# Angiogenesis and the Tumor Vasculature as Antitumor Immune Modulators: The Role of Vascular Endothelial Growth Factor and Endothelin

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**Abstract** Cancer immunotherapies have yielded promising results in recent years, but new approaches must be utilized if more patients are to experience the benefits of these therapies. Angiogenesis and the tumor endothelium confer unique immune privilege to a growing tumor, with significant effects on diverse immunological processes such as hematopoietic cell maturation, antigen presentation, effector T cell differentiation, cytokine production, adhesion, and T cell homing and extravasation. Here, we review the role of angiogenesis and the tumor endothelium on regulation of the antitumor immune response. We place particular emphasis on the role of vascular endothelial growth factor (VEGF) in the suppression of numerous immunological processes that control tumor progression. Further, we

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describe the unique crosstalk between the VEGF and endothelin systems, and how their interactions may shape the antitumor immune response. These insights establish new targets for combinatorial approaches to modify existing cancer immunotherapies.

## Abbreviations

| | |
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| ACT | Adoptive cell transfer |
| APC | Antigen-presenting cell |
| CD | Cluster of differentiation |
| CTLA-4 | Cytotoxic T-lymphocyte antigen-4 |
| DC | Dendritic cell |
| ECE | Endothelin-converting enzymes |
| $EDB^+$ FN | Extra domain-B containing fibronectin |
| ET | Endothelin |
| $ET_AR$ | Endothelin receptor A |
| $ET_BR$ | Endothelin receptor B |
| GM-CSF | Granulocyte macrophage colony stimulating factor |
| GPCR | G protein-coupled receptors |
| HUVEC | Human umbilical vascular endothelial cell |
| ICAM-1 | Intercellular adhesion molecule-1 |
| IFN-γ | Interferon-gamma |
| IL | Interleukin |
| NF-κB | Nuclear factor-kappa B |
| NO | Nitric oxide |
| PD-1 | Programmed death-1 |
| PlGF | Placenta growth factor |
| TGF-β | Transforming growth factor-beta |
| Th | T helper |
| TIL | Tumor-infiltrating lymphocyte |
| TNF-α | Tumor necrosis factor-alpha |
| Treg | Regulatory T cell |
| VCAM-1 | Vascular cell adhesion molecule-1 |
| VEGF | Vascular endothelial growth factor |
| VEGF-R | Vascular endothelial growth factor receptor |

## 1 Introduction

In recent years, cancer immunotherapy has had promising successes, resulting in objective clinical responses in patients with melanoma and other tumors (a historical perspective of cancer immunotherapy has been reviewed in detail by Rosenberg

et al. 2008 and Gattinoni et al. 2006b). Conventionally, investigational approaches have centered on nonspecific immune modulation capitalizing on intrinsic tumor immunogenicity (e.g., therapeutic use of interleukin-2 [IL-2] or blocking antibody against cytotoxic T-lymphocyte antigen-4 [CTLA-4]) (Phan et al. 2003; Rosenberg et al. 1985, 1998), cancer vaccines (using tumor antigens or tumor antigen-pulsed antigen-presenting cells) (Chiang et al. 2010), or adoptive cell therapy (ACT) using expanded, autologous tumor-infiltrating lymphocytes (TILs) (Gattinoni et al. 2006a; Rosenberg et al. 2008). Several of these therapies have yielded substantial results (particularly ACT), while other strategies have been less successful (cancer vaccines), or have produced significant adverse effects (severe autoimmunity in anti-CTLA-4 treated patients) (Phan et al. 2003). Therefore, new combinatorial approaches toward cancer immunotherapy must be considered to improve the clinical outcome for all patients.

Although generating more effective antitumor immune response is extremely pertinent to the success of future immune therapies, a major obstacle impeding the success of cancer immunotherapy is the tumor microenvironment itself. The tumor microenvironment consists of the tumor cells, blood vessels, stromal cells, immune cells, extracellular matrix components, cytokines, and proteases (Hanahan and Weinberg 2000). The tumor microenvironment can impede the success of immune-based therapies through the suppression of homing, extravasation, and effector functions of effector lymphocytes (Witz 2008). In this review, we describe the underappreciated role of tumor angiogenesis, and vascular endothelial growth factor (VEGF) in particular, in modulating the antitumor response. Additionally, we review the crosstalk between VEGF and the endothelin signaling pathways, and its relationship to antitumor immunity.

