Preclinical and clinical studies on the co-regulation of tumor-induced angiogenesis and dendritic cell suppression
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Preclinical and clinical studies on the co-regulation of tumor-induced angiogenesis and dendritic cell suppression

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CHAPTER 1

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1. Introduction

A series of stochastic events has been identified to occur in virtually all human solid tumors (1), generally resulting in seven functional hallmarks that cancer cells acquire: the ability to become resistant to inhibitory growth factors, proliferate in the absence of exogenous growth factors, invade and metastasize, achieve limitless replication potential, evade apoptosis, recruit a blood supply through angiogenesis, and evade tumor immune surveillance. Each of these processes is regulated through signal transduction pathways that normally control cellular and tissue homeostasis. Tumorigenesis can be viewed as a disruption of these pathways, through genetic, epigenetic, or somatic alterations. To design new and effective therapies in order to improve the outcome for cancer patients, understanding tumor biology and the interplay between tumor cells and their microenvironment is of utmost importance. This chapter will describe the emerging relationship between angiogenesis and tumor immune escape and its importance in anticancer therapies. Angiogenesis is the formation of new blood vessels from the existing vasculature, and tumor immune escape includes a wide variety of active mechanisms employed by tumors to prevent or frustrate anti-tumor immune responses. Both processes are employed by virtually all solid tumors to initiate and continue tumor progression. Before describing the relationship between angiogenesis and tumor immune escape, these processes will be discussed separately in more detail.

2. Angiogenesis

Solid tumors cannot grow beyond 2-3 mm³ unless an adequate blood supply is provided (2). Tumor hypoxia induces the multi-step process of angiogenesis, which is regulated by several stimulating and inhibitory growth factors (Figure 1). Angiogenesis starts with an increased vascular permeability leading to a temporary fibrinous exudate. Before proliferating endothelial cells will migrate in the direction of specific stimuli, loosening of interendothelial cell contacts and remodeling of the extracellular matrix (ECM) involving many proteinases have to take place. Finally, tube formation occurs resulting in new blood vessel loops, which have to be stabilized. Normal vasculature matures and stabilizes rapidly. Tumor angiogenesis, however, is characterized by an ongoing pro-angiogenic environment, and as a result, tumor-associated vasculature is structurally and functionally abnormal as compared to normal vasculature. Tumor-associated vasculature consists of large and leaky vessels and has a disorganized structure (3). Frequently, pericytes supporting the endothelial cells are detached or absent (4-6). Furthermore, the basement membrane is often too thick or absent (7). These morphological characteristics of tumor vessels contribute to a pronounced heterogeneity, a high vascular permeability, tumor
hypoxia, and a high interstitial pressure (4;8). Hypoxia-inducible transcription factors (HIF-1α, -1β and 2α) expressed by tumor cells trigger a coordinated response of angiogenesis by inducing expression of VEGF, VEGFR1, neuropilin-1, Ang2, nitric oxide synthase, etcetera (9). Metabolic stimuli such as hypoglycaemia and low pH also stimulate vessel growth but their mechanisms remain to be identified (10;11).

Figure 1 – Tumor-induced angiogenesis

2.1 Pro-angiogenic factors
Vascular endothelial growth factor (VEGF) is the most extensively studied stimulator of angiogenesis. Also naturally occurring inhibitors of angiogenesis have been identified, for example endostatin and angiostatin. It has been suggested that angiogenesis only occurs when the balance of the stimulators and the inhibitors is in favor of the stimulators (12). Identification of the factors involved in angiogenesis has led to a new area of drug development. Some of the pro-angiogenic factors and their receptors will be discussed in more detail in this paragraph.

2.1.1 Vascular endothelial growth factors and receptors
VEGF (also referred to as VEGF-A), is a member of a broader family, which also includes VEGF-B, -C, -D, and -E, and placental growth factor (PIGF)-1 and -2 (Figure 2) (13). In addition, alternative exon splicing results in the generation of four VEGF isoforms: VEGF121, VEGF 165, VEGF189, VEGF206 (14), which all have different binding affinities
for heparin. Acidic VEGF121 does not bind heparin and is secreted as a diffusible protein, whereas basic VEGF189 and VEGF206 bind heparin with high affinity and are sequestered to the ECM. VEGF165, the predominant isoform, can be secreted freely, but a considerable portion remains cell- or ECM-bound via heparan sulphate proteoglycans. Plasmin (15) and matrix metalloproteinase (MMP)-9 (16) can release the ECM-bound isoforms, creating the diffusible, bioactive fragment VEGF110.

![Figure 2](image_url)  
**Figure 2** – Family of vascular endothelial growth factors (VEGF) and their receptors

The production of VEGF by tumors is associated with tumoral hypoxia and genetic tumor aberrations, e.g. loss of tumor suppressors like p53, PTEN and Von Hippel Lindau (VHL) (17-20), and mutation or amplification of oncogenes (21). Growth factors such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) can also induce VEGF expression (22;23). Activated tumor-associated cells like immune cells and fibroblasts are also involved (24;25).

Members of the VEGF family exert their effects via three VEGF tyrosine kinase receptors VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1), and VEGFR-3 (Flt-4). VEGFR-1 and -2 are non-exclusively expressed on vascular endothelial cells. VEGFR-3 is present on lymphatic endothelium. All VEGFRs are characterized by seven extracellular immunoglobulin-like domains, of which the second and third are critical for ligand binding, a transmembrane domain and a cytoplasmatic domain, which contains tyrosine kinase residues important for activating the intracellular signaling transduction pathway. The ligands can form anti-parallel homodimers optimizing binding to their preferred receptor and facilitating receptor dimerization (26). The VEGF/VEGFR-2 complex seems to be the most important signaling
route for the activation of endothelial cells. Interestingly, several heterodimers of both receptor and ligands can be formed leading to different signaling properties. In addition, different affinity of the ligands for neuropilins, co-receptors lacking tyrosine kinase activity, can affect signal transduction. Neuropilin (NRP)-1 and -2 potentiate the binding and activity of VEGF165 to VEGFR-2 (27).

Mitogenesis is one of the best documented biological activities of VEGF (28). Furthermore, it induces tube formation in three-dimensional in vitro models and angiogenesis in a variety of in vivo models including the chick chorioallantoic membrane (29). In addition, VEGF has a pro-survival effect on endothelial cells (30) and it increases the vascular permeability (31). Hypotension was a dose-limiting toxicity when VEGF was administered intravenously to humans, indicating a role of VEGF in regulating vascular tone. This might be mediated by endothelial nitric oxide (32). VEGF can also have an effect on bone marrow cells: it induces monocyte chemotaxis (33), it mobilizes hematopoietic stem cells from the bone marrow (34), and it impairs dendritic cell differentiation (35;36). This last effect will be discussed in more detail later in this chapter. Anti-cancer therapies targeting angiogenesis by interfering with VEGFR tyrosine kinase activity are discussed in chapter 2.

2.1.2. Fibroblast growth factors and receptors

The family of fibroblast growth factor (FGF) receptors comprises 4 different receptors, which can bind more than 20 different heparin-binding FGFs with varying affinity (37). Most cells in tissue culture express FGF receptors (FGFR); however, it remains to be established whether the ubiquitous expression in vitro reflects the actual expression in vivo, and which cells express FGFRs under normal or pathological conditions. FGFs are characterized by their strong affinity for heparin and heparan-like glycosaminoglycans. This feature serves several physiologically relevant goals, among which the formation of a local tissue reservoir of growth factors and the increase of the half-life of the FGF-FGFR complex. Binding of FGF leads to homo- or heterodimerization of FGFRs resulting in the phosphorylation of cytoplasmatic tyrosine kinase domains, initiating activation of the intracellular signal transduction pathway.

The prototype members of the FGF-ligands, acidic FGF (FGF-1) and basic FGF (FGF-2), are mostly studied for their angiogenic potential. FGF-1 and FGF-2 can induce endothelial cell proliferation, migration and protease production. Furthermore, in vivo models have clearly established the pro-angiogenic role of FGFs (37). FGFs can exert their effects on endothelial cells via a paracrine mechanism as a result of their release by tumor or stromal cells and/or by their mobilization from the ECM. In addition, FGF may play an autocrine role in endothelial cells.

Several experiments suggested a cross-talk between the pro-angiogenic factors, FGF and VEGF. The FGF-FGFR system can induce angiogenesis indirectly by activation of the VEGF-VEGFR system (38) and blocking of VEGFR-2 actually reduced FGF-2-induced angiogenesis
Both growth factors, however, retain distinct biological effects on endothelial cells during angiogenesis (40).

2.1.3. Platelet-derived growth factors and receptors
Platelet-derived growth factor receptors (PDGFRs) are composed of two chains (α or β) resulting in three distinct receptors (αα, αβ, or ββ) containing tyrosine kinase domains. Some structurally related receptors, such as c-Kit and Fms-like tyrosine kinase (Flt)3, belong to the same family of PDGFRs. The family of PDGF-ligands consists of four polypeptide chains: PDGF-A, -B, -C, and -D. The polypeptide chains are linked with an amino-acid disulfide bond resulting in the homodimers, PDGF-AA, -BB, -CC, and –DD, and the heterodimer PDGF-AB. These dimers bind to α- and β-receptors with different specificities (41). Ligand binding results in receptor dimerization and activation and phosphorylation of the tyrosine kinase domain, which in turn leads to initiation of the downstream signaling cascade. Eventually, activation of the receptors promotes cell migration, proliferation and survival.

Gene knockout studies have shown the importance of PDGFR signaling in angiogenesis and embryonic development. In adult life, PDGF-PDGFR signaling plays a role in blood vessel development and wound healing and it maintains interstitial fluid pressure. PDGF-B is expressed by endothelial cells while PDGFR-β is expressed by pericytes and smooth muscle cells covering the blood vessels, suggesting a role of PDGFR signaling in recruitment of perivascular structures (42). Aberrant expression has been demonstrated in several pathological conditions, including glioblastoma and ovarian cancer. Besides an autocrine role for PDGF in tumor cells, PDGFs can also stimulate tumor progression via angiogenesis. It has been demonstrated that PDGF can stimulate angiogenesis in several in vivo models, like the chick chorioallantoic membrane or the mouse cornea (43), although it has been suggested that this angiogenic effect on endothelial cell is an indirect one (44). Furthermore, knockout mouse models showed that PDGF might play a role in tumor pericyte recruitment required for the development of capillaries (45). In addition, PDGF-expressing B16 melanoma cells induced increased tumor pericyte numbers and tumor growth, but did not increase tumor vessel density suggesting that the increased pericytes influenced the tumor vasculature in a more functional than quantitative manner (46). Inhibition of PDGF signaling confirmed the involvement of PDGF in tumor-associated angiogenesis (47). Treatment with PDGFR inhibitors (imatinib) reduced the pericyte coverage of tumor vessels possibly rendering the endothelial cells more susceptible to metronomic chemotherapy in a preclinical model (48).

2.1.4. Angiopoietins
The angiopoietin (Ang)-Tie ligand-receptor family consists of four ligands, Ang-1, -2, -3, and -4, and two receptors, Tie1 and Tie2 (49). Tie1 and Tie2 are tyrosine kinase receptors
mainly expressed on endothelial cells. Ang-1 and Ang-2 are the best characterized ligands for Tie2, while no specific ligand has been identified yet for Tie1. Ang-1 is constitutively expressed in many different cell types, including some tumor cells. Ang-2, however, is almost exclusively expressed by endothelial cells and it is significantly up-regulated at sites of vascular remodeling, like the female reproductive tract and tumors. Various factors, including VEGF, bFGF, and hypoxia, can induce Ang-2 expression. Both Ang-1 and Ang-2 bind to the same site in the extracellular domain of Tie2 with similar affinities. The Ang-1 binding results in autophosphorylation of the receptor, promoting endothelial cell migration and survival. By contrast, Ang-2 does not result in Tie2 autophosphorylation, suggesting that Ang-2 acts as a natural occurring antagonist (50).

Genetic studies targeting Ang-1, Ang-2, Tie1 or Tie2 have demonstrated the involvement of the Ang-Tie system in vascular remodeling and maturation of vessels. Of note, vasculogenesis proceeded normally in knockout mice. Other studies showed that Ang-1 induced migration, tube formation, sprouting and survival, but not proliferation, in endothelial cells in vitro (49). Ang-2 alone is a vessel destabilizing factor by inducing endothelial cell apoptosis, while Ang-2 in the presence of VEGF de-stabilizes mature vessels, providing the opportunity for proliferation, migration and sprouting of endothelial cells (51). These complementary actions of the Ang-Tie signaling and VEGF-VEGFR signaling can induce angiogenesis in vivo in a synergistic manner (52) and can co-opt existing host vessels in certain tumors (53).

2.1.5. Interleukin-8
As a member of the CXC chemokine family, interleukin (IL)-8 plays an important role in activating and attracting neutrophils and monocytes. IL-8 is produced by normal and malignant cells. IL-8 produced by monocytes has been demonstrated to be involved in angiogenesis (54). Identification of IL-8 receptors on endothelial cells in vitro further supports the role of IL-8 in angiogenesis (55).

While numerous stimuli, like lipopolysaccharides, tumor necrosis factor, hypoxia and acidosis, can induce tumor-cell derived IL-8 production, many tumors constitutively express IL-8. Beside autocrine mitogenic effects on tumor cells, it has been demonstrated that IL-8 is involved in tumor-associated angiogenesis. Tumor-cell derived IL-8 was able to stimulate angiogenesis in various in vitro models (56).

2.1.6. Transforming growth factor-β
In mammals, transforming growth factor beta (TGF-β) exists as 3 isoforms: TGF-β1, -β2, and -β3. Each is encoded by a unique gene and expressed in a tissue-specific and developmentally regulated manner (57). TGF-β1 is the most abundant and widely studied isoform. TGF-β is secreted into the extracellular matrix as a latent protein complex and needs activation for biologic activity. Once activated, the TGF-β ligands exert their effect
via binding to ubiquitously expressed TGF-β type I and type II receptors (TβRI and TβRII) containing serine/threonine kinases in their intracellular domains. In endothelial cells, TGF-β signals via ALK1 or ALK5, both type I receptors. A third type of TGF-β receptors, endoglin and betaglycan, lack the intracellular enzymatic motif. Betaglycan binds TGF-β ligand and presents it to the signaling receptors, TβRI and TβRII. The role of endoglin is less well understood.

Upon heteromeric complex formation between type I and type II receptors, TβRI is transphosphorylated by TβRII. This activation of the TβRI subsequently results in phosphorylation of intracellular effector molecules, Smads, which transduce the TGF-β signal from the membrane bound receptor to the nucleus. TGF-β signaling functions mainly via Smad-dependent pathways, however Smad-independent pathways have also been reported.

