
ephrinB2	B11, 2B1	human single--chain variable fragments [37]	suppresses endothelial cell migration and tube formation *in vitro,* reduces tumor growth and inhibits lymph- and angiogenesis in animal models [37]	preclinical, animal models [37]
VEGF-D	cVE199	chimeric IgG1 [38]	inhibits VEGF-D binding to VEGFR-3 [38]	preclinical studies, animal models[38]
VEGFR-3	2E11	–	inhibits formation of VEGFR-3 homodimers and VEGFR-3/VEGFR-2 heterodimers but does not inhibit binding of VEGF-C to VEGFR-3 [39]	preclinical studies, animal models[39]
Nrp2	–	–	blocks VEGF-C binding to Nrp2 and disrupts lymphatic endothelial	preclinical studies, animal models
(Neuropilin-2)			cell migration [40]	[40]
VEGF-C	VC2	human single--chain variable fragment [41]	dose-dependently inhibits the binding of VEGF-C to VEGFR-2 and VEGFR-3 [41]	*in vitro* studies [41]

MT1-MMP (MMP-14, matrix metallo-protease-14)	9E8	murine IgG1 [42]	blocks the enzymes ability to activate proMMP-2 without interfering with the general proteolytic activity of MT1-MMP, inhibits outgrowth of lymphatic endothelial cells *in vitro* and lymphatic vessel sprouting *ex vivo* [42]	*in vitro* studies [42]
VEGFR-2 and VEGFR-3	–	human diabody constructed of two human single--chain variable fragments [43]	directed against VEGFR-2 and VEGFR-3, inhibits activation of both receptors [43]	*in vitro* studies [43]

[1] (Roche, Avastin prescribing information, 2013; access on 26.03.2013; www.gene.com/download/pdf/avastin_prescribing.pdf), [2] (Zhu *et al.*, 2003), [3] (ClinicalTrials.gov identifiers: breast cancer NCT00703326, gastric cancer NCT00917384, hepatocellular carcinoma NCT01140347, NSCLC NCT01168973, gastric cancer NCT01170663, colorectal cancer NCT01183780), [4] (Lee, 2011), [5] (Ridgway *et al.*, 2006), [6] (Stewart *et al.*, 2011), [7] (Song *et al.*, 2004), [8] (Khan *et al.*, 2010), [9] (Krasnoperov *et al.*, 2010), [10] (Desselle *et al.*, 2012), [11] (Faibish *et al.*, 2011), [12] (Thomas *et al.*, 2013), [13] (Leow *et al.*, 2012), [14] (Holopainen *et al.*, 2012), [15] (Sato *et al.*, 2012), [16] (Merc, Erbitux prescribing information, 2012), [17] (Perrotte *et al.*, 1999), [18] (Amgen, Panitumumab prescribing information, 2013; access on 26.03.2013; www.pi.amgen.com/united_states/vectibix/vectibix_pi.pdf), [19] (ClinicalTrials.gov identifiers: head and neck cancer NCT00460265, pancreatic cancer NCT01175733), [20] (Roche, Herceptin prescribing information, 2010; access on 26.03.2013; www.herceptin.com/pdf/herceptin-prescribing.pdf), [21] (El-Sahwi, 2010), [22] (Roche, Perjeta prescribing information, 2012; access on 26.03.2013; www.accessdata.fda.gov/drugsatfda_docs/label/2012/125409lbl.pdf), [23] (Wu *et al.*, 2006), [24] (ClinicalTrials.gov identifiers: colorectal cancer NCT01111604, breast cancer NCT01234402, bladder, urethra, ureter, and renal pelvis carcinoma NCT01282463), [25] (Shah *et al.*, 2010), [26] (Gerber *et al.*, 2012), [27] (ClinicalTrials.gov identifiers: NSCLC NCT00918203,soft tissue sarcoma NCT00918203, prostate cancer NCT01204710), [28] (Bhaskar *et al.*, 2008) , [29] (Besse *et al.*, 2013), [30] (Wu *et al.*, 2010), [31] (Zhou *et al.*, 2011), [32] (Shen *et al.*, 2011), [33] (Persaud *et al.*, 2004), [34] (ClinicalTrials.gov identifier: NCT01288989), [35] (Hajrasouliha *et al.*, 2012), [36] (ClinicalTrials.gov identifier: NCT01514123), [37] (Abengozar *et al.*, 2012), [38] (Kashima *et al.*, 2012), [39] (Tvorogov *et al.*, 2010), [40] (Caunt *et al.*, 2008), [41] (Rinderknecht *et al.*, 2010), [42] (Ingvarsen *et al.*, 2013), [43] (Jimenez *et al.*, 2005).

immune cells (Berezhnaya, 2010). They also control leukocyte recruitment and may contribute to the immune escape of tumors (Bussolati *et al.*, 2003; Chouaib *et al.*, 2010). For many years, it was thought that biological properties of TECs are identical or similar to those of endothelial cells from healthy tissue. However, recent studies on isolated TECs revised this hypothesis. TECs are described as "chronically activated or inflamed" (Dudley, 2012). Tumor cells produce many factors, such as TNF, which stimulate expression of adhesion molecules Thy-1, E- selectin, NCAM (Bussolati *et al.*, 2003) on the endothelial cells. Activated endothelium recruits proinflammatory cells, in particular macrophages, which might contribute to angiogenesis and tumor progression.