## 2 Angiogenesis and Cancer

Proposed in 1971 by Judah Folkman (Folkman 1971) as an important mechanism for tumor growth, angiogenesis is now a well-established facet of tumor biology and is key to the progression of cancer. Angiogenesis is important for the supply of oxygen, nutrients, growth factors, and additional survival factors necessary for the cellular function and subsistence of tumors. Angiogenesis is considered a balance between pro- and antiangiogenic forces, and the "switch" to a proangiogenic phenotype is one of the hallmarks of malignant processes involved in cancer (Hanahan and Weinberg 2000). Importantly, increased vascularization and the expression of proangiogenic factors are commonly associated with an advanced tumor stage and a poor prognosis in cancer patients (Dvorak et al. 1995; Hicklin and Ellis 2005).

Angiogenesis is a multistep, complex process that begins with the recruitment of sprouting vessels from the existing vasculature and incorporation of endothelial progenitor cells into the newly developing vascular bed (Hicklin and Ellis 2005; Rafii et al. 2002). Endothelial cells proliferate, migrate, and invade the new area

forming functional tubular structures that mature into fully formed vessels. Although the development and maturation of new vessel growth is multifaceted, requiring the precise and coordinated activation of a multitude of ligands and receptors (e.g., PDGF, Tie-1, Tie-2), the most pivotal regulator in both physiologic and pathologic angiogenesis is the VEGF and VEGF-receptor (VEGF-R) system (Hicklin and Ellis 2005; Rafii et al. 2002). VEGF signaling remains a critical rate-limiting agent in angiogenesis with pleiotropic effects controlling a multitude of angiogenic processes (Ferrara 2004).

VEGF overexpression is associated with tumorigenesis and a poor prognosis in a multitude of cancers, including gastric carcinoma (Maeda et al. 1996), colorectal carcinoma (Lee et al. 2000; Takahashi et al. 1995), lung cancer (Fontanini et al. 1997), melanoma (Gorski et al. 2003), prostate cancer (George et al. 2001), breast (Berns et al. 2003), and ovarian carcinoma (Paley et al. 1997). VEGF is upregulated in cancer cells *in vivo* by hypoxia and starvation (Zhang et al. 2002), and also by oncogene activation, which drives constitutive VEGF overexpression (Zhang et al. 2003b). VEGF directly promotes tumor angiogenesis through multiple mechanisms such as endothelial cell proliferation and survival, endothelial cell migration, vessel destabilization via Tie-2 (Zhang et al. 2003c), and enhancing chemotaxis of bone marrow-derived vascular precursor cells (e.g., endothelial cells, pericytes, vascular leukocytes) (Conejo-Garcia et al. 2004; Ellis and Hicklin 2008). In addition, VEGF promotes tumorigenesis through autocrine signaling, regulating tumor cell functions and driving tumor metastases (Ellis and Hicklin 2008). Important for cancer immunotherapy, VEGF has significant roles in modulation of the immune system and tuning the vascular endothelium, leading to the immune evasion by the tumor.

## 3 Vascular Endothelial Growth Factor

The mammalian VEGF family is comprised of five proteins: VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placenta growth factor (PlGF). The most well-studied family member is VEGF-A (frequently referred to as simply VEGF) (Ellis and Hicklin 2008; Hicklin and Ellis 2005). Alternative splicing of VEGF leads to the expression of multiple functional isoforms of the VEGF protein containing 121, 165, 189, and 206 amino acids. $VEGF_{165}$ is the predominant functional isoform (Ellis and Hicklin 2008; Hicklin and Ellis 2005). The VEGF ligands bind and activate three structurally similar receptors, tyrosine kinases, VEGF-R1 (also referred to as FLT1), VEGF-R2 (or KDR), and VEGF-R3 (or FTL4). The different VEGF ligands have unique binding specificities for each of these receptors, leading to a complex diversity of function following ligation (Ferrara 2004). In addition, neuropilins (NP-1 and -2) act as coreceptors, increasing the binding affinity of VEGF for VEGF-Rs (Soker et al. 1998). It has been proposed that NPs may signal independently of VEGF-Rs, but this has not been definitively demonstrated.