Although TGF-β can serve as an anti-angiogenic factor, its function as a pro-angiogenic molecule is more widely accepted. Knockout studies in mice have demonstrated the role of TGF-β signaling in vascular development. Targeted deletion of TGF-β1, TβRII, ALK1, ALK5, endoglin and Smad5 resulted in embryonic lethality due to aberrant vascular development and angiogenesis (58). Furthermore, mutations in TGF-β receptors (endoglin or ALK1) have been linked to the human vascular disorder hereditary hemorrhagic telangiectasia (59;60). In addition, TGF-β1 regulates the expression of matrix metalloproteases 2 and 9, which are both involved in ECM degradation, thereby initiating angiogenesis (61). Alternatively, TGF-β1 stimulates monocyte chemotaxis and the release of pro-angiogenic cytokines that can subsequently induce the activation of endothelial cells. TGF-β1 has also been linked to tumor-associated angiogenesis: endoglin expression on endothelial cells is highly upregulated during tumor-associated angiogenesis (62) and neutralizing antibodies against TGF-β1 has been shown to inhibit tumor-associated angiogenesis (63).

2.1.7. Gangliosides

Gangliosides are ubiquitous, membrane-associated glycosphingolipids containing at least one sialic acid. Beside regulatory roles in normal physiological processes, gangliosides have been implicated in tumor development and progression (64). The composition and production of gangliosides is altered in many tumor cells and tumors can shed gangliosides in the tumor-microenvironment. It has been reported that gangliosides are involved in tumor-associated angiogenesis. Small amounts of the monosialic ganglioside GM3 relative to the di-sialic ganglioside GD3, have been demonstrated to stimulate angiogenesis (65;66) and GM3 was the only investigated ganglioside found not to increase endothelial cell responsiveness to VEGF (67). Although these observations suggest an anti-angiogenic role for GM3, the opposite has also been demonstrated: GM3 synergistically increased basic FGF-induced proliferation of bovine aortic endothelial cells (68).
2.2. Inhibiting angiogenesis as an anti-cancer strategy

In normal adult life, angiogenesis occurs mainly during wound healing, in the female reproductive cycle and during ischemia. Therefore, targeting tumor-associated angiogenesis is expected to be a promising anti-cancer therapy with limited toxicity in adults. Anti-angiogenic therapy should selectively affect the immature, disorganized tumor vasculature. The remaining tumor vasculature functions more effectively with a better perfusion (3;4). The incapability of tumors to form new vessels during anti-angiogenic therapy, limits tumor cell proliferation.

Among the redundancy of pro-angiogenic growth factors and their receptors, VEGF is probably the most important factor sustaining angiogenesis, and therefore the most promising target. Proof-of-principle was provided by xenograft mouse models, in which neutralizing VEGF-antibodies or VEGFR interference substantially inhibited tumor growth and even induced tumor regression (69-71). These early findings were confirmed and extended in a variety of animal models. Bevacizumab, a humanized monoclonal antibody directed against VEGF, was the first anti-angiogenic agent to be registered for the treatment of advanced colorectal cancer patients (72). Alternatively, via inhibition of the VEGFR kinase activity, the VEGFR pathway can be hampered. Chapter 2 focuses on clinical issues regarding the use of VEGFR tyrosine kinase inhibitors (TKIs), which are low-molecular weight, ATP-mimetic molecules that bind to the intracellular site of the tyrosine kinase domain of VEGFRs, resulting in blockade of the intracellular pathway.

3. Tumor immune escape

According to the cancer immuno-editing theory, the immune system protects the host from tumor development, but also selects for tumor variants which can develop into clinically apparent tumors (73). Tumors employ several strategies to frustrate immune responses, defined as tumor immune escape, and thereby facilitate tumor progression and hinder effective tumor immunotherapies (74). Tumor-induced defects in the host immune system can be found at the effector level of the anti-tumor immune response (mediated by cytotoxic T cells and natural killer cells) or may involve suppression by regulatory T cells. The next paragraph focuses on another important component of anti-tumor immune responses: antigen presentation by dendritic cells (DCs). DCs, the most potent antigen-presenting cells, play a central role in the immune system and are essential in generating a proper anti-tumor immune response, but they often fail to do so, since tumors are known to progress and metastasize. After a discussion of normal DC development and functions, the mechanisms by which tumors hamper DC differentiation and function are highlighted below.
3.1 Normal dendritic cell differentiation

DCs are generated in the bone marrow from hematopoietic progenitor cells (HPCs) under the control of a complex network of soluble growth factors that are produced by bone marrow stroma, and through direct cell-cell contact with bone marrow stromal cells. Several soluble factors, such as granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-3, stem-cell factor and fms-related tyrosine kinase 3 ligand (FLT3L), have been directly implicated in DC precursor expansion and DC differentiation (Figure 3) (75). HPCs (CD34+ FLT3+ KIT+ M-CSFR+) can differentiate via the myeloid lineage pathway into common-myeloid progenitors and immature myeloid cells (ImCs), which eventually give rise to granulocytes, macrophages and conventional myeloid DCs (MDCs). Although previously thought to derive from a lymphoid precursor, plasmacytoid DCs (PDCs) can also derive from a common myeloid precursor (76). PDCs express lymphoid antigens and produce large amounts of interferon (IFN)-α in response to viruses. They are less able to process and present antigens and to stimulate T cells than MDCs. In humans, MDCs are
characterized by the expression of CD11c and they lack the expression of lineage-specific markers, whereas PDC do not express CD11c, but express the receptor for IL-3 (CD123) and (at least in the immature, resting state) the C-type Lectin CD303/BDCA-2 (Figure 4). For survival in vitro, MDCs are dependent on GM-CSF, while PDC are dependent on IL-3 and Interferon (IFN)-α (77).

Figure 4 – Differentiation of human myeloid and plasmacytoid DC subsets

After leaving the bone marrow, DCs are still in an immature state: they have little or no expression of MHC class II and co-stimulatory molecules, such as CD40, CD80 and CD86, and they produce little or no IL-12, which is required to support T cell proliferation and activation. These immature DCs have a high capacity to capture and internalize antigens. In tumor tissues, antigens might derive from dying tumor cells and might be taken up by immature DCs. Appropriate activation (e.g. by endogenous pro-inflammatory or “danger” signals) results in up-regulation of MHC class II and several co-stimulatory molecules, increased production of IL-12 and loss of the ability to process other antigens. After migration towards the lymphoid tissues, these activated, mature DCs will present tumor
antigen-derived epitopes in the context of MHC class I and II molecules to CD8\(^+\) and CD4\(^+\) T cells, respectively. Only DCs can stimulate naive T cells, and they are also the most potent cells for the activation of secondary T cell responses.

### 3.2 Abnormal dendritic cell differentiation in cancer

In the blood of cancer patients, decreased numbers of DCs are present as compared to the blood of healthy donors. Both PDCs and MDCs can be affected, although some studies have reported that only the myeloid subset is affected (78-81). Tumor removal has been reported to result in a restoration of the numbers of blood DCs. Reduced number of DCs were also observed in tumor tissue and tumor-draining lymph nodes. Additionally, these tumor-associated DCs were minimally activated and had a low expression of co-stimulatory molecules CD80 and CD86 (82). If these minimally activated DCs do not provide a proper co-stimulatory signal to T cells, this might lead to T cell anergy or tolerance instead of anti-tumor immune responsiveness (83). Concomitant with the reduced number of DCs, the population of immature myeloid cells (ImCs) can be increased in the blood of cancer patients, consisting of early stage myeloid cells, immature monocytes and DCs (84). ImCs have been found to suppress antigen-specific CD8\(^+\) T cells in cancer, mediated by the production of reactive oxygen species, particularly H\(_2\)O\(_2\) (85-87). Recently, another myeloid cell population, myeloid suppressor cells (MSC), with immunosuppressive effects has been found to be increased in cancer patients (78;88). MSC are defined as CD14\(^+\)HLA-DR\(^{\text{neg/low}}\) cells and are thought to exert their T cell immunosuppressive effects via TGF-\(\beta\) production (88;89).

### 3.3 Tumor-derived factors implicated in defective dendritic cell differentiation

The tumor-induced effects on DC differentiation are systemic, indicating that the effects are mediated by soluble factors. Several years of research could not appoint a single factor, and most likely, an interplay between several factors causes the observed DC suppressive effects. Furthermore, in diverse tumor types, different factors might play a role. The more intensively investigated DC suppressive factors will be discussed in more detail in this paragraph. Of note, the overlap with pro-angiogenic factors may be appreciated.

#### 3.3.1. Vascular endothelial growth factor

The involvement of VEGF in tumor-induced defects in DC differentiation was indicated by \textit{in vitro} experiments in which neutralizing VEGF-specific antibodies abrogated the negative effect of tumor-cell conditioned medium on the differentiation of DC from HPCs (36). Subsequent \textit{in vivo} experiments confirmed the role of VEGF in DC differentiation: recombinant VEGF administration in mice resulted in inhibition of DC differentiation, which
was consistent with an increase in Gr1+ ImCs (35). In addition, the stimulatory effects of FLT3L on DC production was abrogated when VEGF was co-administered (90) and tumor-bearing mice showed improved DC differentiation when antibodies neutralizing the effects of VEGF were administered (91). Correlative data obtained in humans have supported the role of VEGF in defective DC differentiation in cancer: expression of VEGF has been found to negatively correlate with DC frequencies in tumor tissue (92;93) and in peripheral blood of cancer patients (84;94).

3.3.2. Interleukin-6 and macrophage-colony stimulating factor

IL-6 and macrophage-colony stimulating factor (M-CSF) have also been described to mediate tumor-induced inhibition of DC differentiation. Renal cell cancer cell lines were found to release large amounts of IL-6 and M-CSF that inhibited the differentiation of CD34+ precursors into DCs, but induced the production of monocyctic cells (95). Antibodies neutralizing the effects of IL-6 and M-CSF could abrogate the inhibitory effect of renal cell cancer cell lines on DC differentiation (95). Park et al demonstrated in vivo that IL-6 suppresses DC maturation (96). Human data confirmed the role of IL-6 in DC suppression. Defective DC functioning in multiple myeloma patients was shown to be partially due to IL-6 (97). In chapter 6 we describe the correlation between high serum IL-6 levels and reduced rates of peripheral blood DCs in renal cell cancer patients.

3.3.3. Interleukin-10

The inhibitory effect of IL-10 on DC differentiation has been well recognized. IL-10 has been shown to block the differentiation from monocytes into DCs, but it promotes their maturation into macrophages (98;99). Furthermore, functional activities of Langerhans cells, which are professional antigen-presenting cells localized in the epidermis, and of DC differentiated from CD14+ and CD34+ precursors were inhibited by IL-10 (100-102). In tumor-bearing mice, tumor-induced IL-10 appeared to be responsible for suppressing the function of activated DCs. Additional evidence was provided by the improvement of splenic DC function in tumor-bearing IL-10 deficient mice (103). Besides tumor cells, tumor-associated lymphocytes can produce IL-10 (104) with a subsequent negative effect on DC differentiation. Increased circulating levels of IL-10 have been reported in cancer patients as compared to healthy donors (105;106). However, no correlation was observed between serum IL-10 levels in cancer patients and defects in DC differentiation (84). One should keep in mind that a very short half-life of some cytokines, such as IL-10, might be responsible for the observed lack of such a correlation.

3.3.4. Transforming growth factor-β

TGF-β knockout mice suffer from a lethal inflammatory disease that demonstrates the importance of TGF-β in immune homeostasis (107). TGF-β has a broad inhibitory effect on
several immune cells and its role in controlling T cell function has been widely studied (108;109). In addition to its inhibitory effects on the proliferation and the function of conventional T cells, TGF-β further contributes to immunosuppression by generation of T regulatory cells (Tregs). Tregs are suppressive T cells that are frequently observed at higher frequencies in the peripheral blood, lymph nodes and tumors of cancer patients (110). TGF-β inhibits the up-regulation of MHC class II molecules and co-stimulatory molecules, and of IL-12 production in lipopolysaccharide (LPS)-stimulated DCs (111). Beside tumor cells, several other tumor-associated cell types, including Tregs and stromal cells, can produce TGF-β. DCs are affected by Treg-produced TGF-β and IL-10, demonstrated by markedly down-regulated expression of co-stimulatory molecules CD40, CD80, and CD86. These DCs also displayed markedly decreased release of tumor necrosis factor (TNF)-α, IL-12, and CCL5/RANTES, a chemokine important for the attraction of effector T cells (112).

Elevated levels of TGF-β correlate with advanced disease stage and poor clinical outcome of cancer patients (113;114). However, to date no correlation between blood TGF-β levels and decreased DC frequencies has been observed.

3.3.5. Gangliosides
Tumor-derived gangliosides are potent modulators of myeloid cell proliferation and differentiation into antigen-presenting cells (115). The ganglioside GM3 has been described to impair differentiation and function of both CD34+ and CD14+-derived DCs (115). When purified from melanoma, GM3 and GD3 gangliosides also inhibited phenotypic and functional differentiation of DCs from blood-derived monocytes in a dose-dependent manner (116). Furthermore, these gangliosides induced DC apoptosis (116).

3.3.6. Prostaglandins
Prostaglandins are endogenous immunomodulators, which are normally secreted in the course of immune responses by many cell types, including macrophages and DCs. Prostaglandin E2 is one of the best characterized prostaglandins in relation to immune modulation. It can exert both pro- and anti-inflammatory effects, particularly on DCs, depending on the nature of maturation signals (117). Both endogenous and exogenous prostaglandin E2 might play a role during the immune response via modulation of IL-12 production by DCs (118).

It has been demonstrated that prostaglandins (mainly prostaglandin E2, PGE2), are involved in tumor-induced immune suppression. Over-expression of cyclo-oxygenase-2 (COX-2), an enzyme involved in prostaglandin production, is a common feature in human lung, colon, breast and prostate cancers (119;120). Primary colon carcinoma-conditioned media hampered the differentiation of CD14+ and CD34+ precursors more effectively than conditioned media of established colon carcinoma cell lines due to PGE2 produced by
primary colon carcinoma, but not by colon carcinoma cell lines (121). The DC inhibitory effects of these primary tumor conditioned media were abrogated when the conditioned media were prepared in the presence of indomethacin, a COX-1/-2 inhibitor (121).

3.4 Mechanisms of abnormal DC differentiation: Signal transducer and activator of transcription-3 and NF-κB

Although tumor-derived factors with known inhibitory activities on DC differentiation, bind to different cellular receptors, their effects may converge at the level of intracellular signal transduction. One possible candidate pathway is the Signaling Transduction and Activator of Transcription (STAT)3-pathway, down-stream of the Janus-activated kinase-(JAK)2 protein (Figure 5) (122). The STAT-family comprises seven members: STAT1 to 4, STAT5a, STAT5b, and STAT6 (123).

![Figure 5 – The JAK2/STAT3 signal transduction pathway](image)

Normally, STAT proteins transmit cytoplasmic signals from polypeptide cytokines or growth factors that have receptors with intrinsic or associated tyrosine kinase activity. Following ligand binding, STAT proteins become phosphorylated, which leads to homodimerization of STAT and subsequent translocation into the nucleus, where they modulate the expression of target genes. Persistent STAT activation has been identified in many human tumors (124). In particular STAT3 and STAT5 have been implicated in tumor cell proliferation,
apoptosis-resistance, sustained angiogenesis and tumor immune escape. Besides its expression in tumor cells, JAK2/STAT3 activity is required in early hematopoietic cell differentiation because this is one of the main pathways used by cytokines that support myeloid cell differentiation (125). During normal DC differentiation, however, it has been demonstrated that STAT3 activity is decreased (122). This decrease in STAT3 activity of DCs was prevented when precursor cells were differentiated in tumor-conditioned media. Inhibition of STAT3 activity abrogated the suppressive effects of tumor-conditioned medium on DC differentiation (122;126;127).