The role of VEGF/VEGFR signaling in tumor development and metastasis

New blood vessel formation is a complex process regulated by numerous proangiogenic modulators, among which the vascular endothelial growth factor-A (further referred to as VEGF) seems to play a predominant role. VEGF, also known as vascular permeability factor, is a key angiogenic factor that regulates both normal and tumor angiogenesis (Ferrara & Gerber, 2001). It belongs to the VEGF family of ligands that consists of seven members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F and the placental growth factor (PlGF). These ligands exert their biological activity upon binding to tyrosine kinase receptors VEGFRs (VEGFR-1, VEGFR-2 and VEGFR-3). Activation of VEGFR-1 and VEGFR-2 signaling leads to migration of hematopoietic cells and growth, migration and survival of vascular endothelial cells (Ferrara *et al.*, 2003; Ferrara, 2004).

VEGF is overexpressed in tumors compared to normal tissues. It is secreted not only by tumor cells but also by tumor-associated stromal cells in response to hypoxic conditions in pathologically expanding tumor tissue. VEGF induces angiogenesis by activating signaling through VEGFR in the blood vessels surrounding the tumor (Fukumura *et al.*, 1998).

Expression of VEGF in solid tumors stimulates new blood vessel formation, which is crucial for the continued tumor growth as well as for metastasis. Additionally, expression of VEGFR-2, a major receptor involved in malignant angiogenesis, is upregulated several-fold in tumor vascular endothelial cells (Plate *et al.*, 1994). VEGFR-2 is also expressed on immune cells and, after its activation by VEGF, plays an important role as a factor suppressing anti-tumor immunity (Ohm & Carbone, 2001; Suzuki *et al.*, 2010). Many studies demonstrated that a high level of plasma VEGF correlated with disease progression, resistance to chemotherapy and poor prognosis for cancer patients (Lee *et al.*, 2000; Poon *et al.*, 2001).

VEGF is a critical survival factor for tumor endothelial cells; therefore a constant supply of VEGF to the tumor is essential not only for the development of new tumor vasculature but also for the maintenance of blood vessels that already exist within the tumor. In the light of these data it has been proposed that the VEGF/VEGFR axis could be a potential target for developing antiangiogenic and anticancer therapy. However, it is clear that antiangiogenic therapy should not only focus on tumor endothelium; other cells participating in new blood vessel formation like pericytes, fibroblasts, stromal cells and bone marrow-derived endothelial progenitor cells also seem to constitute valuable targets. It should also be noted that a growing body of evidence indicates the significant role not only of blood vessels but also of the lymphatic vessel system in tumor progression. Tumor cells infiltrating the draining lymph nodes (dLNs) are regarded as an important diagnostic factor indicative of the stage and malignancy of the disease.

ROLE OF LYMPHANGIOGENESIS IN CANCER PROGRESSION

The lymphatic vessel system is composed of blind-ended initial lymphatics, precollecting vessels, collecting vessels and the thoracic duct. One of its major physiological roles is to maintain tissue fluid balance by draining protein-rich fluid from the interstitial space and driving it back to the blood circulation. Lymphatic vessels are also involved in the immune response. They are re-

sponsible for the transport of antigens and constitute a route for antigen-presenting dendritic cells leading from peripheral tissues to the dLNs (Lund & Swartz, 2010). Tumor lymphatic vessels are also engaged in the transport of tumor-derived growth factors and other proteins to the local lymph nodes. There they can exert immunosuppressive or immunomodulatory effects, facilitating evasion of tumor cells from the immune surveillance (Lund *et al.*, 2012).

Formation of the lymph vessel system takes place during embryonic development. In adults, the growth of new lymphatic vessels occurs during inflammation and wound healing. Excessive lymphangiogenesis is observed in chronic inflammation, autoimmune disorders, graft rejection and, as it will be discussed in this review, in tumor progression (Alitalo, 2011).

Although the importance of the lymphatic system for tumor development and spreading has been generally accepted for many years, deeper insight into the biology of the lymphatics has long been hampered due to the lack of defined lineage-specific markers.

The discovery of such markers for the lymphatic endothelial cells (LECs): Lyve-1 (Banerji *et al.*, 1999), Prox1 (Wigle & Oliver, 1999), and podoplanin (Breiteneder-Geleff *et al.*, 1999), enabled significant progress in understanding the mechanisms responsible for lymphatic-dependent metastasis. It is now well documented that lymphangiogenesis induced by the growing tumor allows cancer cells to metastasise to the dLNs (Christiansen & Detmar, 2011). Thus, lymphatic vessels are now considered one of the major routes for tumor cell spreading from primary sites to lymph nodes and, further, to distant tissues.

Prolymphangiogenic growth factors

Data from *in vitro* as well as from *in vivo* experiments point at VEGF-C and VEGF-D as major growth factors driving tumor-dependent lymphangiogenesis through activation of VEGFR-3 and its coreceptor neuropilin 2 (Nrp2) (Makinen *et al.,* 2001; Mandriota *et al.,* 2001; Karpanen *et al.,* 2006; Xu *et al.*, 2010; Chen, *et al.,* 2012). Since VEGF-C and VEGF-D can also activate VEGFR-2, they are considered proangiogenic factors as well (Leppanen *et al.*, 2010). VEGF-C and VEGF-D produced by tumor cells along with tumor-infiltrating macrophages stimulate LEC proliferation, survival, and migration (Makinen *et al.,* 2001; Schoppmann *et al.*, 2002; Otrock *et al.*, 2007). VEGF-C has also been shown to stimulate LECs to express CCL21 and CCL19, chemokines which attract CCR7-positive tumor cells to the lymph vessels. As a result of this observation, LECs are now considered active players in tumor metastasis (Issa *et al.*, 2009). The roles attributed to VEGF-C regarding tumor development are presented in Fig. 1.