Ligation of the VEGF-Rs initiates multiple signal transduction cascades unique to each individual VEGF-R, and is responsible for activating the appropriate gene

networks (Kowanetz and Ferrara 2006). VEGF-R2 is expressed primarily in the vasculature, and is the key mediator of VEGF-induced angiogenesis. VEGF-R1 is also expressed on the vasculature, and can also be found on other cell types. Although VEGF-R1 has a higher binding affinity relative to VEGF-R2, it induces less activation than VEGF-R2 (Waltenberger et al. 1994). Therefore, VEGF-R1 may act as a functional inhibitor of VEGF-R2 mediated angiogenesis through competitive binding (Hiratsuka et al. 1998). VEGF-R3 primarily binds VEGF-C and -D, and has important roles in cardiovascular development as well as lymphangiogenesis (Ellis and Hicklin 2008; Kukk et al. 1996).

## 4 Direct Effects of VEGF on Leukocytes

### *4.1 Dendritic Cell Defects in Cancer Patients and Mouse Models: A Role for VEGF*

Dendritic cells (DCs) are central to the generation of an antitumor response. As professional antigen-presenting cells (APC), they present tumor antigens to both B cells and T cells, generating an antigen-specific antitumor response. Defective DC function, combined with a failure of DC maturation, is frequently observed in cancer patients and in tumor-bearing mice. These defects occur in DCs found in the blood, tumor tissue, or draining lymph nodes (Almand et al. 2000; Gabrilovich et al. 1996a, b, 1997; Nestle et al. 1997). The effects of defective DC function (i.e., defective antigen presentation) on the antitumor response are somewhat clear; lack of tumor antigen presentation means lack of effective antitumor response or even worse, active tolerance. Indeed, it has been speculated that immature or incompletely matured DCs may mediate tumor tolerance, inducing T cell anergy or the expansion of regulatory T cells (Tregs) (Lutz and Schuler 2002; Mahnke et al. 2002).

The clinical significance of DC dysfunction has been demonstrated in a study of patients with breast, neck/head, and lung cancer (Almand et al. 2000); DCs isolated from cancer patients were functionally impaired in a mixed leukocyte reaction, and this functional impairment corresponded to a more severe cancer diagnosis (higher stage) (Almand et al. 2000). Further, both the percentage and the total number of DCs were significantly reduced in the peripheral blood of cancer patients, and this observation correlated with an increase in the total number of immature hematopoietic cells. The increase of immature cells in the blood was closely correlated to serum VEGF levels, but not transforming growth factor-beta (TGF-β), IL-6, or granulocyte macrophage colony stimulating factor (GM-CSF) (Almand et al. 2000). Importantly, these aberrations in DCs were somewhat corrected following chemotherapy and anti-VEGF therapy (Almand et al. 2000).

DC defects can be induced by tumor-derived TGF-β (Geissmann et al. 1999) and IL-10 (Steinbrink et al. 1999). However, VEGF plays a significant role in the suppression of DC maturation and function. Although DC defects in cancer patients

and tumor-bearing mice had been appreciated for several years prior, Gabrilovich and colleagues were the first to identify a soluble factor, released from tumor cells, that was capable of impairing both DC function and DC maturation from CD34+ hematopoietic precursors (Gabrilovich et al. 1996a). By using neutralizing blocking antibodies, the tumor-derived soluble factor was discovered to be VEGF, and antibodies against IL-10 or TGF-β were unable to reverse the suppression (Gabrilovich et al. 1996a). Similar observations of defective DCs in cancer patients, with a dependence or association with VEGF, have since been made (Della Porta et al. 2005; Takahashi et al. 2004; Yang and Carbone 2004). Experimentally, these finding have been recapitulated in the mouse, suggesting a common mechanism and inherent role for VEGF in the antitumor response. In particular, Ishida and colleagues demonstrated that tumor-bearing mice displayed defects in DC numbers as well as function, and that VEGF blocking antibody reversed these defects (Ishida et al. 1998).

Although several mechanisms may be involved in the generation of DC defects, VEGF can exert its immunosuppressive effects through the disruption of normal hematopoiesis. VEGF continually infused in mice, at levels commonly associated with cancer pathology, resulted not only in defects of DC maturation and function, but also in widespread changes in the differentiation of multiple hematopoietic lineages. For example, VEGF infusion induced a significant increase in B cells and Gr-1+ immature myeloid cells (Della Porta et al. 2005; Gabrilovich et al. 1996a, 1998; Ishida et al. 1998; Ohm and Carbone 2001; Ohm et al. 1999; Takahashi et al. 2004; Yang and Carbone 2004). It has been discovered that VEGF mediates the suppression of DC maturation through the impairment of normal nuclear factor-kappa B (NF-κB) signaling during hematopoiesis (Oyama et al. 1998), mediated through VEGF-R1 signaling on CD34+ hematopoietic progenitor cells (Dikov et al. 2005).