VEGF is the pro-angiogenic factor most extensively studied for its role in immunosuppression. Research into the mechanism by which VEGF exerts its effects on DC, has principally targeted the transcription factor nuclear factor kappaB (NF-κB) (35;128). NF-κB activity is required for the development of DCs. VEGF and tumor cell conditioned medium have been shown to inhibit NF-κB activity both in vitro and in vivo (35;128). Because VEGF can signal via STAT3, some studies have been performed to identify possible cross-talk between STAT3 and NF-κB, or its sub-units. Indeed, STAT3 has been demonstrated to inhibit NF-κB through direct binding (Figure 6) (129;130).

Figure 6 – Proposed model of inhibited NF-κB gene transcription by phosphorylated STAT3
The role of cellular receptors in VEGF-mediated immune suppression has also been examined, although with less conclusive results. VEGFR-2 signaling has been found to be important in regulating early hematopoiesis, but not in final maturation (131). Dikov et al suggested that VEGFR-1 is crucial in inhibiting DC maturation (132). SU5416, a tyrosine kinase inhibitor of VEGFR-1, -2 and c-Kit, only partially rescued VEGF-mediated inhibition of DC maturation (132). This implies that tyrosine kinase dependent and independent pathways mediate VEGF-induced inhibition of DC differentiation.

4. Co-regulation of angiogenesis and dendritic cell maturation in the tumor microenvironment

As illustrated by the previous paragraphs, angiogenesis and tumor-induced immune suppression are co-regulated by a number of the same tumor-derived factors. In addition, immune cells in a tumor-microenvironment may contribute to angiogenesis by the production of angiogenic factors and/or their trans-differentiation into endothelial-like cells. Alternatively activated DCs (i.e. in the presence of LPS and PGE2, or IL-10) showed increased production of biologically active VEGF and produced less IL-12, an inflammatory cytokine with anti-angiogenic activity, as compared to classically activated DCs (in the presence of LPS only) (133). These findings were extended to PDCs in ovarian cancer, where tumor-associated PDCs produced TNF-α and IL-8, which contributed to neovascularisation in an in vivo matrigel assay (134). Alternatively activated DCs may produce both pro-angiogenic and anti-angiogenic factors (135), but the pro-angiogenic activity of these DCs exerted in vivo suggests that, at least under defined experimental conditions, the balance of these factors favors angiogenesis. Apart from the production of pro-angiogenic factors, DC (precursors) may also contribute to angiogenesis through their trans-differentiation upon certain environmental cues into endothelial-like cells, which display similar phenotypic and functional features as true endothelial cells. In a murine model of ovarian cancer, β-defensin, an antimicrobial inflammatory peptide, was observed to recruit DC precursors to the tumor, where VEGF transformed them into endothelial-like cells contributing to vasculogenesis and tumor progression (136). In addition, it has been reported that a tumor micro-environment characterized by the presence of cytokines and lactate will induce the differentiation of monocytes into tumor-associated DCs which, in the presence of pro-angiogenic factors, will further trans-differentiate into endothelial-like cells (137). Indeed, immature DCs can also trans-differentiate into endothelial-like cells when cultured in the presence of a cocktail of pro-angiogenic factors, including VEGF (138). These data were supported by the identification of a novel cell population expressing both endothelial cell and leukocyte markers in human tumors. These so-called vascular leukocytes might originate from leukocytes which are recruited to the tumor-environment,
where they acquire an endothelial-like cell phenotype with corresponding functional properties (139;140).

5. Immune potentiating effects of anti-angiogenic therapies

Due to the interconnected processes of angiogenesis and tumor-induced DC suppression, it has been proposed that therapies targeting angiogenesis, in particular VEGF as the most prominent pro-angiogenic factor, might also result in improved immune responses. Fricke et al showed in a heterogeneous population of advanced cancer patients that inhibition of VEGF signaling with VEGF-Trap, a fusion protein neutralizing VEGF and placental growth factor, increased the frequency of mature DC precursors in the blood, but did not alter frequencies of immature myeloid cells (141). Despite this increase in mature DC precursors, VEGF-Trap did not improve antigen-specific immune responses. Sub-analysis of the study population showed that in patients with stable or decreasing MSC (Lin− CD33+ HLA-DR−) frequencies upon VEGF-Trap treatment, immune responses were improved (141). This finding suggests that the potential beneficial effect of increased mature DC precursors might be negated by the presence of MSC. Another study examining the DC immune potentiating effects of bevacizumab, a monoclonal antibody against VEGF, showed a decrease in immature myeloid cells, but no significant increase in mature DC precursors in the blood of cancer patients (142).

Tumor-associated endothelial cells have a suppressed expression of adhesion molecules like intercellular adhesion molecule (ICAM)-1, vascular adhesion molecule (VCAM)-1, E-selectin and CD34, leading to decreased leukocyte-vessel wall interactions and subsequent decreased infiltration of CD45+ cells into tumor tissue making tumors an immunoprivileged site. It has been demonstrated that increased tumor-derived angiogenic factors are responsible for the suppressed expression of the adhesion molecules (143). Normalization of adhesion molecules on tumor-associated endothelial cells by the use of angiogenesis inhibitors, e.g. anginex, endostatin, chemotherapeutics, and anti-VEGF antibodies, resulted in an increased infiltration of leukocytes into the tumor (144).

6. Clinical studies combining anti-angiogenic therapy with immunotherapy

Preclinical and clinical studies combining VEGF-interfering therapy and immunotherapy have been performed to overcome the VEGF-induced immune suppression and thus increase the anti-tumor effect of immunotherapy. Vice versa, immunotherapy may also be applied to sustain the responses seen with VEGFR-interfering agents. Clinical studies combining VEGFR-interfering agents with IFN-α suggested higher response rates compared
to either agents alone. However, toxicity of the combination regimen also exceeded that of either agent alone (145-149).

Combining VEGFR-interfering agents with vaccination strategies seems an attractive approach. In preclinical settings anti-angiogenic therapies, mostly targeting the VEGF pathway, appeared to improve anti-tumor efficacy of vaccination strategies (91;150-157). The first clinical study employing this combination reported Prostate-Specific Antigen (PSA) responses and immune responses in hormone-refractory prostate cancer patients treated with DC-based vaccines and bevacizumab (158).

7. Outline of this thesis

In the current chapter we summarized the literature regarding the relation between angiogenesis and tumor-induced DC suppression. In this thesis the cross-regulation between both tumor-associated processes will be further described. Clinically available agents with anti-angiogenic properties were studied for their potential to overcome DC suppression in cancer patients and tumor-associated factors were studied for their possible role in angiogenesis and/or DC suppression.

Part 1 of this thesis includes chapters describing the clinical experience with VEGFR and epidermal growth factor receptor (EGFR) TKIs. In chapter 2, we describe the clinical evaluation of VEGFR tyrosine kinase inhibitors with a special emphasis on efficacy, response evaluation, typical toxicities and their management, and pharmacology and dosing. In chapter 3, the arguments to combine strategies targeting EGFR signaling pathway with anti-VEGF(R)-therapies are discussed. The results of a phase I study employing these two anti-cancer strategies are reported in chapter 4.

Part II concerns the immunmonitoring, especially of the peripheral blood DCs, in cancer patients treated with VEGFR tyrosine kinase inhibitors (TKIs). In chapter 5, the effects of cediranib (a selective VEGFR TKI) and gefitinib (an EGFR TKI) on DC differentiation in advanced cancer patients are described. The clinical efficacy and toxicity of this combination therapy are reported in chapter 4. In chapter 6, the effects of sunitinib, another VEGFR TKI, on several myeloid cell populations, including DCs, are investigated in advanced renal cell cancer patients.

The specific aims of Part III were to report preclinical studies investigating the impact of tumor-derived factors on DC differentiation and angiogenesis. Chapter 7 describes an immunohistochemical study investigating two tumor cell characteristics in non-small cell lung cancer, i.e. ganglioside expression and STAT3 activation, and their putative involvement in angiogenesis and tumor-induced DC suppression. In chapter 8, we characterize the DC suppressive profile of glioblastoma soluble factors in an in vitro study and delineate the involved subcellular signaling events.
Finally, the results described in this thesis will be discussed and put into perspective in chapter 9.

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CHAPTER 2

Tyrosine kinase inhibitors of VEGF receptors: clinical issues and unanswered questions

Hester van Cruijsen, Astrid AM van der Veldt and Klaas Hoekman

Frontiers in Bioscience 2009, 14: 2248-2268
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4. Response evaluation
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7. Conclusion
1. Abstract

The number of VEGFR tyrosine kinase inhibitors (TKIs) used as an anti-cancer agent is rapidly increasing, but several issues in clinical practice remain to be elucidated. VEGFR TKIs are multikinase inhibitors that have additional targets such platelet-derived growth factor receptors, which may result in an increased efficacy as well as an increased toxicity. Efficacy in several cancers has been shown, but acquired resistance also occurs during treatment with this new class of drugs. Tumor response evaluation can be a challenge, because VEGFR TKIs can cause extensive tumor necrosis without a marked decrease in tumor size. Therefore, new response criteria and functional imaging techniques are required. In this review we will also focus on the specific toxicities and their management: hypertension, proteinuria, cardiac toxicity, fatigue, hypothyroidism, voice changes, gastrointestinal toxicity, cutaneous reactions, wound healing, hemorrhage and thromboembolic events, hematological toxicity and cerebral toxicity. Furthermore we will discuss some issues regarding the pharmacology and dosing of these drugs. This review may provide important information to clinicians who prescribe VEGFR TKIs to their patients.

2. Introduction

Angiogenesis, the formation of new blood vessels from the existing vasculature, is essential for tumor growth and metastasis formation. Angiogenesis is a multi-step process where endothelial cells proliferate and migrate in the direction of specific stimuli. This can only happen with a concurrent remodeling of the extracellular matrix (ECM). Finally, tube formation occurs resulting in new blood vessel loops, which have to be stabilized. Several stimulating and inhibitory growth factors regulate this complex process (1). A key stimulator of angiogenesis is vascular endothelial growth factor (VEGF), which induces proliferation, differentiation and migration of endothelial cells (2). In addition, VEGF has a pro-survival effect on endothelial cells of newly formed vessels (3) and it increases vascular permeability (4). Compared to normal vasculature, tumor-associated vasculature consists of large and leaky vessels and has a disorganized structure with high interstitial pressure (5).

VEGF (also referred to as VEGF-A), is a member of a broader family, which includes VEGF-B/-C/-D/-E, placental growth factor (PIGF)-1 and -2 (2). In addition, alternative exon splicing generates four VEGF isoforms: VEGF121, VEGF165, VEGF189, VEGF206 (6), which all have different affinities for heparin binding. Acidic VEGF121 does not bind heparin and is secreted as a diffusible protein, whereas basic VEGF189 and VEGF206 bind heparin with high affinity and are sequestered in the ECM. VEGF165, the predominant isoform, can be
secreted freely, but a considerable portion remains cell- or ECM-bound via heparan sulphate proteoglycans. Plasmin (7) and matrix metalloproteinase (MMP)-9 (8) can release the ECM-bound isoforms, creating the diffusible, bioactive fragment, VEGF110.

The production of VEGF by tumors is associated with tumoral hypoxia and genetic tumor aberrations, e.g. loss of tumor suppressors like p53, PTEN and Von Hippel Lindau (VHL) (9-11), and mutation or amplification of oncogenes (e.g. Ras) (12, 13). Growth factors like epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) can also induce VEGF expression (14, 15). Within tumors, the VEGF production of activated immune cells and fibroblasts may also be substantial (16, 17).

Members of the VEGF family exert their effects via three VEGF tyrosine kinase receptors VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1), and VEGFR-3 (Flt-4). VEGFR-1 and -2 are mostly expressed by vascular endothelial cells whereas VEGFR-3 is present on lymphatic endothelium. All VEGFRs are characterized by seven extracellular immunoglobulin-like domains, of which the second and third are critical for ligand binding, a transmembrane domain and a cytoplasmatic domain, which contains tyrosine kinase residues important for activating the intracellular signaling transduction pathway (Figure 1a). The ligands can form anti-parallel homodimers optimizing binding to their preferred receptor and facilitating receptor dimerization (18). The VEGF/VEGFR-2 complex seems to be the most important signaling route of activating endothelial cells. In addition, different affinity of the ligand for neuropilins, which are 130-kD transmembrane receptors expressed by a variety of cells including endothelial cells, can affect signal transduction (19). Neuropilin (NRP)-1 and -2 are no tyrosine kinase receptors, but they act as a co-receptor and potentiate the binding and activity of VEGF165 to VEGFR-2 (Figure 1a).

Other receptor tyrosine kinases and their ligands are also involved in angiogenesis: PDGF receptors (PDGFR), Tie receptors, fibroblast growth factor receptors (FGFR), hepatocyte growth factor receptors (HGFR) and ephrin receptors (EphR). Briefly, PDGFR and FGFR will be described, since some of the compounds discussed in this review inhibit these pathways. PDGFRs are composed of two chains (α or β) resulting in three distinct receptors (αα, αβ, or ββ). Some structurally related receptors, such as c-Kit and Fms-like tyrosine kinase (Flt3), belong to the same family as PDGFRs. The family of PDGF-ligands consists of four homodimers, PDGF-AA, -BB, -CC, and –DD, and the heterodimer PDGF-AB, which bind to α- and β-receptors with different specificities (20). PDGFR signaling is involved in blood vessel development and wound healing and is involved in maintaining interstitial fluid pressure. PDGF-B is expressed by endothelial cells while PDGFR-β is expressed by pericytes and smooth muscle cells covering the blood vessels, suggesting a role of PDGFR signaling in recruitment of perivascular structures (21). The family of FGFRs comprises 4 different receptors, which can bind more than 20 different heparin-binding
Figure 1 - Schematic representation of the vascular endothelial growth factor receptors (VEGFR) on endothelial cells, VEGFR ligands and the intracellular signaling pathway leading to angiogenesis (a). Strategies to inhibit the VEGFR signaling pathways, leading to anti-angiogenic activity (b). For reasons of legibility only the most important molecules and connections are included in this figure. 
R - receptor (extracellular domain), P - phosphorylation site (intracellular domain), NRP - neuropilin.
FGFs with different affinity. FGF-1 and -2 induce endothelial cell proliferation, migration and protease production (22). Although there is a redundancy of proangiogenic growth factors and their receptors, VEGF is supposed to be the most important factor sustaining angiogenesis. Proof-of-principle was provided by xenograft mouse models, in which neutralizing VEGF-antibodies or VEGFR interference inhibited tumor growth substantially and even induced tumor regression (23, 24). These early findings were confirmed and extended in a variety of animal models.