The results of *in vivo* experiments are even more convincing. VEGF-C significantly increased intratumoral lymphangiogenesis in a breast cancer model and the extent of this process correlated with the dLN and lung metastasis (Skobe *et al.*, 2001). In B16 melanoma model, overexpression of VEGF-C resulted in increased lymphangiogenesis around the tumor and metastasis to the dLNs (Lund *et al.,* 2012). Similar correlation between VEGF-D-stimulated lymphangiogenesis and dLN metastasis was also reported (Stacker *et al.*, 2001). These observations are in accordance with the results of *in vivo* experiments in which the VEGF-C/VEGFR-3 axis has been blocked. The silencing of VEGF-C and/or VEGFR-3 expression resulted in decreased metastasis in mouse models of breast-, bladder- and lung cancers (Chen *et al.*, 2005; Wang *et al.*, 2010; Feng *et al.*, 2011).

Recent data showed that the tumor-derived lymphangiogenic growth factors are transported through the lymphatics and lead to expansion of the lymphatic network in the draining lymph nodes before the actual metastasis occurs, thereby preparing a premetastatic niche, enabling the seeding of tumor cells to the dLN (Liersch *et al.*, 2012).

In addition to animal models, there are an impressive number of studies on patients suffering from gastric carcinoma, melanoma and breast cancer, showing a close

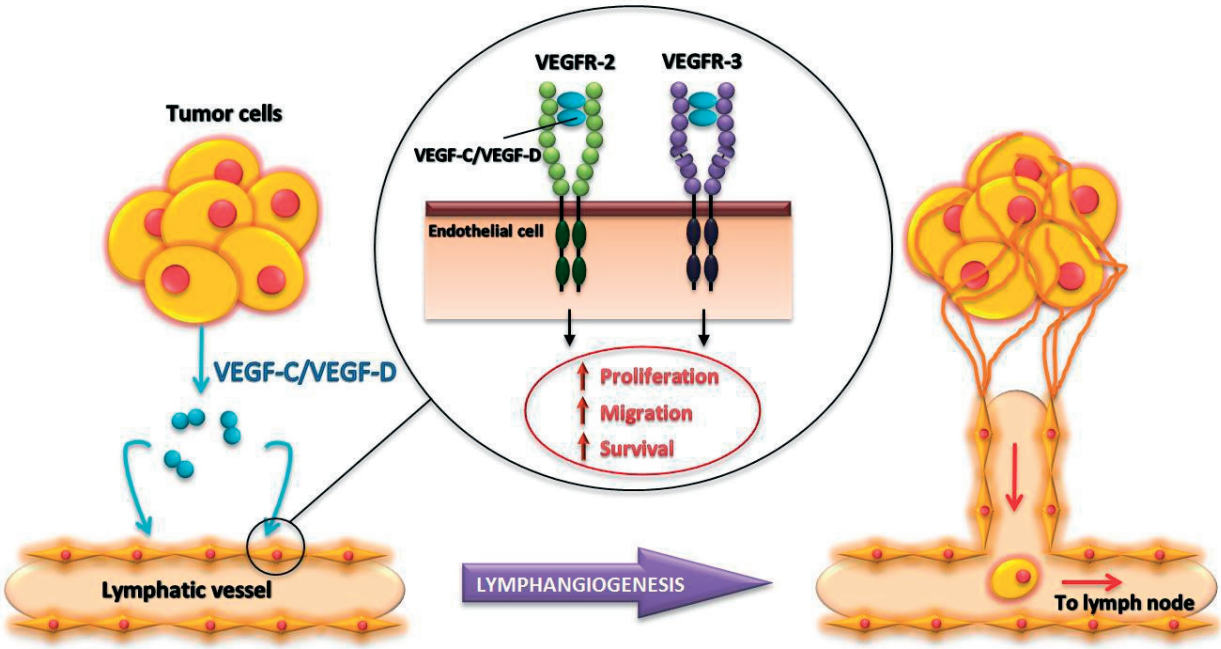

Figure 1.The role of VEGF-C in cancer metastasis is the promotion of tumor-associated lymphangiogensis. Based on Alitalo *et al.*, 2005.

correlation between the serum level of VEGF-C, the density of lymphatic vessels and the incidence of lymph node metastases (Gao *et al.*, 2009; Cianfarani *et al.*, 2012; Ding *et al.*, 2012). In addition to VEGF-C and VEGF-D, also other factors, such as VEGF-A, FGF, ANG-2, and HGF may control the process of lymphangiogenesis and lymphatic metastasis (Achen & Stacker, 2006).

Today, it is clear that both angiogenesis and lymphangiogenesis may serve as excellent targets for cancer therapies. The antiangiogenic therapies are already evaluated in medical procedures while antilymphangiogenic therapies are still at the stage of preclinical and clinical trials.