The effects of VEGF on DC maturation and function can be partially reversed through VEGF blockade. Treatment of patients with the VEGF blocking antibody, Bevacizumab, has been shown to partially reverse some of the DC defects. In an initial study by Almand et al., cancer patients receiving anti-VEGF antibody demonstrated a reversal of maturation defects of their DCs, and this observation has also been observed by others (Almand et al. 2000; Fricke et al. 2007; Osada et al. 2008). These observations have also been recapitulated experimentally in mouse tumor models (Gabrilovich et al. 1999; Nair et al. 2003; Roland et al. 2009). Therefore, VEGF blockade may be critical to the success of any cancer immunotherapeutic strategy.

VEGF likely exerts effects on the immune system beyond its role in the suppression of hematopoiesis. B7-H1 is expressed on tumor cells, but it is also highly expressed on tumor-associated myeloid DCs in ovarian cancer patients (Curiel et al. 2003). Interestingly, incubation of blood myeloid DCs with VEGF induced robust expression of B7-H1 on the cell surface (Curiel et al. 2003). B7-H1 is a cell surface protein belonging to the B7 family of costimulatory molecules. B7-H1 may inhibit T cell growth by ligation of the programmed death-1 (PD-1) receptor, as well as promote programmed cell death of effector T cells through

an unknown mechanism (Curiel et al. 2003). Therefore, expression of B7-H1 is associated with suppression of T cell effector functions. Thus, VEGF has potential roles in multiple aspects of immunosuppression mediated through DCs.

## *4.2 Effects of VEGF on T Cells*

In the context of cancer immunotherapy, T cells have a well-appreciated role in the antitumor response, and cancer immunotherapies rely on the use of autologous, tumor-reactive T cells to mediate tumor regression (Rosenberg et al. 2008). In ovarian cancer, our lab has demonstrated that the presence of intratumoral T cells (also called intraepithelial T cells) was significantly associated with an increase in the five-year overall survival rate (Zhang et al. 2003a). Specifically, the five-year overall survival rate was 38% for patients with intratumoral T cells, and only 4.5% in patients whose tumor islets contained no T cells (Zhang et al. 2003a). This observation is not unique to ovarian cancer as the infiltration of T cells into tumors has been associated with positive clinical outcomes in breast (Marrogi et al. 1997), prostate (Vesalainen et al. 1994), esophageal (Schumacher et al. 2001), and colorectal cancers (Naito et al. 1998). The effects of VEGF extend to many cell types in the hematopoietic system, and are not exclusive to DCs (Gabrilovich et al. 1998; Huang et al. 2007). VEGF-Rs are expressed on many additional cell types, notably T cells. Interestingly, we observed that ovarian tumors expressing high levels of VEGF were rarely associated with intratumoral T cells (Zhang et al. 2003a). Whether this observation is mediated by VEGF through direct or indirect action on T cells remains to be determined.

Thymic atrophy is a common characteristic of cancer patients (Ohm et al. 2003). Although most cancer patients tend to be older, premature thymic atrophy occurs in many childhood cancers, which is partially reversible upon treatment (Ohm et al. 2003). Further, thymic involution occurs in tumor-bearing mice, suggesting a common mechanism (Ohm et al. 2003). In addition to negative effects on DC maturation, VEGF is also believed to suppress proper T cell development (Huang et al. 2007; Ohm et al. 2003). Treatment of mice with pathologic levels of VEGF, comparable to that seen in cancer patients, induced a robust thymic atrophy, and a significant reduction in CD4+ and CD8+ T cells (Ohm et al. 2003). Further, VEGF blockade in tumor-bearing mice partially reversed the thymic atrophy (Ohm et al. 2003). The immunosuppressive effects of VEGF on T cells occurred on bone marrow precursors, as VEGF did not appreciably disrupt maturation of T cells already in the thymus (Ohm et al. 2003). These effects likely occur through VEGF-R2 signaling on bone marrow precursor cells (Huang et al. 2007). Although pathologic levels of VEGF clearly influence the proper development of T cells, the relevance of these findings and their impact on the antitumor response remain undefined.