In mature tissues, angiogenesis occurs mainly during wound healing, in the female reproductive cycle and during ischemia. Therefore targeting tumor-associated angiogenesis represents a promising anti-cancer therapy with reduced toxicity in adults. Anti-angiogenic therapy affects mostly the immature, disorganized tumor vasculature leaving the remaining tumor vasculature to function better with a subsequent improved perfusion (5, 25). In glioblastoma patients, magnetic resonance imaging (MRI) established a rapid, but reversible, normalizing effect of the anti-angiogenic agent cediranib (AZD2171) on tumor vessels, resulting in a promising response rate. In addition, reduction in permeability correlated with a decrease of tumor-associated brain edema (26).

Bevacizumab, a monoclonal humanized antibody directed against VEGF, was the first anti-angiogenic agent to be registered (27). Inhibition of the VEGFR kinase activity is another strategy to inhibit the VEGFR pathway. This review focuses on VEGFR tyrosine kinase inhibitors (TKIs), which are low-molecular weight, ATP-mimetic proteins that bind to the intracellular site of the tyrosine kinase domain of VEGFRs, resulting in a blockade of the intracellular pathway (Figure 1b). The number of VEGFR TKIs in clinical trials is rapidly increasing and some are already registered (Table 1). In this review we address remaining questions regarding response evaluation, specific toxicities, dosing and scheduling of these agents, and rational combinations with other anti-cancer therapies.
### Table 1. IC<sub>50</sub> (µM) of indicated receptors determined by kinase assay unless stated otherwise

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* Biochemical IC<sub>50</sub> (µM), values were determined in biochemical kinase assays using recombinant enzymes

** Cellular IC<sub>50</sub> (µM), values were determined by measuring intrinsic or ligand-stimulated kinase activity (phosphorylation) in cell lines expressing a given target

*** Clinical trials—gov

T1/2, half-life

Phase, most advanced phase of clinical testing

Reg, registered as standard anti-cancer treatment for indicated tumor type; RCC, renal cell carcinoma; GIST, gastrointestinal stroma tumor
3. Efficacy

The VEGFR TKIs sunitinib (SU11248), sorafenib (BAY 43-9006) and axitinib (AG-013736) have demonstrated their efficacy in metastatic renal cell cancer (RCC). In a randomized phase III clinical trial sunitinib had an objective response rate of 31% which was significantly higher than the 6% in the interferon-alpha (IFN-α) group (28). The progression-free survival on sunitinib was 11 months, while that on IFN-α was 5 months (28). As compared with the placebo group, treatment with sorafenib prolonged the progression-free survival with almost 3 months in RCC patients, resistant to standard cytokine therapy (29), but failed to improve the progression-free survival in comparison to that of IFN-α as studied in the first-line setting (30). Recently, a phase II study has been reported demonstrating the efficacy of axitinib in cytokine-refractory RCC patients (31). The success of VEGFR TKIs in clear cell RCC can be explained by its tumor biology. Clear cell RCC is characterized by a defect in the VHL tumor suppressor gene leading to stabilization of the hypoxia-inducible factor (HIF)-1-α protein and subsequently to overexpression of VEGF and PDGF resulting in tumor progression and angiogenesis (32). Additional targeting of PDGFR by VEGFR TKIs might explain the higher response rate of VEGFR TKIs in RCC when compared to bevacizumab (33).

Efficacy data of VEGFR TKIs concerning other tumor types are still immature, but interesting response data in (early) clinical trials have been observed. For example, sunitinib in phase II clinical trials had promising activity in metastatic breast cancer (34) as well as advanced non-small-cell lung cancer (35). Sorafenib showed antitumor activity in prostate cancer (36-38) and improved overall survival with 44% in hepatocellular carcinoma patients when compared with placebo (39). Sorafenib and axitinib have shown objective response rates of 33% and 22% respectively in thyroid cancer (40, 41). In some settings, the inhibitory activity of VEGFR TKIs to receptors other than VEGFRs (Figure 2) may be responsible for their efficacy: inhibition of c-Kit by sunitinib may be responsible for its success in imatinib-resistant gastrointestinal stromal tumors (GIST) (42) and inhibition of Raf and RET by sorafenib may explain the clinical efficacy in thyroid cancer (40) (Figure 2). In settings where chemotherapy is considered standard of care, VEGFR TKIs are being added to increase efficacy. VEGFR TKIs may improve the tumoral uptake of anticancer agents by a vessel normalization effect. In mice bearing glioma xenografts, sunitinib has demonstrated to increase the temozolomide tumor distribution (43). Most advanced data are available from phase III studies which investigated the potential benefit of adding vatalanib (PTK787/ZK 222584) to chemotherapy (FOLFOX 4) in colorectal patients (CONFIRM-1 and -2 (44, 45)). Although the results indicate that patients with high baseline serum lactate dehydrogenase levels benefit from vatalanib treatment, this did not
Clinical issues of VEGFR inhibitors

Figure 2 - Additional targets of vascular endothelial growth factor receptor (VEGFR) tyrosine kinase inhibitors, which may contribute to their anti-tumor efficacy either by targeting tumor-associated vessels or by targeting the tumor cells themselves. RCC, renal cell cancer; GIST, gastrointestinal stromal tumor; BC, breast cancer; NSCLC, non-small cell lung cancer; GBM, glioblastoma multiforme

Increase survival in the whole population (46). A high drop-out rate in the vatalanib arm of the CONFIRM-1 study, due to toxicity, might have contributed to the unsatisfactory results. Furthermore, vatalanib administrated as a single daily dose might be less effective due to a short half life (i.e., ~6 hours). Bevacizumab, which has a longer half life (i.e., ~20 days) and is administered once in three weeks, did increase the efficacy of chemotherapy in colorectal cancer patients (27). VEGFR TKIs have also been combined with other targeted therapy such as bevacizumab (47, 48) and agents that target the EGF receptor (49-51) or the mammalian target of rapamycin (mTOR) (52), however, efficacy data are still incomplete. Two phase II studies combining sorafenib with IFN-α in RCC patients have recently been reported (53, 54). These studies suggested higher response rates for the combination, however, toxicity also exceeded that of either agent alone.

Angiogenesis inhibitors might ultimately increase the radioresistance of tumor cells, by inducing more tumor hypoxia (55). However, preclinical studies have shown that anti-angiogenic therapy can increase anti-tumor effects of radiotherapy (56-58). Preclinical data have suggested that angiogenic factors, like VEGF, being a survival factor for endothelial cells, are upregulated in tumors during radiotherapy (59, 60). Even after an initial VEGFR TKI-induced regression, tumors eventually progress. Preclinical studies have demonstrated that this acquired resistance may be associated with upregulation of VEGF and other angiogenic factors (61, 62). Increased tumor hypoxia upon administration of VEGF(R) interfering agents has been demonstrated in preclinical models.
and this may, via stabilization of HIF-1α result in increased production of a variety of angiogenic factors, like HGF and FGF (61, 62).

4. Response evaluation

Most VEGFR TKI trials use the bi-dimensional World Health Organization (WHO) criteria or the uni-dimensional Response Evaluation Criteria in Solid Tumors (RECIST) (65). The RECIST criteria are most widely used and are based upon the sum of the longest diameters of the appointed target lesions in the transversal plane. Objective responses may be missed or underestimated by RECIST (66), since VEGFR TKIs can cause direct and rapid anti-vascular effects, leading to secondary tumor necrosis without a marked decrease in tumor size (66-68). Furthermore, tumor markers used for response monitoring during therapy with conventional agents may lack efficacy to monitor VEGFR TKI response, as it has been suggested for the prostate-specific antigen (PSA) in prostate cancer patients treated with sorafenib (36, 37) and CA125 in ovarian cancer patient treated with sorafenib (69). Choi response criteria have recently been defined to evaluate responses in GIST patients treated with imatinib (70, 71). In this setting, the Choi criteria correlated better with disease-specific survival than RECIST. According to these criteria a response is defined as a ≥10% decrease in one-dimensional tumor size or a ≥15% decrease in tumor density on computed tomography. The Choi response criteria may also be of value to evaluate tumor responses after treatment with VEGFR TKIs.

Instead of conventional morphologic imaging, functional imaging can be applied to measure the efficacy of VEGFR TKIs. Several vascular end-points such as tumor blood volume, tumor blood flow rate, perfused/non-perfused tumor fractions and vascular permeability-surface area can be determined by techniques such as Dynamic Contrast Enhanced Magnetic Resonance Imaging (DCE-MRI), Perfusion Computed Tomography (CTP), Dynamic Contrast Enhanced Ultrasound (DCE-US) and Positron Emission Tomography (PET) using different PET tracers (72). In only a few trials such techniques have been applied to evaluate the tumor response to VEGFR TKIs.

DCE-MRI has been used in assessing tumor vascularity and permeability following treatment with the agents sorafenib, vatalanib and axitinib (73-75). In sorafenib-treated RCC, DCE-MRI seemed to be a promising tool to predict progression-free survival (73). In colon cancer vatalanib caused a reduction in DCE-MRI contrast enhancement parameters within 26 to 33 hours of administration of the first dose (75), and in patients with advanced solid tumors an immediate decrease in tumor vascular parameters as measured by DCE-MRI was seen on day 2 after axitinib administration (74). A decrease in tumor perfusion has been demonstrated by CTP after administration of cediranib (76). In RCC patients treated with sorafenib an early reduction in tumor vascularization/tumor volume
Clinical issues of VEGFR inhibitors

measured by DCE-US was shown to correlate with response and progression-free and overall survival (77, 78). The obvious advantage of DCE-US is that it is simpler, more patient-friendly and cheaper than the other imaging modalities.

PET is gaining increased interest by the development of new tracers. Although 2-deoxy-2-[18F]fluoro-D-glucose (FDG)-PET has been validated to assess treatment efficacy of conventional cytostatic agents (79), the use of FDG-PET in detecting VEGFR TKIs responses is limited. An early metabolic response by FDG PET has been demonstrated for semaxanib (SU5416) within 2 weeks of therapy in a patient with metastatic RCC (80). Using oxygen-15 [15O] labeled tracers such as 15O-labeled water ([15O]H2O) and carbon monoxide ([15O]CO2), tissue perfusion and blood volume can be quantified (81). Also anticancer agents are increasingly being labeled with radioisotopes to serve as PET tracers. Sunitinib is the first VEGFR TKI that has been labeled with 18F as PET tracer (82), however [18F]sunitinib has not been evaluated in patients yet. In the future, radiolabeled VEGFR TKIs will most likely provide new information on their pharmacokinetic and pharmacodynamic action.

The data on functional imaging of the tumor response to VEGFR TKIs are limited, preliminary and not well validated. Rapid changes in vascular parameters are seen during treatment with VEGFR TKIs, which makes these parameters interesting markers for early prediction of tumor response and possibly progression-free and overall survival. Long term vascular consequences of therapy with VEGFR TKIs are largely unknown. Regarding tumor response evaluation, clinical benefit should be kept in mind. Our experience is that some patients have clinical benefit from VEGFR TKIs even while they have progressive disease. This may therefore be a reason to continue treatment.

5. Toxicity

VEGFR TKIs have a distinct profile of side-effects. Toxicity observed with VEGFR TKIs has overlap with the toxicity associated with bevacizumab, indicating that these side effects are largely caused by inhibiting the same pathway. The differences in toxicity profiles between several VEGFR TKIs can be explained by differences in specificity and affinity. For some side-effects the etiology remains poorly understood. Typical class specific side-effects will be discussed.

5.1. Hypertension

Administration of inhibitors of VEGF signaling often results in elevation of blood pressure with an incidence up to 50% in clinical trials (83-85). Grade 3 and 4 hypertension has especially been reported for VEGFR TKIs. This dose-dependent toxicity can occur within days and is reversible upon discontinuation of treatment. Insight into the mechanisms of
VEGF-blood pressure effects is necessary for optimal clinical handling of this adverse event.

Bevacizumab treatment has resulted in a reduced density of microvessels and endothelial dysfunction, two mechanisms that may be responsible for VEGFR TKI-induced hypertension (86). Evidence suggests that VEGFR-2 is involved in the regulation of the vascular tone (Figure 3a). It has been shown in vivo and in vitro that VEGFR-2 predominantly mediates the vasodilative and hypotensive effects of VEGF (87).

**Figure 3** - Role of vascular endothelial growth factor receptor (VEGFR)-2 in the vascular tone (a). Inhibition of VEGFR-2 by tyrosine kinase inhibitor (TKI) might result in a dysbalance between vasodilatation and vasoconstriction, leading to increased peripheral resistance and subsequent hypertension (b,c). R - receptor (extracellular domain), P - phosphorylation site (intracellular domain), PI-3K - phosphoinositide 3-kinase, eNOS - endothelium-derived nitric oxide synthase, NO - nitric oxide, ET-1 - endothelin-1

So, it is conceivable that blocking VEGFR-2 causes vasoconstriction of the microcirculation. Activation of VEGFR-2 via phosphoinositide 3-kinase (PI3K) and its downstream serine protein kinase Akt induces endothelium-derived nitric oxide synthase (eNOS), resulting in the production of the potent vasodilator nitric oxide (NO) (Figure 3a) (88-92). Conversely, vasoconstriction is induced by endothelin-1 via protein kinase C and the Ras-Raf-ERK1/2 cascade (Figure 3a) (93). The VEGFR-2 TKI SU1498 blocks VEGF-induced activation of two
intracellular pathways, namely endothelial Akt, eNOS and NO production, and ERK1/2 (Figure 3b) (94). SU1498, however, only blocks flow-induced activation of the Akt, eNOS and NO production, but not the activation of ERK1/2 (Figure 3c) (94). This suggests that the ERK1/2-endothelin-1 cascade dominates when VEGFR-2 is inhibited. It can be hypothesized that blocking VEGFR-2 by inhibitors of the TK domain will cause hypertension by inducing an imbalance between the PI3K-Akt-eNOS and ERK1/2 pathways (Figure 3c). Tension control can be obtained with standard oral anti-hypertensive drugs, such as calcium-antagonists or beta-blockers (95). Angiotensin-converting enzyme (ACE) inhibitors or angiotensin-receptor blockers are more rational to apply when proteinuria is also observed. Patients treated with VEGFR TKIs should have their blood pressure regularly measured, especially patients with pre-existent hypertension. Home blood pressure monitoring can be valuable to evaluate blood pressure during treatment with VEGFR TKIs (96).

Retrospective studies have identified grade 3 hypertension as a predictive factor for response to sunitinib (97, 98). This should be confirmed in large, prospective trials.

5.2. Proteinuria

Administration of bevacizumab is associated with an increased risk for proteinuria (99). Proteinuria is usually asymptomatic, rarely resulting in serious renal dysfunction. Less data on the incidence of proteinuria in VEGFR TKI trials are available. A preeclampsia-like syndrome characterized by hypertension and proteinuria has been described in seven patients treated with sunitinib or sorafenib (100). In advanced thyroid cancer patients treated with axitinib, proteinuria was observed in 27% of the patients (41). Grade 3 proteinuria appeared to be a dose-limiting toxicity in a phase I study of KRN951 (101). Furthermore, in a phase I study of AMG706 grade 3 and 4 proteinuria was observed in 4% and 1% respectively (102).