ANTIBODY-BASED THERAPIES TO INHIBIT ANGIOGENESIS

Several preclinical studies demonstrated that monoclonal antibodies specific for VEGF completely suppressed tumor angiogenesis and inhibited the growth of human-derived tumor cell lines injected into nude mice. These results strongly suggested that VEGF inhibitors blocked both angiogenesis and tumor growth (Kim *et al.*, 1993; Warren *et al.*, 1995; Borgstrom *et al.*, 1998) and led to development of therapeutic anti-VEGF monoclonal antibodies.

Monoclonal antibody neutralizing VEGF

Bevacizumab (produced by Genentech/Roche and distributed under the name Avastin) is a humanized monoclonal antibody that specifically recognizes, removes from the circulation and suppresses biological activity of human VEGF. In 2004 bevacizumab was approved by FDA for treatment of metastatic colorectal cancer (mCRC) and thus became the first antiangiogenic drug accepted for cancer therapy. Bevacizumab is generally well tolerated by patients but sometimes it has specific adverse effects, most commonly hypertension, proteinuria, bleeding, wound healing complications, and thromboembolism (Fox *et al.*, 2007).

After the unsatisfactory results of the initial trials with bevacizumab as a single agent (Giantonio *et al.,* 2007) it is now most commonly used in combination with cytotoxic therapy for treatment of mCRC, non-small cell lung cancer, and renal cell cancer (Roche, Avastin prescribing information, 2013[1]), while the use of bevacizumab as a single agent is limited to the palliative treatment of patients suffering from glioblastoma multiforme (Agha *et al.*, 2010, Chamberlain, 2011). In combination with chemotherapy, bevacizumab prolonged progression-free survival compared to chemotherapy alone, and showed meaningful clinical benefits in patients with different tumor types (Kabbinavar *et al.*, 2003; Hurwitz *et al.*, 2004; Miller *et al.*, 2007). In 2008 FDA approved bevacizumab for treatment of patients with HER2-negative metastatic breast[1] cancer on the basis of previous clinical studies that demonstrated an increase in progression-free survival of patients submitted to combined therapy of bevacizumab and paclitaxel (O'Shaughnessy, 2010; Ocana *et al.*, 2011). However, further clinical trials showed that combination of bevacizumab with cytotoxic agents did not lead to any increase in overall survival of patients with breast cancer and, additionally, this therapy was associated with a high risk of severe life threatening events, the reasons of which are discussed below. The synergistic effect of bevacizumab and chemotherapeutic agents observed in some types of tumors is probably associated with normalization of the tumor vasculature by inhibition of VEGF/VEGFR signaling. Elevated level of VEGF in tumors leads to disorganization and lack of typical hierarchy of tumor blood vessels. It is also reported that high permeability of the tumor vasculature and high interstitial fluid pressure (IFP) in tumors results from a high VEGF level in the tumor microenvironment. It is believed that VEGF inhibition by bevacizumab causes a decrease in IFP and thus improves transfer of chemotherapeutic agents into the tumor (Jain & Carmeliet, 2001; Gerber & Ferrara, 2005).

The efficiency of bevacizumab in combined therapies is constantly under investigation. Preclinical studies demonstrated that bevacizumab might act as a biological enhancer of radiation therapy. Experiments performed by Gorski and colleagues showed that exposure to ionizing radiation (IR) strongly induced VEGF expression in the tumor (Gorski *et al.*, 1999), which is regarded as an important element of tumor resistance to radiation therapy. Blockade of tumor angiogenesis with bevacizumab could improve the therapeutic efficacy of IR. This strategy, however, requires verification in clinical trials (Kim *et al.*, 2013).

Apart from its role in angiogenesis, VEGF is also an important immunomodulatory factor that might have a suppressive effect on antitumor immune response. In this respect, VEGF induces the activity of regulatory T cells (Tregs) (Li *et al.*, 2006) and strongly inhibits differentiation of hematopoietic progenitor $CD34^+$ cells into conventional dendritic cells by inactivating NF-$\varkappa$B transcription factor through VEGFR-1 (Gabrilovich *et al.*, 1998; Oyama *et al.*, 1998). Since Tregs can promote immune suppression and the dendritic cells play an important role in host anti-tumor response, high levels of VEGF could contribute to tumor immune escape. Therefore, inhibition of the VEGF/VEGFR-1 signaling pathway with bevacizumab could be a strategy to improve dendritic cell maturation and enhance host immune response to tumor (Osada *et al.*, 2008). Adoptive cell transfer (ACT) immunotherapy is a new approach involving transfusion of different leukocyte subsets into patients in order to eradicate tumor cells with the use of immune mechanisms (June, 2007). Based on the fact that the ACT strongly depends on the extravasation of the leukocytes from tumor vessels into the tumor stroma, it can be supposed that the immunological cells administered to patients during ACT treatment would be most effective in interaction with endothelial cells in normalized vessels. Therefore, it is proposed that treatment with anti-VEGF antibodies prior to the ACT procedure may have a beneficial effect on the success of the therapy. Such an approach was investigated on the model of metastatic melanoma. As shown in studies on animal models, mAb that blocks VEGF can also normalize tumor vasculature, upregulate endothelial adhesion molecules in tumor vessels, and increase the number of tumor-infiltrating leukocytes (Mulligan *et al.*, 2009; Shrimali *et al.*, 2010). The efficacy of ACT combined with anti-VEGF has recently been tested in the therapy of human cancer (Kandalaft *et al.*, 2013).