Tregs control peripheral tolerance through the suppression of autoreactivity, but are believed to also suppress antitumor immunity. CD4+CD25+Foxp3+ Tregs isolated from tumors were recently demonstrated to suppress tumor-specific T cell immunity both *in vitro* and *in vivo*, and importantly, an accumulation of tumor Tregs was associated with reduced survival and a high death hazard (Curiel et al. 2004). However, the precise mechanisms controlling the activation and accumulation of Tregs into tumors remain poorly defined. NP-1, a coreceptor that interacts with VEGF-R1 and -R2, has been detected on CD4+CD25+ Tregs (Sarris et al. 2008). Enhanced activation of NP-1 increased CD4+CD25+ Treg interactions with DCs in preference to T helper (Th) cells (Sarris et al. 2008). Although not specifically demonstrated, enhanced VEGF signaling, in conjunction with NP-1, may enhance Treg activation, creating a tolerogenic environment and tumor evasion. Additionally, VEGF treatment of mouse splenocytes during T cell stimulation has been demonstrated to induce IL-10 production from T cells while suppressing IFN-γ production (Shin et al. 2009). This immunosuppressive effect was attributed to VEGF-R1 expressed on T cells (Shin et al. 2009). Therefore, although it remains to be specifically demonstrated, direct VEGF signaling on T cells may enhance T cell regulatory functions, contributing to an immunosuppressive environment.

In contrast to the observations above, it has been suggested that direct VEGF signaling on T cells may enhance T cell functions (Mor et al. 2004). Coincubation with VEGF of concanavalin A or antigen-stimulated T cells supported Th1 differentiation, enhanced IFN-γ production, and suppressed IL-10 production (Mor et al. 2004). Further, VEGF treatment of T cells during peptide stimulation enhanced the severity of an adoptive transfer model of experimental allergic encephalomyelitis (Mor et al. 2004). In addition, both VEGF-R1 and -R2 were expressed in memory phenotype CD4+CD45RO+ cells in human T cells, but not naïve cells (Basu et al. 2010). VEGF treatment of these cells activated the MAPK and the PI3K-Akt pathways and enhanced IFN-γ production. Further, VEGF was chemotactic for the CD4+CD45RO+ T cells (Basu et al. 2010).

Clearly, the direct effects of VEGF on T cell functions remain inconclusive. However, insights into the roles of VEGF on the T cell antitumor response, either direct or indirect, can be gleaned from studies using VEGF blocking antibodies. In one single-arm clinical trial of a tumor vaccine combined with anti-VEGF therapy (Bevacizumab), it has been shown that the combination is associated with a high rate of T cell specific immune response, characterized by increased IFN-γ levels and T cell proliferation following stimulation with antigen (Rini et al. 2006). Supporting this observation, VEGF-R2 blockade in mice using an anti-VEGF-R2 antibody has been demonstrated to induce a de novo T cell-mediated antitumor response in mice (Manning et al. 2007). VEGF-R2 blockade resulted in spontaneous infiltration of CD4+ and CD8+ T cells that produced IFN-γ, and VEGF-R2 blockade protected against subsequent tumor challenge in a tumor vaccine model (Manning et al. 2007). However, VEGF-R2 blockade resulted in a substantial increase in serum VEGF levels. Therefore, it is unknown whether the antitumor T cell response was generated through blockade of tumorigenic angiogenesis, or increased serum VEGF enhanced activation of T cells through VEGF-R1 signaling.

On the other hand, consistent with a role for VEGF signaling in CD4+CD25+ Tregs, VEGF-R2 blockade in this study enhanced T cell effector functions in a tolerized mouse tumor model system (Manning et al. 2007). This observation is supported by the demonstration that anti-VEGF treatment in mice reduced the number of Tregs, decreased Foxp3 expression, enhanced cytotoxic lymphocyte (CTL) induction, and increased tumor vaccine efficacy (Li et al. 2006). In conclusion, VEGF or VEGF-R blockade predominantly enhances T cell antitumor immunity, an effect most consistent with the concept that VEGF has direct immunosuppressive functions on T cells.