The occurrence of proteinuria induced by anti-VEGF(R) therapy indicates the importance of VEGFR signaling in renal function (103). Podocytes express VEGF, which in turn activates glomerular endothelial cells. Inhibition of VEGF-dependent interactions between podocytes and glomerular endothelium by VEGFR TKIs might disrupt glomerular filtration leading to proteinuria. Increased blood pressure may contribute to the VEGFR TKI-induced proteinuria.

Patients treated with VEGFR TKIs should be monitored for proteinuria. In case of proteinuria grade 3 it is advised to reduce the dose or to discontinue treatment temporarily or permanently.
5.3. Cardiac toxicity
Cardiac toxicity has been observed during treatment with sunitinib and sorafenib (104). For sunitinib, two cases of congestive heart failure were reported in a phase I study (105) and an additional case on acute cardiac failure with a fatal outcome has been described (106). A phase III study with sunitinib in patients with metastatic renal cell cancer reported that 10% of patients had declines in left ventricular ejection fraction (LVEF) after a median treatment duration of 6 months (28). Additionally, two retrospective studies have investigated the sunitinib-induced cardiac toxicity (107, 108). Khakoo et al. reported that 6 of 224 (2.7%) patients treated with sunitinib developed heart failure which occurred soon after initiation of sunitinib and was not completely reversible in most patients, even after termination of sunitinib therapy (108). Chu et al. reported that 2 out of 75 patients with GIST had cardiac infarction and six developed congestive heart failure during sunitinib treatment (107). In these studies, hypertension and a decline in ejection fraction also occurred. Chu et al. have recently demonstrated that sunitinib exposure induced mitochondrial injury and cardiomyocyte apoptosis in mice and in cultured rat cardiomyocytes (107). Furthermore, during sorafenib treatment, increased cardiotoxicity has been observed in sunitinib-pretreated patients with metastatic renal cell cancer (109). These results indicate that patients on VEGFR TKIs should be monitored carefully. Follow-up may consist of electrocardiogram and longitudinal measurements of LVEF. Special attention should be paid to patients with severe heart disease and coronary artery disease. With regard to cardiac toxicity, hypertension should be treated promptly.

5.4. Fatigue
Fatigue, asthenia and malaise are frequent symptoms observed in advanced cancer patients. Administration of VEGFR TKIs has been associated with the development of fatigue with an increased intensity. Mild to moderate fatigue has been reported in almost all phase I studies using VEGFR TKIs, and in some studies fatigue has been found to be dose-limiting. Establishing the cause of treatment-related fatigue is difficult. Anemia and renal or adrenal failure are not common events during VEGFR TKI treatment, and therefore not an obvious cause of the observed fatigue. Hypothyroidism, occurring during VEGFR TKI treatment, may be involved in some patients.

5.5. Hypothyroidism
The incidence of hypothyroidism in advanced cancer patients treated with sunitinib ranges from 2% to 80% (28, 110-113). The wide range can be explained by differences in defining hypothyroidism and the retrospective design of most of the studies. Other VEGFR TKIs are also capable of inducing hypothyroidism (114, 115). Increased TSH concentrations are far more common than a change in T3/T4 levels during sunitinib therapy. A modest TSH increase with no related symptoms does not require
supplemental therapy. In patients treated with VEGFR TKIs, T3/T4 monitoring is recommended. Profound TSH increases associated with low T3/T4 levels and overt hypothyroid symptoms should guide levothyroxine therapy. In some patients treated with sunitinib TSH increase was preceded by a short period of TSH decrease and T3/T4 increase, suggestive of thyroiditis (110) which may be associated with transient thyrotoxicosis (116).

Several mechanisms can be involved in VEGFR TKI-induced hypothyroidism. The thyroid gland is a hypervascularized tissue in which follicular cells have a close relationship with surrounding capillaries (117, 118). VEGFR TKIs might cause a significant regression of thyroidal capillaries, but also a disappearance of endothelial fenestrations (119), a decrease of vascular permeability (120), and capillary vasoconstriction (94). These effects may disturb delivery of iodide to the follicular cells, and/or result in regression of thyroid tissue, subsequently leading to reduced biosynthesis of thyroid hormones and an increased TSH response. TSH itself is known to increase the expression of VEGF and its receptors in the thyroid gland (118), which will induce proliferation of thyroid vessels and an increase in vascular permeability and vascular dilatation. This compensatory mechanism of TSH will increase the thyroid blood flow and facilitate iodide uptake. When the compensatory mechanism fails, overt hypothyroidism may develop.

Recently, two other underlying mechanisms of hypothyroidism have been proposed. In patients treated with sunitinib, hypothyroidism may be caused by impaired iodine uptake (121) as well as inhibition of thyroid hormone synthesis (112). With regard to the latter process, Wong et al. demonstrated in vitro that sunitinib inhibits peroxidase, the enzyme involved in the production of T4/T3 (112).

It is conceivable that VEGFR TKI induced hypothyroidism is multifactorial. Prospective studies to further evaluate the incidence of VEGFR TKI-induced hypothyroidism and its relationship with fatigue and other possibly related symptoms like voice changes, cold intolerance and constipation, are needed.

5.6. Voice changes

Voice changes, hoarseness, or dysphonia have been reported in a number of clinical studies using VEGFR TKIs (115, 122-124). Disturbing the mucosal integrity by the use of VEGF(R) interfering agents might result in hoarseness. Incidental laryngoscopic examinations did not reveal any functional abnormalities of the vocal cords.

Voice changes may be a sign of hypothyroidism. However, voice changes occur already in the first week of treatment with VEGFR TKIs, while TSH increases usually occur after 8 weeks of treatment. Voice changes are reversible after discontinuation of VEGFR TKI treatment.
5.7. Gastrointestinal toxicity
Almost all clinical studies using VEGFR TKIs report mucositis of the upper and/or lower gastrointestinal tract leading to pain and diarrhea in a subset of patients. Stomatitis, usually described as a sore mouth without any overt blistering, is rather therapy-resistant, necessitating dose reduction or treatment interruption in severe cases. It is reversible after treatment discontinuation. Diarrhea has been reported in 43% of patients treated with sorafenib compared with 13% of patients treated with placebo (29). The diarrhea is usually manageable, but some patients need dose reduction or treatment discontinuation. Mechanisms of the observed gastrointestinal toxicity remain unclear. It has been suggested that VEGF plays a role in physiological mucosal turnover and mucosal healing (125). It is also a possibility that TKIs affect other tyrosine kinases involved and this remains to be studied.

Bowel perforation is a less common but a serious side effect of anti-VEGF treatment. It occurred in 1.5% of colorectal cancer patients treated with bevacizumab (27) and in 11-15% of ovarian cancer patients treated with bevacizumab with or without erlotinib, an EGFR TKI (126, 127). Potential risk factors for this complication are previous irradiation, bowel metastasis, abdominal carcinomatosis, peptic ulcers, diverticulosis and recent surgery (128, 129). Local ischemia due to decreased perfusion may cause localized necrosis and subsequent perforation. When radiotherapy is combined with anti-angiogenic agents, the gastrointestinal tract is particular vulnerable (130, 131). In case of bowel perforation VEGFR interfering therapies should be withdrawn immediately. Although tumor cavitations and fistula formation upon VEGFR TKI treatment has been described (132), gastrointestinal perforations upon VEGFR TKI treatment have not been reported.

5.8. Cutaneous reactions
Hand-foot syndrome (HFS) is a painful palmar or plantar erythema associated with some cytostatic agents, such as doxorubicin, docetaxel and fluorouracil/capecitabine. In phase III trials using sorafenib or sunitinib, HFS has been observed in 30% and 20% of the patients, respectively (28, 29). HFS appears to be reversible after drug discontinuation. Bevacizumab is not related with cutaneous toxicity, suggesting that other TK receptors which are inhibited by sunitinib and sorafenib, like PDGFR and c-Kit, might be involved in the development of HFS. Since both VEGFR TKIs and some cytostatic agents can induce skin reactions of hands and feet, combining these agents might aggravate these reactions. Other cutaneous side-effects like skin coloration (132, 133), subungal splinter hemorrhages (132, 134), and hair depigmentation (132, 135) have been described in patients treated with VEGFR TKIs. Yellow skin coloration can already appear after 1 week of sunitinib treatment and relates to yellow coloration of the urine. This might be due to the local deposition of the drug and its metabolites (132). Hair depigmentation has been
observed in patients using sunitinib and pazopanib (GW786034) (132, 135), and might be due to the inhibitory effects on c-Kit (136).

5.9. Wound healing
Angiogenesis is thought to be critical for wound healing and blocking VEGF(R) signaling could interfere with this process (137, 138). An increased, but not significant, rate of wound healing complications has been observed in patients treated with bevacizumab (128, 139, 140).
In mouse models several VEGFR TKIs have been shown not to impair wound healing (141-143). This is a paradoxical finding which needs to be confirmed in clinical studies. Considering the short half life of VEGFR TKIs (i.e., 24 hours) compared with bevacizumab (i.e., 2-3 weeks), normally one week treatment interruption of VEGFR TKIs is recommended in the peri- or postoperative period.

5.10. Hemorrhage and thromboembolic events
Bevacizumab treatment has been associated with mostly non-serious bleeding events in patients with various tumor types. Serious, and even fatal, pulmonary hemorrhage was observed in lung cancer patients with central localization of squamous cell cancers (144, 145). Other infrequently occurring but serious bleeding events attributed to bevacizumab include gastrointestinal hemorrhages (146). VEGFR TKI studies also report bleeding events. In a phase III study comparing sorafenib with placebo in RCC patients, mild bleeding, like epistaxis, occurred more often in the sorafenib group (29). Studies with chemotherapy plus or minus vatalanib in patients with advanced colorectal cancer did not show an increase in bleeding events (45). Phase I trials using VEGFR TKIs, including vatalanib, have reported incidental bleeding events, which was fatal in some patients (101, 115, 147). Controlled trials are awaited to elucidate this association. Most of the bleedings do not occur in primary tumors or metastases, suggesting other vulnerable targets. Attention should be paid to patients with cerebral tumor lesions and patients on anticoagulant therapy.
Although thromboembolic events have not been reported for VEGFR TKIs administered as monotherapy, when combined with chemotherapy, semaxanib, a VEGFR TKI no longer in clinical development, resulted in increased incidence of thromboembolic events (148). VEGF stimulates endothelial cells to produce tissue factor (149), an important regulator of the coagulation cascade, inducing thrombin and eventually clot formation. On the other hand, a low concentration of VEGF is needed to keep endothelial cells in a quiescent, anti-coagulant state. This may be explained by the fact that VEGF induces anti-apoptotic genes, and blockade of the VEGF pathway may lead to endothelial cell apoptosis, and consequently to a pro-coagulant state (150, 151). In addition, VEGF inhibition reduces the proliferation rate of endothelial cells, and hence the capacity to cope with vascular
damage. This may cause an increased exposure of the underlying ECM, which may lead to hemorrhage or thrombosis. This all supports that VEGF is also a maintenance factor for endothelial cells. Endothelial cells deprived of VEGF might be more vulnerable to prothrombic activity of cytostatic agents (152). Caution should therefore be taken when inhibitors of VEGF signaling are combined with chemotherapy (148, 153).

5.11. Hematological toxicity

A typical side-effect of classical cytotoxic agents is myelosuppression. Sunitinib and CHIR-258 can also cause neutropenia and this seems to correlate with inhibition of Flt3 (28, 154), since VEGFR TKIs lacking inhibitory activity against this target are not associated with neutropenia (102, 115, 122, 155). Sunitinib also induced moderate or severe thrombocytopenia in 40% of the patients compared to 4% in the placebo-treated patients. The Flt3-ligand is involved in early hematopoiesis, and primitive hematopoietic cells express its receptor (156). One should therefore be cautious in combining VEGFR TKIs, targeting Flt3, with classical cytotoxic agents. Increased serious neutropenia was observed with 50 mg sunitinib in combination with a FOLFOX-regimen compared to 37.5mg sunitinib (157).

Many advanced cancer patients have pre-existing disease- or therapy-related anemia before entering trials. In clinical trials comparing sunitinib or sorafenib with placebo, no increased anemia was observed in the experimental arm (29, 42). Preclinical experiments demonstrated that neutralizing VEGF signaling resulted in an increased production of erythropoietin (Epo) by the liver, leading to enhanced red blood cell counts (158). The impact of increased Epo levels during anti-angiogenic treatment is currently not known (159). Erythrocytosis and increased Epo levels have not yet been reported in the human setting.

5.12 Cerebral toxicity

Reversible posterior leukoencephalopathy syndrome (RPLS), has been associated with the use of VEGFR TKIs and bevacizumab, albeit at a low frequency (45, 160-164). RPLS is associated with headache, seizures, altered consciousness, and visual changes in association with characteristic posterior cerebral white matter edema on neuroimaging. Prompt recognition of the medical symptoms and discontinuation of the treatment is important in preventing permanent damage. The pathogenesis of RPLS remains unclear, but it appears to be related to disordered cerebral autoregulation and endothelial dysfunction (165).

In elderly patients on sunitinib treatment cognitive disorders have been described which disappeared promptly upon discontinuation of this drug (166). These elderly patients had pre-existent cerebral vascular abnormalities visualized as subcortical arteriosclerotic
encephalopathy, which suggests that sunitinib may decrease the cerebral blood flow. Attention should therefore be paid to cognitive function in elderly patients on VEGFR TKIs.

6. Pharmacology and dosing

The intestinal absorption of most VEGFR TKIs is quick and peak plasma concentrations are observed within 1 to 7 hours. The plasma half-life (T1/2) differs among these agents (Table 1). For example, T1/2 of sunitinib is 41-86 hours and T1/2 of vatalanib is 3-6 hours (132, 167). This may indicate that the exposure to target receptors is more steady for the first agent and it might be a reason to give the second drug twice daily (167). The affinity of VEGFR TKIs to the three VEGF receptors is high (Table 1), but the clinical impact of differences in VEGFR-2 affinity between the various agents needs to be established. The affinity for other growth factor receptors, especially PDGFR, FGFR, c-Kit, Flt3, RET, differs substantially, which may significantly affect clinical activity and toxicity. Currently, data about the reversibility of binding to the receptors are lacking.

Compared to monoclonal antibodies, like bevacizumab, VEGFR TKIs are small molecules and penetration into any tissue, even brain tissue, is thought to be easy. However, the effects of VEGFR TKIs on brain tumors remain controversial (26, 168-171). Cediranib has proven to be effective in glioblastoma patients (26), sorafenib reduced the incidence of brain metastases in RCC patients (170) and response of brain metastases from RCC has been described for sunitinib (169, 171). On the other hand, a report on advanced RCC patients treated with sunitinib has suggested that brain metastases can be the first and/or only sign of tumor progression (168). An explanation may be that VEGFR TKIs are substrates for an upregulated P-glycoprotein mediated cellular efflux at the blood-brain barrier (172), resulting in a reduced penetration of these compounds.