High expectations associated with bevacizumab after the positive results of preclinical studies were not fulfilled. The best clinical effects of bevacizumab are obtained in combination therapy of mCRC. Nevertheless,

[1][online], access on 26.03.2013; www.gene.com/download/pdf/avastin_prescribing.pdf

even in these cases, bevacizumab is not capable of arresting the progression of the disease and the possible long-term effect of the inhibition of VEGF is the development of therapy resistance mechanisms in the tumor. However, it should be emphasized that bevacizumab is recommended for patients with advanced and metastatic cancers and it cannot be excluded that its effectiveness could be greater if the therapeutic was applied at less advanced stages of the disease.

Monoclonal antibodies blocking VEGFR

Another strategy for suppression of VEGF/VEGFR signaling involves monoclonal antibodies targeting receptors for VEGF. Animal studies with a rat MF1/IMC-18F1 mAb (developed by ImClone Systems) specific to VEGFR-1 demonstrated that suppression of this receptor effectively blocked tumor angiogenesis and metastasis (Luttun *et al*., 2002). Later, the same company generated a fully human mAb (IMC-18F1) specific to VEGFR-1, the antiangiogenic and tumoricidal activity of which was tested both *in vitro* and *in vivo* on human breast carcinoma models. Results of these studies showed that IMC-18F1 inhibited VEGF, VEGF-B and PlGF binding to VEGFR-1, thus suppressing tumor growth (Wu *et al*., 2006). The antiangiogenic and tumor-suppressing activity of IMC-18F1 is being evaluated in an ongoing Phase 2 clinical trial. Encouraging results of studies on VEGFR-1 inhibitor inspired ImClone Systems to develop mAb specific to VEGFR-2: IMC-1121B (ramucirumab) is a human mAb which binds to the extracellular domain of VEGFR-2. Anticancer efficacy of ramucirumab was verified in completed Phase 1 clinical trials, which were carried out on patients with melanoma, gastric adenocarcinoma, uterine leiomyosarcoma and renal cancer. At present, ramucirumab is tested in Phase 2 and 3 clinical trials as a single agent or combined with paclitaxel, docetaxel, carboplatin, or other cytotoxic drugs (Zhu *et al*., 2003).

In October 2012 Eli Lilly, Inc. (ImClone Systems) announced the results of the REGARD trial, which was one of the two ramucirumab Phase 3 studies on advanced gastric cancer. It showed that ramucirumab used as a single agent statistically improved overall survival and prolonged progression-free survival of patients with advanced gastric cancer. The ongoing Phase 3 RAINBOW study is carried out to verify clinical activity of ramucirumab combined with paclitaxel in metastatic gastric cancer[2]. It is noteworthy that AVAGAST, a similar study in which bevacizumab was tested in combination with first-line chemotherapy on gastric cancer, did not meet its primary endpoint of extending overall survival in patients. The still unanswered question is whether the therapy with ramucirumab could show actual clinical benefit in cancer types where bevacizumab was not successful (Ohtsu *et al.,* 2011).

Tumor resistance to antiangiogenic therapy targeting VEGF/VEGFR

Resistance to therapy is a major clinical problem that strongly affects successful treatment of patients with cancers (Lee *et al*., 2012). Antiangiogenic therapy was developed as a novel anticancer strategy in the hope to avoid the problems of tumor resistance because it targets stable endothelial cells instead of unstable tumor cells (Kerbel, 1991; Boehm *et al*., 1997). Since clinical studies with bevacizumab revealed limited therapeutic efficacy of this drug, at least two important questions were raised: firstly, whether the tumor could develop resistance to antiangiogenic agents; and secondly, if such resistance might be considered as a major reason for the failure of this therapy. Recent data indicate that tumor progression after antiangiogenic treatment can be the result of different mechanisms of adaptation depending on tumor type and microenvironment, which involve both tumor cells and stromal components (for review see Grepin & Pages, 2010; Loges *et al*., 2010).

Selection of more invasive tumor cells[2]

Several months after FDA revoked the approval for bevacizumab for treatment of metastatic breast cancer, Conley and colleagues from the University of Michigan Department of Internal Medicine published results of studies performed in order to elucidate potential reasons of the limited efficacy of antiangiogenic drugs. They hypothesized that the highly metastatic cancer stem cells (CSCs) play a crucial role in the acceleration of tumor growth, observed very often after antiangiogenic treatments (Burstein *et al*., 2008). Using mouse model of human breast cancer, they demonstrated that administration of antiangiogenic agents such as bevacizumab or sunitinib (receptor tyrosine kinase inhibitor) leads to the generation of hypoxia within the tumor tissue. This activates hypoxia-inducible factor 1α, which increases the number of CSCs in the tumor. Accelerated regrowth of breast tumors results from an increase in the aggressive breast CSCs population (Conley *et al*., 2012). Authors of the abovementioned studies claim that a combined therapy targeting both the tumor vasculature and CSCs might overcome tumor resistance to antiangiogenic agents.

Angiogenic redundancy

The VEGF/VEGFR signaling pathway plays a predominant role in tumor angiogenesis. However, it is clear that this process is also regulated by many other factors. When VEGF-dependent angiogenesis is blocked by agents such as bevacizumab or sunitinib, the tumor adaptation involves upregulation of other proangiogenic stimulators including VEGF-B, PlGF, TNF, fibroblast growth factors (FGFs), plateled-derived growth factor (PDGF), angiopoietin 1 and 2, ephrins, TGF-α, TGF-β, or HB-EGF (Casanovas *et al*., 2005; Fischer *et al*., 2008; You & McDonald, 2008; Acevedo *et al*., 2009; Lieu *et al*., 2011). It is thought that targeting more than one angiogenic pathway at the same time could prevent angiogenic redundancy and increase anticancer activity of antiangiogenic therapies (Gossage & Eisen, 2010).