## 5 VEGF, the Tumor Vascular Endothelium, and Immune Evasion

### *5.1 The Vascular Endothelium*

The tumor vascular endothelium presents a significant challenge to the success of immune therapy, as it provides a physical barrier through which tumor-reactive T cells must extravasate, recognize tumors, and exert their cytotoxic effects. The vascular endothelial barrier, frequently prohibitive to tumor-reactive T cells, is maintained by locally expressed cytokines, growth factors, and the nature and quantity of adhesion molecules expressed by the endothelium (Zitvogel et al. 2006). In many of the T cell immune therapies that have been conducted, it has been noted that while activated T cells could be found in the periphery, they often failed to infiltrate the tumor itself (Boon et al. 2006; Dudley et al. 2002; Lurquin et al. 2005). Thus, successful transmigration through the tumor endothelial barrier is required for activated or administered lymphocytes to execute their effector functions, resulting in tumor regression. Precisely how the tumor vasculature establishes immune privilege is not well known, but the ongoing processes of angiogenesis may participate in immune escape. Specifically, tumor-derived VEGF may play a pivotal role in reducing leukocyte homing to and extravasation through the vascular endothelium.

### *5.2 VEGF and Adhesion Molecule Expression*

T cells extravasate through the endothelium to the tumor in a multistep process that includes binding to adhesion molecules expressed on endothelial cells, and is followed by diapedesis. VEGF has been demonstrated to increase the expression of many endothelial cell adhesion molecules (CAMs), particularly in the context of angiogenesis (reviewed in detail by Francavilla et al. 2009). In agreement with this observation, VEGF-induced enhancement of CAM expression has been associated

with increased leukocyte adhesion both *in vitro* and *in vivo* (Detmar et al. 1998; Min et al. 2005). However, understanding the role of VEGF in leukocyte adhesion is complicated by reports that demonstrate VEGF may actually inhibit adhesion molecule expression on endothelial cells (Bouzin et al. 2007; Detmar et al. 1998; Dirkx et al. 2003; Griffioen et al. 1996a,b; Min et al. 2005).

Although the role of VEGF signaling and leukocyte adhesion may be difficult to discern, in the context of a proinflammatory environment the emerging concept is that angiogenic growth factors impair immune cell adhesion (Bouzin et al. 2007; Griffioen et al. 1996a, b). For example, Griffioen and colleagues demonstrated reduced expression of adhesion molecules intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule (VCAM-1) after treatment of tumor necrosis factor-alpha (TNF-α) stimulated HUVEC with basic fibroblast growth factor (bFGF) or VEGF (Griffioen et al. 1996a). In a similar manner, Bouzin and colleagues observed reductions in ICAM-1 and VCAM-1 expression in TNF-α stimulated HUVECs as early as 2 h after VEGF addition (Bouzin et al. 2007). Although these effects on adhesion molecule expression were transient, longer treatment times demonstrated a disruption of adhesion molecule organization and clustering on the cell surface (Bouzin et al. 2007). This response was associated with a perturbation of the spatial organization and clustering of ICAM-1, and was dependent on caveolin-1 and nitric oxide (Bouzin et al. 2007).

## 6 The Tumor Endothelium and VEGF Crosstalk: A Role for the Endothelin System

### *6.1 The Endothelin System*

Members of the endothelin system have been identified in a broad array of tissue types, including neuronal, renal, and vascular tissues, and regulate a number of critical physiological processes including reproduction, embryonic development, and cardiovascular homeostasis (Grant et al. 2003; Kedzierski and Yanagisawa 2001; Meidan and Levy 2007; Yanagisawa et al. 1998). The endothelin system has well-known roles in regulating vasoconstriction and mediates both cardiovascular and renal disorders (Bagnato and Rosano 2008; Nelson et al. 2003). Particularly, the endothelin system is an important regulator of physiologic and pathogenic angiogenesis, and VEGF signaling is intimately involved in dynamic crosstalk with the endothelin system (Bagnato and Rosano 2008; Nelson et al. 2003).

The endothelin system is comprised of four endothelin (ET) peptide ligands, ET-1, ET-2, ET-3, and ET-4 (Saida et al. 1989; Yanagisawa and Masaki 1989) that signal through their two G protein-coupled receptors (GPCR), $ET_AR$ and $ET_BR$ (Frommer and Muller-Ladner 2008; Meidan and Levy 2007). Biologically active ETs are derived from precursor proteins following cleavage by membrane-bound metalloproteinases termed endothelin-converting enzymes (ECE) (Valdenaire et al.

1995). Amongst the four endothelin ligands, ET-1 is the most potent ligand and is widely expressed in multiple cells types, notably endothelial cells (Luscher and Barton 2000). Binding of the $ET_AR$ and $ET_BR$ by ET peptides triggers downstream signal transduction pathways, including, but not limited to, the RAF/MEK/MAPK pathway and PI3K/AKT pathway (Nelson et al. 2003).