VEGFR TKIs are predominantly metabolized in the liver by cytochrome P450 (CYP3A4). Therefore, the blood concentrations of the VEGFR TKIs can be influenced by co-medication with CYP3A4 modulators, which may affect the tumoral exposure to VEGFR TKIs (173). Since there is an interindividual variation in activity of CYP3A4, identification of factors that predict VEGFR TKI exposure are required to optimize treatment with VEGFR TKIs. The oral midazolam test measures the CYP3A4 activity and may be useful to predict whether patients are predisposed to be overdosed or underdosed (174).

Since a large number of TKIs are substrates of CYP3A4, combining VEGFR TKIs with other TKIs increases the chance of drug-drug interactions. Significant pharmacokinetic interactions between VEGFR TKIs and TKIs targeting other receptors have been described (50, 175), although not in all studies (49, 51). Many combination regimens of VEGFR TKIs and cytostatic agents are currently under investigation. Pharmacokinetic analyses, performed so far, do not show significant drug-drug interaction with cytostatic agents.
Pharmacokinetic evaluation in combination regimens is important to reassure sufficient plasma levels without the risk of accumulation or increased toxicities. Most VEGFR TKIs are administered daily in a continuous dosing schedule, since it is widely believed that continuous inhibition of the VEGF receptors is needed for an optimal effect. Sunitinib is administered in an intermittent schedule of 4 weeks followed by a 2-week rest period meant to recover from toxicities. However, in the 2-week rest period some patients, especially those with an initial tumor response, experience a rapid clinical deterioration (within days), which may necessitate continuous administration of sunitinib. The rapid rebound during the rest period is intriguing and may be due to early regrowth of the tumor vasculature (176), or, more likely, to tumoral edema (177). In several studies VEGF levels increase during VEGFR TKI therapy (26, 178). This may have consequences when the VEGFR TKI is temporarily discontinued. Increased vascular permeability leading to tumoral edema will be the first symptom.

7. Conclusions

VEGFR TKIs are increasingly being integrated in treatment regimens for cancer patients and efficacy data of these regimens are promising. Understanding the role of various ligand-receptor pathways in tumor biology in general and in a specific tumor type in a specific patient will guide future applications. Regimens combining VEGFR TKIs with other anti-cancer strategies must be evaluated and optimized for each agent and in each setting. This is even true for any co-medication which modulates CYP3A4. In settings where chemo- and/or radiotherapy is considered the standard of care, anti-angiogenic agents must be introduced carefully to minimize toxicity. Although VEGFR TKIs are generally well tolerated and are associated with manageable side-effects, they have distinct toxicity profiles which are partly due to their activity towards other, may be unknown, TK receptors. It is important to realize that their long-term effects on normal human cell and tissue physiology are largely not known. Considering the promising efficacy in adjuvant settings, VEGFR TKIs will be administered to cancer patients for longer periods. At present it is not clear which long-term and secondary consequences can be expected from VEGFR TKI-induced side-effects such as hypertension. Moreover, long-term effects of VEGFR TKIs on tumor biology remain to be investigated, since VEGFR TKIs generally does not result in complete tumor remission. Focus should also be given to long term consequences of inducing tumor hypoxia. Hypoxia may induce a rebound complex angiogenic response which also may affect tumor metastasis. A better understanding of these effects can help to reduce acquired resistance and to design better combination regimens that finally will improve patient outcome.
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CHAPTER 3

Epidermal growth factor receptor and angiogenesis: opportunities for combined anti-cancer strategies

Hester van Cruijsen, Giuseppe Giaccone and Klaas Hoekman

Abstract

Tumour-induced angiogenesis is essential for malignant growth. This mini-review focuses on the role of vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF) and their receptors in this process, and the rationale to combine inhibitors of these growth factors as anti-cancer therapy. Concomitantly targeting the VEGF(R) and the EGF(R) signalling pathway may circumvent the problem of acquired resistance to EGFR inhibitors. By targeting both pathways the anti-angiogenic effect may be more pronounced, which may lead to greater anti-tumour activity. Preliminary efficacy data from clinical trials encourage further exploration of this combined anti-cancer strategy.
**Introduction**

Tumour development and progression depend mainly on cellular changes like overexpression of oncogenic tyrosine kinase receptors. The activity of the epidermal growth factor receptor (EGFR), a tyrosine kinase receptor of the ErbB family, is abnormally elevated in most human solid tumours and has been associated with progression and poor prognosis. Multiple mechanisms are involved in the activation of EGFR signaling: production of EGFR-ligands by tumour cells or stromal cells in their environment, high expression of EGFR associated with enhanced sensitivity for EGF-like ligands, and constitutively active EGF-receptor mutations which lead to ligand-independent activation of the receptor. A mutation of the EGFR gene has recently been described, resulting in a more active, ligand-dependent receptor with increased sensitivity to gefitinib and erlotinib, both tyrosine kinase (TK) inhibitors of EGFR. We refer to some excellent reviews which describe the ligands and receptors of the ErbB family and the formation of homo- and heterodimers leading to signal amplification and diversity.

Therapeutic agents directed against EGFR induce anti-proliferative effects in tumour cells cultured in vitro. This most frequently results in cytostatic effects, more than cytoreduction and apoptosis. In contrast, in several in vivo models anti-EGFR therapy frequently induces tumour regression, which may not be solely explained by inhibition of the tumour target. This discrepancy suggests that EGFR signalling affects both biologic characteristics of the tumour cell and tumour-host interactions like angiogenesis.

Angiogenesis, the formation of new blood vessels from the existing vasculature, is essential for tumour growth and metastasis formation. Several inhibitors and stimulators regulate this complex multi-step process. A key stimulator of angiogenesis is vascular endothelial growth factor (VEGF), which induces proliferation, differentiation, and migration of endothelial cells. In addition, VEGF increases the vascular permeability and induces the production of a variety of proteases, which are involved in the modification of the extracellular matrix (ECM).

The production of VEGF can be disproportionately up-regulated in tumours via hypoxia or loss of tumour suppressors like PTEN and von Hippel Lindau. A functional relationship between oncogenes and tumour angiogenesis has been shown by Rak et al and Grugel et al in 1995. They showed that ras oncogenes upregulate VEGF expression and therefore could promote tumour growth indirectly by stimulating angiogenesis. Petit et al reported in 1997 that the oncogeneity of the EGF receptor might partially be mediated through promotion of angiogenesis by up-regulating VEGF. Since then there has been growing evidence that stimulation or inhibition of EGFR also has significant consequences for tumour-induced angiogenesis.

Here we will review the angiogenesis-related aspects of EGFR function and the effect of EGFR-targeted therapies on tumour-induced angiogenesis. Moreover, we will discuss the
possible mechanisms of acquired resistance to anti-EGFR therapies and the rationale for the combination of an EGFR-antagonist with anti-angiogenic agents as anti-cancer therapy.

**Preclinical studies**

**EGFR signalling and endothelial cells: in vitro studies**

The most frequently used endothelial cells for in vitro studies are human microvascular endothelial cells (HMVECs) and human umbilical vein endothelial cells (HUVECs). The former migrate in the presence of EGF or TGF-α and VEGF. HUVECs do not migrate in the presence of EGF or TGF-α. Different characteristics of endothelial cells like growth, migration and tube formation, have been investigated in order to evaluate the direct and indirect effects of EGF and the effect of blocking EGFR.

It has been shown that microvascular endothelial cells express ErbB1. Stimulation of MVECs with EGF or TGF-α, cultured on collagen type I gels, induced tube formation, and treatment of MVECs with gefitinib dose-dependently inhibited EGF-induced migration and tube formation. HUVECs on the other hand express ErbB2, ErbB3, and ErbB4, but do not express ErbB1. Stimulation of HUVECs with recombinant neuregulin (ligand for ErbB3 and ErbB4) induced rapid calcium fluxes, receptor tyrosine phosphorylation and cell proliferation, and stimulation of HUVECs with betacellulin resulted in phosphorylation of the ErbB2-4 receptors and proliferation, migration, and tube formation in collagen gels. These results indicate that the angiogenic influence of ErbB signalling is not limited to the EGF-EGFR pathway, since other ligands and receptors play a role in angiogenesis as well.

EGF and TGF-α bind to ErbB1, suggesting that EGF and TGF-α might not have a direct effect on HUVECs. An indirect effect of EGF on HUVECs was demonstrated by Hirata et al. They showed that HUVECs, when cultured alone, did not migrate in the presence of EGF, but when co-cultured with A431 cells, which express high levels of EGFR, EGF enhanced migration of HUVECs. Both gefitinib and SU5416, a selective TK inhibitor of VEGFR-1 and -2 and c-kit, blocked the migration of HUVECs in this context. Hirata et al demonstrated that A431 cells produced VEGF and IL-8 in response to EGF. These pro-angiogenic molecules stimulated migration of the HUVECs. In conclusion, the effect of EGFR signalling on HUVECs or microvascular endothelial cells can be either direct or indirect.

Although stimulated endothelial cells used in in vitro models are accepted as mimicking tumour-associated endothelial cells, it has to be taken into account that endothelial cells isolated from different tumour tissues may vary in their expression levels of ErbB and VEGF receptors. For instance, Baker et al showed that in tumours expressing high
levels of EGF or TGF-α, tumour-associated endothelial cells express EGFR and its activated form, in contrast to tumour-associated endothelial cells within a tumour which did not express EGF or TGF-α.27 Finally, maturation of endothelial tubes needs recruitment of smooth muscle cells (SMCs/pericytes). Important factors involved in vessel maturation are PDGF and TGF-β.30 EGFR signalling might play a role in vessel maturation as well: heparin-binding EGF, which is produced by endothelial cells,31 is a ErbB1 and -4 ligand. It stimulates ErbB receptor phosphorylation of SMCs and migration of SMCs.32 Hence, heparin-binding EGF may be involved in tumour-induced angiogenesis via activation of SMCs.32

**EGFR signalling and tumoral production of angiogenic molecules: in vitro studies**

Activation of the EGFR pathway increases the production of angiogenic molecules in a great variety of tumour cells. This results in an indirect effect of EGFR signalling on angiogenesis. EGF stimulation of glioma cells consistently increased the production of VEGF by these cells. The conditioned medium of the stimulated glioma cells induced activation of HUVECs, which could be inhibited by an anti-VEGF antibody.26 EGF also enhanced VEGF mRNA in several gastric cancer cell lines.33 In a bladder carcinoma cell line EGF increased the production of VEGF and bFGF.34 In gastric and pancreatic cell lines EGF stimulation also caused an enhanced production of neuropilin-1, a co-receptor of VEGFR-2, increasing the affinity of specific isoforms of VEGF to VEGFR-2.33,35

As mentioned previously, like other ligands of the ErbB family other than EGF, different ErbB receptors than EGFR play a role in angiogenesis as well.19 For example, stimulation of breast and lung cancer cells, which had a constitutive or engineered overexpression of ErbB-2, with heregulin-α, which induces ErbB3 and ErbB4 heterodimerization with ErbB2, induced the secretion of VEGF, while no significant effect was observed in normal mammary and bronchial cells.36,37 Overexpression of ErbB2 in human tumour cells is closely associated with increased angiogenesis and expression of VEGF.36,38 This effect may be caused by the increased synthesis of hypoxia-inducible factor 1 alpha (HIF-1α) in these cells.39 Finally, transformation of rat fibroblasts with a mutant of ErbB2 resulted in an increased VEGF production compared to the parental fibroblasts.18

Other than stimulation of the EGFR pathway, inhibition of the EGFR pathway using antibodies or TK inhibitors (see table 1 for an overview of mentioned therapies) confirmed the involvement of EGFR in tumour-induced angiogenesis.18,28,34,40-43 C225 or cetuximab, a monoclonal antibody to EGFR, reduced the production of VEGF and IL-8 by human transitional, pancreatic, colon and epidermoid (A431) cancer cells in vitro,18,34,41,43 whereas antibodies directed against the extracellular domain of ErbB2 resulted in a dose-dependent down-regulation of VEGF production by an ErbB2 positive breast cancer cell line.18 Gefitinib treatment of various cancer cell lines caused a dose- and time-dependent decrease of VEGF and bFGF production in vitro,42 while this agent also inhibited the expression of
cyclooxygenase-2, well-known to be involved in tumour-induced angiogenesis, in squamous cell carcinoma cell lines of the head and neck.\textsuperscript{44}

In summary, we may conclude that stimulation with EGF and/or overexpression of EGFR on tumour cells is associated with the production of angiogenic molecules, while inhibition of EGFR reduces the angiogenic profile of tumour cells.

<table>
<thead>
<tr>
<th>Agent</th>
<th>Type</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gefitinib (ZD 1839)</td>
<td>Tyrosine kinase inhibitor</td>
<td>EGFR</td>
</tr>
<tr>
<td>Erlotinib (OSI 774)</td>
<td>Tyrosine kinase inhibitor</td>
<td>EGFR</td>
</tr>
<tr>
<td>PKI 166</td>
<td>Tyrosine kinase inhibitor</td>
<td>EGFR</td>
</tr>
<tr>
<td>ZD 6474</td>
<td>Tyrosine kinase inhibitor</td>
<td>EGFR, VEGFR</td>
</tr>
<tr>
<td>AEE 788</td>
<td>Tyrosine kinase inhibitor</td>
<td>EGFR, ErbB2, VEGFR</td>
</tr>
<tr>
<td>PTK 787</td>
<td>Tyrosine kinase inhibitor</td>
<td>VEGFR</td>
</tr>
<tr>
<td>Sorafenib</td>
<td>Tyrosine kinase inhibitor</td>
<td>VEGFR, Raf kinase</td>
</tr>
<tr>
<td>AZD 2171</td>
<td>Tyrosine kinase inhibitor</td>
<td>VEGFR</td>
</tr>
<tr>
<td>Cetuximab (C 225)</td>
<td>Monoclonal antibody</td>
<td>EGFR</td>
</tr>
<tr>
<td>Bevacizumab</td>
<td>Monoclonal antibody</td>
<td>VEGF</td>
</tr>
<tr>
<td>DC 101</td>
<td>Monoclonal antibody</td>
<td>Murine VEGFR</td>
</tr>
</tbody>
</table>

\textbf{Table 1 - Several compounds for inhibiting EGFR and/or VEGF(R) signalling mentioned in this article.}

\textit{Angiogenic properties of EGFR signalling: in vivo studies}

ErbB ligands stimulate angiogenesis in preclinical models. This has been shown for EGF in a mouse cornea model,\textsuperscript{41} for heregulin in a chorioallantoic membrane assay\textsuperscript{26} and for heparin-binding EGF in a xenograft model, in which human bladder cancer cells are transplanted in nude mice.\textsuperscript{46}

Many xenograft models have been used to evaluate the effect of EGFR inhibitors on tumour growth, metastasis formation, angiogenesis, and survival. Several human cancer cell lines and several types of targeted therapies (TK inhibitors or antibodies) have been used in these assays. The anti-angiogenic effects of these treatments were measured by investigating apoptosis of tumour-associated endothelial cells and microvessel density (MVD) in tumour tissue. In addition, several angiogenic growth factors (bFGF, VEGF and IL-8) were measured in tumour tissue by immunohistochemistry (IHC).