Many studies indicate that EGFR signaling strongly contributes to tumor angiogenesis (Ellis*,* 2004). The epidermal growth factor receptor (EGFR) family consists of four members (EGFR, ErbB2, ErbB3, and ErbB4; further referred to as EGFRs) that are activated upon binding of the EGF- family ligands (EGF, TGF-α, HB-EGF, amphiregulin, neuregulin). EGFRs, as well as EGF-family ligands, are overexpressed in numerous solid tumor types (Laskin & Sandler, 2004; Laskin & Sandler, 2004) and in tumor endothelium (Amin *et al*., 2006; Amin *et al*., 2008). Activation of EGFRs by their ligands is gener-

[2][online] access on 26.03.2013; www.targetedhc.com/articles/Ramucirumab-Achieves-Primary-Endpoint-as-Single-Agent-in-Metastatic-Gastric-Cancer

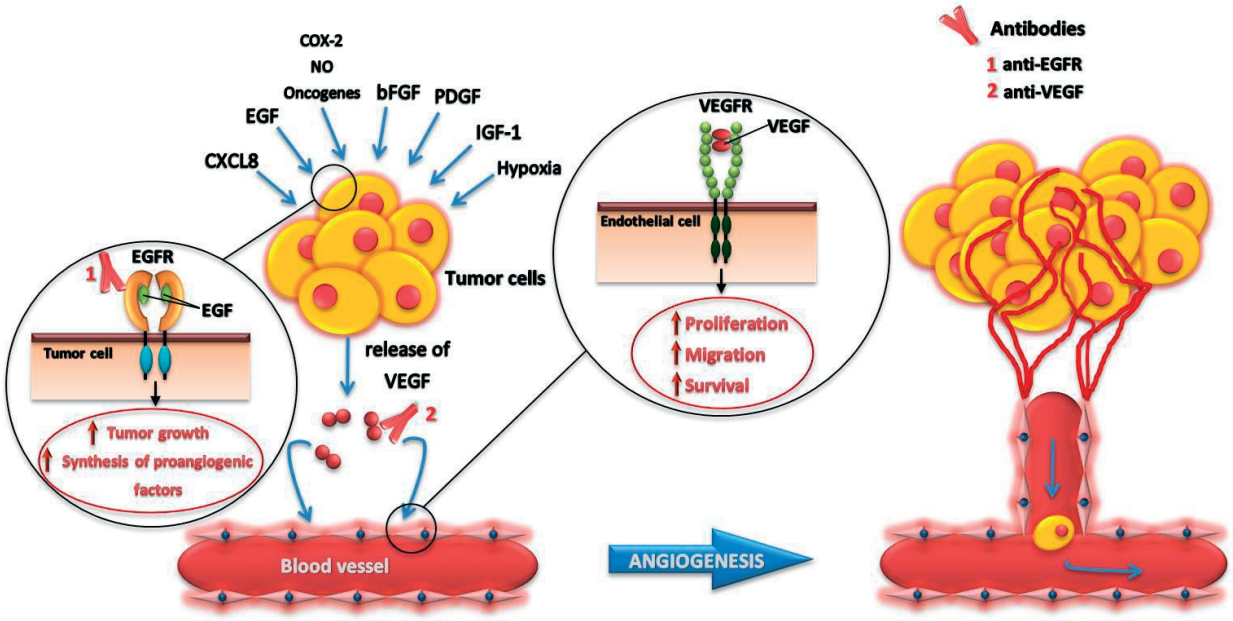

Figure 2. The interconnection of EGFR and VEGF pathways with respect to angiogenesis.
Simultaneous blockade of these signaling pathways by monoclonal antibodies was tested to target tumors in therapies. Based on Alitalo *et al.,* 2005.

ally accepted as crucial for tumor progression and metastasis because it stimulates multiple signaling pathways leading to increased cell survival, proliferation, and motility (Yarden, 2001). EGFRs signaling regulates the expression of several key proangiogenic factors, including VEGF, chemokine (C-X-C motif) ligand 8 (CXCL8), and basic fibroblast growth factor (bFGF) (Goldman *et al.*, 1993), as summarized in Fig. 2. In preclinical models, suppression of EGFR signaling resulted in downregulation of proangiogenic factors and decrease in tumor blood vessel density as well as metastasis (Ellis, 2004). It has been recently demonstrated that EGFR is activated on stromal cells in bevacizumab-resistant tumors and that the combined therapy with bevacizumab and erlotinib (small-molecule EGFR tyrosine kinase inhibitor) may delay tumor adaptation to antiangiogenic treatment (Cascone *et al.*, 2011).

Presently, two therapeutic monoclonal antibodies specific to EGFR, cetuximab and panitumumab, are approved for treatment in patients with cancer. Cetuximab (distributed as Erbitux, marketed by Merck Serono) is a human-murine chimeric monoclonal antibody that binds to the extracellular domain of human EGFR hindering its activation. It was approved by FDA in 2004 for treatment of patients with EGFR-expressing, KRAS wild-type mCRC as well as head and neck cancer (Jonker *et al.*, 2007; Bonner *et al.*, 2010).