The endothelin axis has been speculated to play significant roles in tumorigenesis. Endothelin or the endothelin receptors or both are upregulated in a number of cancers including ovarian, breast, renal, colon, and prostate cancer (Bagnato and Rosano 2008; Nelson et al. 2003). Importantly, the use of specific endothelin receptor antagonists has been demonstrated to slow tumor growth in patients, or prevent tumor growth in mouse models (Bagnato and Rosano 2008; Nelson et al. 2003). In addition to its role in angiogenesis described in more detail below, the endothelin axis is believed to activate autocrine/paracrine loops that promote proliferation, protection from apoptosis, immune evasion, vasculogenesis, and invasion and metastatic dissemination of tumors (Bagnato and Rosano 2008; Nelson et al. 2003).

## *6.2 Endothelin and Tumor Angiogenesis*

The interactions between endothelin and VEGF regulate multiple aspects of angiogenesis including endothelial cell proliferation, migration, invasion, vessel formation, and neovascularization (Nelson et al. 2003). Further, endothelin and VEGF signaling influence the regulation of vascular permeability (Nelson et al. 2003). In the context of angiogenesis, ET-1 upregulates the expression of the extra domain-B containing fibronectin ($EDB^+$ FN) in human vascular endothelial cells (Bagnato and Spinella 2003; Khan et al. 2005). $EDB^+$ FN has been suggested as a marker of angiogenesis in human cancers and is believed to control ocular neovascularization in patients with proliferative diabetic retinopathy (Bagnato and Spinella 2003; Khan et al. 2005). Additionally, the expression of endothelins, or their receptors, correlates with high expression of VEGF in a multitude of tumor types (Boldrini et al. 2006; Salani et al. 2000a; Wulfing et al. 2004), and elevated expression of ET-1 and VEGF was associated with lymphatic vessel invasion and poor outcomes in invasive ductal breast carcinoma (Gasparini et al. 1994).

ET-1 induces the expression of VEGF in cancer cell lines *in vitro* (Rosano et al. 2003; Salani et al. 2000b; Spinella et al. 2002, 2007). ET-1 increases VEGF production through HIF-1α (Salani et al. 2000b) by ovarian cancer cells via $ET_AR$ activation (Spinella et al. 2004). Additionally, ovarian tumor growth in nude mice was inhibited after treatment with the $ET_AR$-selective antagonist ABT-627, an effect associated with reduced VEGF expression (Spinella et al. 2004). $ET_BR$ activation counters ET-1/$ET_AR$ activity by increasing production of nitric oxide, promoting ET-1 clearance, triggering apoptotic pathways, and blocking cell growth. However, it is unclear whether this antagonism occurs in tumor

cells (Lalich et al. 2007). As such, there may also be role for $ET_BR$ in tumor angiogenesis and cancer development (Bagnato and Rosano 2008). ET-1 has been shown to directly promote tumor angiogenesis by inducing endothelial cell survival, proliferation, and invasion in an $ET_BR$-dependent manner (Salani et al. 2000b). $ET_BR$ may promote angiogenesis indirectly by upregulating VEGF production in the vasculature (Jesmin et al. 2006). Furthermore, there is a strong correlation between $ET_BR$ and VEGF expression in a number of different tumor specimens (Kato et al. 2001). In summary, the interaction of the endothelin system and angiogenesis, and VEGF in particular, may be a significant regulator of tumorigenesis.

## 6.3 *$ET_BR$ and the Tumor Endothelial Barrier to T Cell Homing*

$ET_BR$ is overexpressed in melanoma and is associated with aggressive tumor phenotype (Bachmann-Brandt et al. 2000). Highlighting the role of $ET_BR$ in melanoma, the receptor antagonist BQ-788 inhibited the growth of human melanoma cell lines and reduced human melanoma tumor growth in a nude mouse model (Lahav 2005; Lahav et al. 1999). $ET_BR$ is also overexpressed in ovarian cancer, Kaposi's sarcoma, glioblastoma, and breast cancer (Bagnato et al. 2004; Egidy et al. 2000; Kefford et al. 2007; Rosano 2003). Interestingly, $ET_BR$ upregulation predicts poor outcome in both breast and ovarian cancers (Grimshaw et al. 2004; Wulfing et al. 2003), and $ET_BR$ overexpression has even been proposed as tumor progression marker (Demunter et al. 2001).