Xenografts of pancreatic carcinoma showed a decreased expression of VEGF and IL-8 after several weeks of treatment with either cetuximab or PKI 166 (a TK inhibitor of EGFR), which was accompanied by decreased EGFR activation. The reduction in VEGF and IL-8 expression was associated with a decrease in MVD and an increase in endothelial cell apoptosis.\textsuperscript{40,41} Established A431 tumours also showed a prominent suppression of VEGF staining shortly after treatment with cetuximab.\textsuperscript{18} Similar results were reported by Perrotte et al, who administered cetuximab to animals with orthotopically established transitional cancer cells. Here, the decrease in VEGF, IL-8 and bFGF, assessed by IHC, preceded the
involution of tumour blood vessels. In a colon carcinoma model, therapy with either cetuximab or gefitinib resulted in a decrease of VEGF, bFGF and TGF expression and a reduction in microvessel count. Finally, Kedar et al reported that in vivo treatment with PKI 166 of human renal cell carcinoma in nude mice down-regulated expression of VEGF, IL-8 and bFGF and decreased MVD.

These studies show that activation of the EGFR pathway is involved in tumour associated angiogenesis. The anti-tumour effect of EGFR inhibitors in vivo might be partially due to anti-angiogenic effects. EGFR inhibitors affect endothelial cells via a direct or indirect pathway. It has been reported that patients whose tumours did not significantly express EGFR, could still respond to EGFR inhibitors. The effect of EGFR inhibitors on tumour-associated endothelial cells might be involved in the responsiveness of these patients.

Resistance to anti-EGFR therapy

Higher responses to gefitinib in non-small-cell lung cancer patients have been seen in Japanese patients, in women, in non-smokers, and in patients with adenocarcinomas. Furthermore, cutaneous rash seems to be a surrogate marker of clinical benefit. It was recently reported that specific EGFR mutations in the ATP binding site of the receptor are predictive for a clinical response to gefitinib. The value of EGFR mutations in predicting sensitivity to small molecule EGFR inhibitors needs to be confirmed on larger patient numbers and in prospective studies. In addition to the EGFR status, the status of specific downstream signalling molecules (Akt, MAPK) may determine the efficacy of EGFR TK inhibitors in vitro and in vivo.

As well as intrinsic resistance, acquired resistance to therapies which target biological processes in tumour cells can be expected, as this therapy deals with genetically unstable cells. Acquired resistance to EGFR inhibitors occurs in clinical studies and can be induced in preclinical models by continuous treatment with anti-EGFR agents. This reduced sensitivity to anti-EGFR therapy cannot be explained by changes in EGFR status, since total protein and phosphorylation of EGFR did not significantly change during treatment in two studies.

The mechanisms of acquired resistance to EGFR-inhibitors have recently been reviewed. Alteration or overactivity of oncogenic signalling pathways independently of EGFR is most likely responsible for resistance to EGFR inhibitors. For instance, increased activation of PI3K due to loss of PTEN expression as a result of mutations and/or gene deletions, or overexpression of other TK receptors (e.g. IGFR-1) led to the activation of the same central signalling pathways involving cell growth and survival. Here the acquisition of other potent oncogenic mutations, such as K-ras mutations, may also play an important role.
An additional mutation in the EGFR kinase domain of tumour cells harbouring the gefitinib or erlotinib sensitive mutation may result in acquired resistance to EGFR inhibitors in these initially sensitive tumour cells.\(^{60}\)

Another mechanism, which may be involved in the acquired resistance to EGFR inhibitors, is the upregulation of VEGF and other angiogenic molecules by tumour cells.\(^{57,58}\) Viloria-Petit et al showed that chronic treatment of mice harbouring A431 tumour explants induced resistance to cetuximab, which was associated with retained high EGFR expression and unaltered sensitivity to cetuximab when these tumour cells were harvested and cultured in vitro. Their increased growth potential in vivo could be explained by an increased production of VEGF, resulting in increased angiogenic potential.\(^{55}\) Ciardiello et al induced resistance to cetuximab and gefitinib by continuous treatment of colon cancer xenografts in athymic mice by these agents.\(^{56}\) These resistant colon cancer cells showed a 5-10 fold increase of activated MAPK and expression of cyclooxygenase-2 and VEGF, compared with parental colon cancer cells.

**Targeting two signalling pathways: EGFR and VEGFR**

If an increase in the angiogenic potential is involved in the acquired resistance to EGFR-inhibitors, it may be appealing to combine anti-EGFR drugs with anti-angiogenic drugs. This has been investigated preclinically using agents with dual inhibition of both EGFR and VEGFR (e.g. ZD6474 and AEE788), and with combination of single EGFR and VEGF(R) inhibitors.

ZD6474, a tyrosine kinase inhibitor of the VEGFR and the EGFR, was effective in colon cancer cell xenografts resistant to cetuximab and gefitinib, and continuous treatment with ZD6474 monotherapy resulted in efficient growth inhibition for up to 150 days in this model.\(^{55}\) In an orthotopical model of gastric or renal cell cancer ZD6474 markedly inhibited tumour growth, which was associated with reduced tumour cell proliferation and reduced microvessel density.\(^{61,62}\) AEE788, inhibiting tyrosine kinase of ErbB-1 and -2 and VEGFR-1 and -2, demonstrated anti-proliferative activity against a range of EGFR overexpressing tumour cell lines and EGF- and VEGF-stimulated HUVECs. In preclinical models this agent had significant anti-tumour activity, induced apoptosis of tumour-associated endothelial cells and decreased the microvessel density.\(^{63-67}\)

Addition of cetuximab to an anti-VEGFR antibody, DC101, resulted in a further significant reduction of tumour vascularity, tumour growth and formation of ascites, and increase in apoptosis of both tumour cells and endothelial cells in a colon peritoneal carcinomatosis model.\(^{68}\) In this model, cetuximab significantly augmented the anti-angiogenic effects of DC101.\(^{68}\) In an orthotopic gastric cancer mice model the combination of both agents was superior in tumour growth inhibition when compared with these agents alone.\(^{69}\) Combining
cetuximab with an antisense VEGF oligonucleotide in mice harbouring human colon cancer also resulted in a prolonged inhibition of tumour growth and reduction in MVD, which was more pronounced when compared to therapy with these agents alone.\textsuperscript{43} Moreover, Hidalgo mentioned additional anti-tumour effects when erlotinib was combined with the anti-VEGF antibody bevacizumab in a variety of preclinical models.\textsuperscript{70} Taken together, these preclinical investigations establish growing evidence that therapy combining anti-EGFR with anti-VEGF(R) could be of interest for cancer patients. A great number of clinical trials based on this concept are running. (Preliminary) results of phase I/II trials combining EGFR and VEGF(R) inhibitors are listed in table 2.

Generally, these trials were performed as second or third line treatment in patients with recurrent/advanced/metastatic cancers, who had undergone intensive therapies and for whom no further standard therapies were available. The toxicity in these studies was limited to rash, diarrhoea, hypertension and fatigue as the most common side effects, which seldom necessitated discontinuation of therapy. So far we may conclude that preliminary response evaluation has supported the preclinical data and that the combination of anti-EGFR and anti-VEGF(R)-therapy seems to be feasible and promising. The results for renal cell cancer patients were of special interest. The combination of erlotinib and bevacizumab resulted in 25% partial responses, 15% minor responses and 47% stable diseases,\textsuperscript{71} while EGFR inhibitors alone are not effective\textsuperscript{72} and bevacizumab alone generated a maximum of 10% partial responses in this disease population.\textsuperscript{73} A next step will be the combination of drugs targeting both EGFR and VEGF(R) together with chemotherapy or radiotherapy. Preclinical evidence supporting this approach is growing. Clinical trials are in preparation or running at many sides.

\textbf{Conclusion}

Strong preclinical evidence points to an important cross communication between activated EGFR pathways and angiogenesis in tumours. Therefore, the combination of inhibitors of both pathways is logical and may be more effective than blocking one or the other. Prolonged or repeated treatment with anti-EGFR therapy may cause resistance, partially due to the induction of a higher angiogenic potential. In preclinical studies, resistance to anti-EGFR therapy could be circumvented and survival could be improved by combining anti-EGFR with anti-angiogenic therapy. This encourages clinical exploration of this combination. Results of clinical studies combining angiogenesis inhibitors and EGFR inhibitors have been reported and they appear promising in a number of tumour types. Larger studies are being performed at this time and chemotherapy and/or radiotherapy will be added to this combination. Properly designed studies will try to dissect the
Table 2. Completed or ongoing clinical trials inhibiting EGFR and VEGF(R) with multiple or single agents in patients with advanced cancer.

<table>
<thead>
<tr>
<th>Phase</th>
<th>Regimen</th>
<th>Tumour type</th>
<th>Response</th>
<th>Response rate</th>
<th>Stable disease</th>
<th>Outcome</th>
<th>MST</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>II</td>
<td>B 5 mg/kg/2wks and C 400 mg/m2, 250mg/m2/wk</td>
<td>mCRC</td>
<td>8/35 (23%)</td>
<td>19/35 (54%)</td>
<td>-</td>
<td>-</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>B 10 mg/kg/2wks and E 150 mg/d</td>
<td>AOUJ</td>
<td>1/19 (5%)*</td>
<td>10/19 (53%)*</td>
<td>-</td>
<td>-</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>U/II</td>
<td>B 15 mg/kg/3wks and E 150 mg/d</td>
<td>SCC of HN</td>
<td>7/48 (14%)*</td>
<td>26/48 (54%)*</td>
<td>3.8 mo</td>
<td>6.8 mo</td>
<td>76</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>B 15 mg/kg/3wks and E 150 mg/d</td>
<td>MBC</td>
<td>1/9 (11%)*</td>
<td>2/9 (22%)*</td>
<td>-</td>
<td>-</td>
<td>77</td>
<td></td>
</tr>
<tr>
<td>U/II</td>
<td>B 15 mg/kg/3wks and E 150 mg/d</td>
<td>NSCLC (IIIb or IV)</td>
<td>8/40 (20%)</td>
<td>26/40 (65%)</td>
<td>6.2 mo^</td>
<td>12.6 mo^</td>
<td>78</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>B 10 mg/kg/2wks and E 150 mg/d</td>
<td>RCC</td>
<td>15/59 (25%)</td>
<td>36/59 (61%)</td>
<td>11 mo</td>
<td>22.8 mo</td>
<td>79</td>
<td></td>
</tr>
<tr>
<td>U/II</td>
<td>B 10 mg/kg/2wks and E 150 mg/d</td>
<td>RCC</td>
<td>4/44 (9%)*</td>
<td>27/44 (61%)*</td>
<td>At 9 mo 66%</td>
<td>At 9 mo 70%</td>
<td>79</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>AEE788 25-550 mg/d</td>
<td>-</td>
<td>1/69</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>Sorafenib 200 or 400 mg BID and Gefitinib 250 mg/d</td>
<td>- (large number NSCLC)</td>
<td>1/22</td>
<td>20/32 (63%)</td>
<td>18 wks</td>
<td>-</td>
<td>81</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>AZD2171 20-45 mg/d and Gefitinib 250 or 500 mg/d</td>
<td>-</td>
<td>1/15*</td>
<td>5/15*</td>
<td>-</td>
<td>-</td>
<td>82</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>AEE788 50-600 mg/d</td>
<td>GBM</td>
<td>0/26</td>
<td>2/26</td>
<td>At 6 mo 14%</td>
<td>-</td>
<td>83</td>
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</tr>
<tr>
<td>I</td>
<td>ZD6474 100-400 mg/d</td>
<td>- (NSCLC, n=9)</td>
<td>4/9 (all NSCLC)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>84</td>
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<tr>
<td>II</td>
<td>ZD6474 100 or 300 mg/d</td>
<td>MDC</td>
<td>0/46</td>
<td>-</td>
<td>7.5 weeks (both cohorts)</td>
<td>-</td>
<td>85</td>
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</tr>
</tbody>
</table>

contribution of each inhibitor, and identification of an optimal combination and sequence will eventually lead to improved outcomes for cancer patients.

References


64. Yigitbasi OG, Younes MN, Doan D, Jasser SA, Schiff BA, Bucana CD, Bekele BN, Fidler IJ, Myers JN. Tumor cell and endothelial cell therapy of oral cancer by dual tyrosine kinase receptor blockade. Cancer Res 2004;64:7977-84.


CHAPTER 4

Phase I evaluation of cediranib, a selective VEGFR signaling inhibitor, in combination with gefitinib in patients with advanced tumors

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Submitted for publication
Abstract

Cediranib is a highly potent and selective inhibitor of VEGFR signaling. Preclinical and clinical data suggest that inhibition of the VEGFR and EGFR pathways may be synergistic. Combination treatment with cediranib and gefitinib, an EGFR signaling inhibitor, was evaluated in patients with advanced solid tumors.

Ninety patients received treatment in this four-part, open-label study. Patients received once-daily oral doses of cediranib (20–45mg) and gefitinib 250mg (part A1; n=16) or 500mg (part B1; n=44). A cohort expansion phase investigated the potential pharmacokinetic interaction of cediranib 30mg with gefitinib 250mg (part A2; n=15) or 500mg (part B2; n=15). The primary objective was to assess the safety and tolerability of cediranib with gefitinib. Secondary assessments included pharmacokinetics, efficacy and pharmacodynamics.

Combination treatment was generally well tolerated; the protocol-defined maximum tolerated dose of cediranib was 30mg/day with gefitinib 250mg/day (part A1) and cediranib 45mg/day was the maximum dose investigated with gefitinib 500mg/day (part B1). The most common adverse events were diarrhea (84 [93%]), anorexia (63 [70%]) and fatigue (60 [67%]). Cediranib pharmacokinetic parameters were not substantially different when given alone or in combination with gefitinib. Efficacy results included eight (9%) confirmed partial responses and 38 (42%) patients with stable disease. Pharmacodynamic assessments demonstrated changes in levels of VEGF and soluble VEGFR-2 following treatment.