In vitro and *in vivo* studies showed that the anticancer activity of cetuximab probably results from direct suppression of EGFR signaling and involves inhibition of cell cycle progression (Wu *et al.*, 1995), angiogenesis and invasion (Perrotte *et al.*, 1999). The Fc fragment of cetuximab (an IgG1 class antibody) may also trigger immune mechanisms such as antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytolysis (CDC). Intravenous administration of cetuximab may result, in some patients, in a severe infusion reaction due to the presence of mouse protein sequences (Patel & Goldberg, 2006). However, anti-EGFR therapy can be also conducted with a fully human antibody, panitumumab (distributed as Vectibix, marketed by Amgen) (Heun & Holen, 2007; Keating, 2010). Both these anti-EGFR mAbs have proven their clinical usefulness. Nevertheless, tumor drug resistance remains a serious clinical problem that strongly affects their therapeutic efficacy. The mechanism of resistance to anti-EGFR therapy is not yet understood but some data indicate a potential role of VEGF in that process. Viloria-Petit *et al.* demonstrated that tumors that developed resistance to cetuximab overexpressed VEGF (Viloria-Petit *et al.*, 2001). Other studies showed increased expression of VEGFR-1 and VEGFR-2 on resistant tumor cells and also confirmed an important role of the VEGF/VEGFR signaling pathway in the development of resistance to the anti-EGFR antibody (Ciardiello *et al.*, 2004; Bianco *et al.*, 2008). Interference and cross-talk between the VEGFR and EGFR signaling axes led to the assumption that they might constitute a very attractive target for combined therapy. Preclinical studies confirmed that simultaneous suppression of EGFR signaling by a monoclonal antibody and inhibition of VEGF secretion by VEGF antisense oligonucleotides prolonged tumor growth inhibition as compared to controls (monoclonal anti-EGFR antibody alone, or VEGF antisense oligonucleotide alone) (Ciardiello *et al.*, 2000). The results of the BOND2 trial, which tested the anticancer activity of cetuximab combined with bevacizumab in patients with advanced mCRC, were also promising (Saltz *et al.*, 2007). Alas, further studies did not confirm clinical benefits of such a strategy: results of the CAIRO2 study (combination of bevacizumab and cetuximab with chemotherapy) and the PACCE study (combination of bevacizumab and panitumumab with chemotherapy) revealed that the combined anti-VEGF and anti-EGFR monoclonal antibody- based treatment merged with chemotherapy resulted in a statistically decreased progression-free survival in patients with advanced colorectal cancer (Hecht *et al.*, 2009; Tol *et al.*, 2009).

To summarize, despite significant progress in understanding the mechanism of angiogenesis and in pro-

duction of different types of antibodies to inhibit this process, success in overcoming angiogenesis-dependent tumor growth and metastasis is a still unattained goal. In the context of tumor metastasis, merging antiangiogenic therapies with strategies targeting another route of cancer dissemination, the lymphatic vessel system, might have favorable outcomes. Inhibition of tumor metastasis through blood vessels, while the lymphatic vessel system is still unimpaired by treatment, may not be enough to prevent cancer dissemination.

ANTIBODY-BASED THERAPIES TO INHIBIT LYMPHANGIOGENESIS

Monoclonal antibodies blocking VEGFR-3

Since VEGF-C and VEGF-D, and their receptors were the first identified factors that drive tumor lymphangiogenesis, the first neutralizing antibodies were directed towards the VEGF-C/VEGF-D/VEGFR-3 signaling axis components. Pytowski *et al.* produced mF4-31C1, an anti-VEGFR-3 mAb that antagonized the binding of VEGF-C to this receptor. Administered into mice, this mAb potently inhibited formation of new lymphatic vessels, without affecting the preexisting ones (Pytowski *et al.*, 2005). Another anti-VEGFR-3 mAb, hF4-3C5, was produced by Persaud *et al.* This mAb binds to VEGFR-3 with high affinity and blocks its interaction with VEGF-C (Persaud *et al.*, 2004). Subsequently, Jimenez *et al.* produced a more sophisticated antibody — a bispecific antibody (diabody), which simultaneously binds to VEGFR-2 and VEGFR-3, and blocks their interaction with VEGF and VEGF-C. In *in vitro* studies, this diabody inhibited both VEGF- and VEGF-C-stimulated migration of endothelial cells. Diabodies directed against two different tumor-associated targets, are supposed to have enhanced therapeutic activity (Jimenez *et al.*, 2005).

All mAbs described above inhibit ligand binding to VEGFR-3. The outcome of this inhibition depends on mAb affinity and on the ratio of concentrations of the VEGF family ligand and the antibody, since the antibody competes with the ligand for receptor binding. High ligand-to-antibody ratio could decrease the blocking efficacy of mAb directed against ligand binding sites in the receptor. To overcome this problem, Tvorogov *et al.* (2010) produced an anti-VEGFR-3 mAb that blocks dimerization of the receptor and thus abrogates signal generation in a ligand concentration-independent manner. Since elevated inhibitory activity and better therapeutic outcome is expected from therapies utilizing combinations of antibodies, this group combined an anti-dimerization antibody with an antibody which blocks ligand binding. In this configuration the inhibitory effect on sprouting, migration and *in vivo* tube formation by microvascular endothelial cells was more pronounced (Tvorogov *et al.*, 2010).