Our laboratory has recently demonstrated a novel role for $ET_BR$ in tumor immunotherapy (Buckanovich et al. 2008). Microarray analysis was conducted using the endothelial cells isolated using laser capture microdissection. $ET_BR$ was discovered as one of the few genes overexpressed in the endothelial cells of tumors lacking TILs (Buckanovich et al. 2008). Immunohistochemical staining of ovarian cancer tumors confirmed this result, and $ET_BR$ was localized to the endothelium and the stroma. Importantly, $ET_BR$ overexpression was associated with poor survival, likely due to lack of TILs, which was previously demonstrated as an indicator of a good prognosis (Zhang 2003). Further, recombinant human ET-1 blocked the adhesion of activated T cells to human umbilical vein endothelial cells (HUVECs) *in vitro* (Buckanovich et al. 2008). This effect was reversed if HUVECs were treated with the specific $ET_BR$ antagonist, BQ-788. ET-1 signaling through $ET_BR$ was discovered to block T cell adhesion to the endothelium through suppression of ICAM-1 expression. $ET_BR$ blockade upregulated ICAM-1, promoted ICAM-1 clustering, and restored T cell adhesion (Buckanovich et al. 2008). Thus, these data provide a mechanistic link between the observations made in ovarian cancer patients.

TNF-α is a major inflammatory cytokine implicated in carcinogenesis, tumor angiogenesis, and progression; and it is upregulated in ovarian cancer (Merogi et al. 1997). It has been previously reported that the overall TNF-α mRNA levels are

similar in ovarian tumors with or without intraepithelial T cells (Zhang et al. 2003a). This was counterintuitive, as TNF-α is a major factor activating endothelium and promoting adhesion of T cells. It has now been found that ET-1 efficiently blocks adhesion of T cells to endothelial cells even when endothelial cells are activated with TNF-α (Buckanovich et al. 2008). This observation explains the paradox of how tumors may exhibit inflammation yet be prohibitive to T cell infiltration, thus establishing immune privilege even in the face of inflammation.

Based on the above data, the effectiveness of $ET_BR$ blockade, using the receptor antagonist BQ-788, was determined using a tumor vaccine therapy that controls tumor growth very poorly. In this context, the tumor vaccine had little effect on tumor growth, but $ET_BR$ blockade significantly enhanced the antitumor effect by permitting the infiltration of tumor antigen-specific T cells into the tumor site (Buckanovich et al. 2008). The benefits of $ET_BR$ blockade were attenuated with the use of an ICAM-1 neutralizing antibody, indicating that adhesion molecule interactions between the endothelium and T cells were responsible for the antitumor effects of $ET_BR$ blockade (Buckanovich et al. 2008). Thus, in tumors there is likely a hyperactivation of ET-1/$ET_BR$ signaling that is responsible for the suppression of T cell homing. Furthermore, these results establish a vascular mechanism of tumor immune evasion mediated by the endothelium, and also present a new opportunity to target the $ET_BR$ to prevent tumor growth and enhance cancer immunotherapy.

## 7 Concluding Statements

The mechanisms regulating the overexpression of $ET_BR$ on the tumor endothelium are unknown. However, the overexpression of $ET_BR$ may participate in a feed-forward loop of autocrine/paracrine ET-1 production and ET receptor signaling between the tumor and the vascular endothelium. Thus, enhanced ET-1 signaling in tumor and endothelial cells through $ET_BR$ would lead to enhanced NO and HIF-1α production, followed by increased VEGF production by tumor cells. In the context of inflammation, $ET_BR$ and VEGF signaling on endothelial cells would shut down the capacity of T cells to extravasate through the endothelium to attack the tumor through a reduction in adhesion molecule expression, particularly ICAM-1. Further, enhanced VEGF production would support ongoing maturation defects in DCs and possibly T cells, while enhancing Treg activation, leading to reduced antigen presentation and immune evasion.

If this hypothesis is correct, targeted therapies to break the cyclical enhancement of VEGF, ET-1, and NO production should be the key components of any cancer immunotherapy. The use of $ET_BR$ receptor antagonists combined with anti-VEGF antibody administration may function synergistically to sanction the tumor environment to attack by the immune system. Thus, new complimentary approaches to

existing cancer immunotherapies may enhance the existing therapies and extend their benefits to more patients.

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