Anti-VEGFR-3 therapies are of great importance due to the fact that, in addition to expression on lymphatic vessels, VEGFR-3 is also expressed on tumor microvasculature in angiogenic sprouts. Antibodies which block the ligand binding site of VEGFR-3 significantly reduce blood vessel density and sprouting. Thus, anti-VEGFR-3 mAbs not only reduce lymphangiogenesis but might also be beneficial in antiangiogenic therapies, especially in combination with anti-VEGFR-2 mAbs (Tammela *et al.*, 2008).

To our knowledge, despite encouraging *in vitro* and *in vivo* results, only one anti-VEGFR-3 antibody, IMC-3C5, produced by ImClone LLC, has entered Phase 1 clinical trials. The results of these studies are supposed to be completed by the end of 2013 (ClinicalTrials.gov identifier: NTC01288989). IMC-3C5 combined with classical chemotherapy demonstrated significant inhibition of tumor growth in animal models of lung and head and neck cancers. The inhibitory effect of combined treatment surpasses that of either agent alone. Thus, IMC-3C5 is supposed to improve today's chemotherapy treatment[3].

Monoclonal antibodies neutralizing VEGF-C and VEGF-D

Neutralization of VEGF-C and VEGF-D is another approach to inhibit lymphangiogenesis through interference with the VEGF-C/VEGF-D/VEGFR-3 signaling axis. As the mature form of VEGF-C can signal through both VEGFR-3 and VEGFR-2 (Plate, 2001), inhibition of VEGF-C may have an impact not only on lymphangiogenesis but on angiogenesis as well. One of the antibodies produced to achieve this goal, an scFv-format anti-VEGF-C antibody binding to an epitope that is important for receptor binding, was generated by Rinderknecht *et al.* (Rinderknecht *et al.*, 2010). Two years later, Kashima *et al.* produced an anti-VEGF-D antibody (cVE199) inhibiting the binding of VEGF-D to VEGFR-3. The inhibitory activity of this antibody was confirmed *in vitro* and *in vivo* on a model of neuroblastoma. cVE199 significantly inhibits both lymphangiogenesis and lymphatic metastasis (Kashima *et al.*, 2012).

Until now, only one anti-VEGF-C antibody has been translated from preclinical studies into a clinical development stage: VGX-100 (produced by Circadian Technologies Limited) entered Phase 1 clinical trials[4] in 2012. VGX-100 combined with bevacizumab and/or chemotherapy significantly decreased the growth of tumors in the animal models of glioblastoma, prostate cancer and pancreatic cancer.

Antibodies binding newly recognized prolymphangiogenic factors[3,4]

With meaningful progress in the field of lymphangiogenesis, more factors stimulating this process have been identified. Since *in vitro* and *in vivo* data confirmed their contribution to lymphatic vessel metastasis, they are now considered potential therapeutic targets. One such newly identified prolymphangiogenic factor is ephrin-B2. Blocking ephrin-B2 with scFv antibody fragments leads to inhibition of endothelial cell migration and tube formation in *in vitro* studies, which is in agreement with the observation that in xenografted mice this approach resulted in reduction in blood and lymphatic vessel density (Abengozar *et al.*, 2012). Another potential target for blocking lymphangiogenesis is Nrp2. Anti-Nrp2 antibody that inhibits the binding of VEGF-C to Nrp2 and blocks the formation of the Nrp2-VEGFR-3 complex significantly suppresses LEC migration as well as tumor-dependent

[3][online] access on 26.03.2013; www.circadian.com.au/sites/default/files/Circadian%20partner%20ImClone%20Systems%20demonstrates%20VEG FR-3%20antibody%20improves%20anti-tumour%20effects%20of%20chemotherapy%20in%20mouse%20tumour%20models.pdf

[4][online], access on 26.03.2013; www.businesswire.com/cgi-bin/mmg.cgi?eid=6250628&lang=en

lymphangiogenesis and lymph node metastasis (Caunt *et al.*, 2008).

Although impressive progress has been made in identifying prolymphangiogenic factors as well as in generation of neutralizing mAbs with evident efficiency in preclinical studies, only two of these products entered Phase 1 clinical trials.

CONCLUSIONS

Although major progress has been made in the vascular biology and theories connecting lymphangiogenesis and angiogenesis with tumor progression and metastasis have been proposed, clinical solutions based on these findings usually show little or no therapeutic effect on cancers when antiangiogenic or antilymphangiogenic factors are used as single agents. Preclinical and clinical studies demonstrate that normalization of tumor blood vessels and inhibition of tumor-induced lymphatic formation with the use of antiangiogenic and antilymphangiogenic therapeutic monoclonal antibodies seems to be a valid direction in cancer treatment when combined with other antitumor agents. This strategy might improve the efficiency of chemotherapy, radiotherapy, and immunotherapy based on the ACT. As the molecular pathogenesis of cancers is a matter of extreme complexity, the changes leading to malignant transformation may involve both (i) extracellular and membrane-associated molecules (such as the growth factors and their receptors), and (ii) intracellular factors and effector molecules responsible for the metastatic properties of tumor cells.

An important issue, resulting directly from the above-described diversity of mechanisms of malignancy, is the problem of proper patient selection for clinical trials. Only patients that, according to the current state of knowledge, can be cured using therapeutics acting on extracellular and membrane-associated targets, should be chosen. This demonstrates the need to find new molecular biomarkers that would ideally give an insight into the pathogenesis of each particular case of the malignant disease and help to assess the chances of recovery, to choose an appropriate therapy, and to monitor changes in the tumor throughout the treatment.

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