Combination treatment was generally well tolerated and showed encouraging antitumor activity in patients with advanced solid tumors. These results merit further exploration.
Introduction

Vascular endothelial growth factor (VEGF) is a key factor in tumor-associated angiogenesis, and exerts its effects via three high-affinity receptors: VEGFR-1, VEGFR-2, both present on vascular endothelium, and VEGFR-3, present on lymphatic endothelium. VEGFR-2 appears to play a predominant role in tumor-associated angiogenesis and therefore is considered an important target for inhibition of tumor growth.¹ The epidermal growth factor receptor (EGFR) tyrosine kinase is expressed at high levels in most human solid tumors and has been associated with progression and poor prognosis in non-small-cell lung cancer (NSCLC).²,³ In addition, EGFR signaling has also been shown to be involved in tumor–host interactions such as angiogenesis.⁴-⁷

Since tumor growth and progression depends on tumor–host interactions as well as tumor cell characteristics,⁸,⁹ combining inhibition of VEGFR- and EGFR-dependent signaling with may be a useful approach to treating cancer. Preclinical data suggest that concomitant targeting of the VEGFR and EGFR signaling pathways may lead to greater antitumor activity than inhibition of either pathway alone.¹⁰-¹³ Furthermore, the results of a recent Phase II study in advanced NSCLC support the combined use of anti-VEGF (bevacizumab) and anti-EGFR (erlotinib) therapy compared with chemotherapy alone.¹⁴

Cediranib (RECENTIN™) is a highly potent and selective inhibitor of all three VEGFRs.¹⁵ Cediranib has shown broad-spectrum antitumor activity in a range of histologically diverse xenograft models.¹⁵ During early clinical evaluation, cediranib monotherapy was generally well tolerated at doses ≤45mg. The most common adverse events (AEs) were fatigue, hypertension and gastrointestinal toxicity, and dose-limiting toxicities (DLTs) were hypertension and diarrhea. There was also encouraging evidence of antitumor activity and the pharmacokinetic profile supported a once-daily oral dosing regimen.¹⁶

Gefitinib (IRESSA™) is an EGFR tyrosine kinase inhibitor that has demonstrated non-inferiority relative to docetaxel in terms of overall survival in unselected patients with pretreated advanced NSCLC in the recently reported Phase III INTEREST study.¹⁷

The primary objective of the present study was to assess the safety and tolerability of combination therapy with cediranib and gefitinib in patients with advanced cancers. Secondary objectives included an investigation of the pharmacokinetics of cediranib when given alone or in combination with gefitinib, a preliminary assessment of the antitumor activity of this regimen, and an exploration of the effects of treatment on biological markers.
Patients and methods

Patient eligibility
Patients with advanced cancer refractory to standard treatment were recruited to one of three centers in the Netherlands. Patients were required to have a life expectancy of at least 12 weeks and a WHO performance status of 0–2. The main exclusion criteria were inadequate bone marrow reserve; renal or hepatic dysfunction; poorly controlled hypertension; co-existing malignancies; symptomatic brain metastases; and spinal cord compression. Patients with a history of interstitial lung disease or significant arrhythmia were also excluded. Written informed consent was provided by all patients. The trial was approved by all relevant institutional ethical committees or review bodies, and was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice.

Study design
This was an open-label, multicenter study conducted in four parts (Figure 1; study code 21711L0004). The first part of the study was a dose-escalation phase in which successive cohorts of at least three patients received fixed daily oral doses of cediranib (20–45mg) combined with fixed daily oral doses of gefitinib 250mg (part A1) or 500mg (part B1). The starting dose of cediranib 20mg was selected based on the safety, tolerability and biological activity data from a previous cediranib monotherapy study.16
To identify the maximum tolerated dose (MTD) of cediranib with gefitinib 250mg or 500mg in parts A1 and B1 patients were considered evaluable for inclusion in dose escalation decisions once they had completed 21 days of continuous daily treatment or if they experienced a dose limiting toxicity (DLT) within 21 days. Providing <33% of patients experienced a DLT the dose was defined as tolerable and dose escalation was continued. If a DLT was observed in ≥33% to <50% of patients, the cohort was expanded to include a further three patients. If a DLT was observed in ≥50% of patients, this dose was considered above the MTD and dose escalation was stopped. The dose one level below was determined to be the MTD. Once the MTD had been defined in parts A1 and B1, the pharmacokinetics of cediranib were explored alone and in combination with gefitinib in the cohort expansion part of the study. Patients (15 per cohort) received once-daily cediranib as monotherapy for 7 days and then in combination with gefitinib 250mg (part A2) or 500mg (part B2) for 14 days.
Phase I evaluation of cediranib and gefitinib

**Safety and tolerability**

A DLT was defined as: an increase from baseline in QT or QTc interval of at least 100ms or a QT or QTc interval of more than 550ms; or two consecutive electrocardiogram (ECG) measurements recorded at least 24 hours apart where both ECGs had a QTc interval of ≥500 but <550ms or both ECGs showed an increase of ≥60ms from baseline QTc to a value of ≥460ms; or grade 3 (of the National Cancer Institute Common Terminology Criteria [CTC] version 3.0) or higher hypertension (using modified CTC as described previously\(^\text{16}\)); or any other CTC AE ≥grade 3 (with the exception of rash or diarrhea, where the safety committee considered whether the frequency of these events was greater than that observed with gefitinib alone from experience and historical data).

Blood samples for determination of clinical chemistry and hematology parameters were taken on day 1 and weekly for the first 4 weeks in part A1/B1, 3 weeks in part A2/B2, and every 2 weeks thereafter. Systolic and diastolic blood pressure (SBP and DBP, respectively) measurements were taken at baseline and day 1 (pre-dose and up to 24 hours post-dose) and every 7 days thereafter.

**Pharmacokinetic assessments**

In parts A2/B2, blood samples were collected for determination of plasma levels of cediranib and gefitinib at the timepoints indicated in Figure 1. Plasma concentrations of cediranib and gefitinib were analyzed using high-performance liquid chromatography with tandem mass spectrometric (HPLC-MS-MS) detection. Maximum and minimum steady-state drug concentration (\(C_{\text{max,ss}}\) and \(C_{\text{min,ss}}\)), and the time to reach maximal concentration...
(T_{max}) were determined by visual inspection of the individual plasma concentration–time profiles. The area under the plasma profiles at steady-state (AUC_{ss}) was estimated by the linear trapezoidal algorithm to the last data point of the dosing interval.

**Tumor response evaluation**

Tumor response was evaluated according to Response Evaluation Criteria in Solid Tumors (RECIST). Baseline imaging was performed no more than 4 weeks before the start of study treatment. Subsequent assessments were performed 4 weeks (part 1)/5 weeks (part 2) from the start of treatment and every 6 weeks thereafter.

**Pharmacodynamic analyses**

Serum and plasma samples were collected at the times shown in Figure 1 for measurement of surrogate markers of angiogenesis and activated endothelial cells. VEGF and basic fibroblast growth factor (bFGF) were measured in plasma samples, with soluble VEGFR-2 (sVEGFR-2) and soluble tunica interna endothelial cell kinase (sTie-2) were measured in serum. Parameters were determined as described previously.

**Statistical analyses**

There was no formal statistical analysis for the assessments of safety and tolerability. Response data were listed and summarized. Biomarker data were plotted and summarized as appropriate.

Pharmacokinetic data collected in Parts A2 and B2 were log_{e}-transformed and analyzed separately and combined using a paired t-test. Results were then back-transformed to provide a point estimate and the corresponding two-sided 90% confidence interval (CI) for the ratio (cediranib + gefitinib/cediranib). The intent of this analysis was to estimate the effect of gefitinib on the pharmacokinetics of cediranib in order to support the safety and tolerability conclusions, and not to formally demonstrate no clinically meaningful effect of gefitinib on the pharmacokinetics of cediranib.

The number of patients in parts A1 and B1 was based on the desire to obtain adequate safety and tolerability data while exposing as few patients as possible to the study medication. In parts A2 and B2 the number of patients was based on obtaining adequate data to assess the pharmacokinetics of cediranib alone and in combination with gefitinib.
Table 1. Patient characteristics

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<th>Study part</th>
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<th>A2</th>
<th>B1</th>
<th>B2</th>
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*Histologies include NSCLC (n=7), bronco alveolair (n=2), mesothelioma (n=2), lung, not otherwise specified (n=2), pancoast (n=1)
Table 2. Adverse events. (a) Number of patients with adverse events occurring during treatment (≥15%), irrespective of causality. (b) Number of patients with adverse events meeting dose-limiting toxicity criteria in part A1 and B1 (all grade 3)

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<th>B2</th>
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<td>500</td>
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ALT, alanine aminotransferase; AST, aspartate aminotransferase; PPE, palmar-plantar erythrodysesthesia syndrome (hand-foot syndrome)
Table 2 (b)

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<thead>
<tr>
<th></th>
<th>A1</th>
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<tr>
<td>Rash</td>
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</table>

As no dose escalation occurred in parts A2 or B2, the DLTs for these patients are not presented. Patients with AEs is more than one category are counted once in each of those categories. *Criteria for DLT was any AE grade 3 not clearly related to disease progression and considered by the investigator to be related to cediranib and or gefitinib treatment (for the purpose of this table, only AEs considered to be related to cediranib are summarized). In order to try and simulate the conditions that dose-escalation decisions would have been made under, the AEs included in this table are only those within 21 days from the first cediranib dose. AEs presented here have been calculated prospectively using study data and are not those highlighted during the study as DLTs. AE, adverse event; PPE, palmar-plantar erythrodysesthesia syndrome (hand-foot syndrome)
Results

Patient characteristics
Between May 2004 and February 2006, 92 patients were enrolled into the study and of these 90 received treatment and were included in the safety analysis (Table 1). Two patients did not receive treatment due to AEs which occurred prior to the first cediranib dose. At the time of data cut off (August 2006) the mean duration of treatment was 113.9 days (range 2–395) in part A and 144.7 days (range 12–365) in part B, with 12 patients still on treatment at the time of database lock.

Safety and tolerability
Overall, the most frequent AEs in both parts of the study were diarrhea, anorexia and fatigue (Table 2a). These were also among the most common AEs considered to be related to either treatment. The other most common AEs attributed by the investigator to study treatment were hypertension (cediranib), and rash and dry skin (both gefitinib).
AEs that met the criteria for DLT in parts A1 and B1 are listed in Table 2b. In part A1, a higher percentage of patients experienced DLTs in the 45mg group (3 patients [38%]) compared with the 30mg group (1 patient [20%]); no patients in the 20mg group experienced a DLT. The only DLT experienced by >1 patient in any one group was hypertension (2 patients [25%] in the 45mg group). On the basis of a higher number of DLTs in part A1 at 45mg, the MTD of cediranib was identified as 30mg in combination with gefitinib 250mg. Once the 30mg dose was declared tolerable in part B1, this dose was taken forward into part B2 (the protocol prevented the dose in part B1 being escalated above the MTD in part A1). However, since the 30mg dose was considered to be well tolerated in part B1 the safety review committee amended the protocol to allow further dose escalation to 37.5mg and then to 45mg in part B1, while part B2 was ongoing. There were no plans to escalate the dose beyond 45mg since this was the MTD in the monotherapy study. In part B the dose response for DLTs was less clear than for part A, with a higher percentage of patients in the 45mg (4 patients [57%]), 37.5mg (7 patients [54%]) and 25mg (5 patients [63%]) groups experiencing DLTs, compared with the 30mg (2 patients [25%]) and 20mg (3 patients [38%]) groups. As a result of the caveat regarding diarrhea in the DLT definition, cediranib 45mg in combination with gefitinib 500mg, the maximum dose reached in part B1, was considered to be a tolerated dose. In total 10 patients died during the study; none of the deaths were considered by the investigator to be related to study treatment and all were considered to be a result of the patients’ underlying disease.
Increases in thyroid-stimulating hormone (TSH) above the normal range (>5 mu/L) were observed in approximately 37% of patients with a baseline and a post-dose reading. All of these increases were seen in patients receiving cediranib ≥30mg. A minority of these patients developed reductions in free or total thyroxine (T4) to levels below the lower limit of normal; there was only one reported AE of symptomatic hypothyroidism (CTC grade
2). With the exception of TSH, there were no clinically relevant trends in laboratory parameters and, with the exception of blood pressure, no clinically relevant trends in vital signs, physical findings or ECG observations. The majority of patients experienced early, dose-dependent increases in systolic and diastolic blood pressure during part A; in part B, the increases were less marked and were not dose dependent (Figure 2).

**Pharmacokinetics**

The pharmacokinetic parameters of cediranib and gefitinib were explored in parts A2 and B2 (Figure 3). In part A2, a 21% reduction in AUCss was observed for cediranib 30mg when received in combination with gefitinib 250mg for 14 days compared with cediranib 30mg when received alone for 7 days. A smaller reduction (8%) in AUCss in part B2 was observed for cediranib 30mg received in combination with gefitinib 500mg compared with cediranib 30mg received alone. Results of a pooled analysis of data from parts A and B showed a statistically significant 16% reduction in AUCss. These results are summarized in Table A1. The same pattern was not observed for Css,max; the Css,max of cediranib 30mg was similar when received in combination with gefitinib 250mg or 500mg compared with cediranib 30mg alone (Table 3a). Results of a pooled analysis of data from Parts A and B showed a 2% reduction in Css,max.

The pharmacokinetic profile of gefitinib following administration of gefitinib (250 or 500mg) in combination with cediranib was similar that seen previously when gefitinib was administered as monotherapy (Table 3b).20,21

**Efficacy**

Preliminary evidence of antitumor activity was observed with eight patients (9%) achieving a partial response with a median duration of response of 4.2 months (range 1.4–12 months); 6/18 patients with renal cell carcinoma (RCC, 20mg/500mg; 25mg/ 500mg; 30mg/500mg [n=3]; 37.5mg/500mg), 1/14 with lung cancer (mesothelioma; 45mg/250mg) and one with a bone tumor (30mg/500mg). Figure 4a shows computed tomography (CT) scans of a 73-year-old male patient diagnosed with metastatic RCC who experienced a partial response. In addition, 38 patients experienced stable disease. These included nine patients with a confirmed minor response (10–30% reduction); four with RCC (20mg/500mg; 25mg/500mg; 30mg/500mg 45mg/500mg), two with colorectal cancer (37.5mg/500mg; 45mg/500mg), two with a primary lung tumor (30mg/250mg; 45mg/500mg) and one with a right orbital tumor (25mg/500mg). Figure 4b shows the best change in target lesion size for each patient in parts A1 and B1 (waterfall plot). The results are suggestive of a dose response.
**Phase I evaluation of cediranib and gefitinib**

### Summary statistics

#### Parameter (units)

<table>
<thead>
<tr>
<th>Parameter (units)</th>
<th>Summary statistics</th>
<th>cediranib 30 mg (n = 12)</th>
<th>cediranib 30 mg + gefitinib 250 mg (n = 12)</th>
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<tbody>
<tr>
<td>$C_{\text{ss, min}}$ (ng/mL)</td>
<td>gmean (CV%)</td>
<td>81.8 (58.3)</td>
<td>81.8 (58.3)</td>
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<td>$C_{\text{ss, max}}$ (ng/mL)</td>
<td>gmean (CV%)</td>
<td>25.7 (78.1)</td>
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<tr>
<td>$t_{\text{max}}$ (h)</td>
<td>Median (range)</td>
<td>3.0 (1.9 to 8.0)</td>
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<tr>
<td>AUC$_{\text{ss}}$ (ng.h/mL)</td>
<td>gmean (CV%)</td>
<td>1170 (84.4)*</td>
<td>918 (54.3)</td>
</tr>
</tbody>
</table>

*Data were only available for 11 patients

### Figure 3

- Geometric mean (± standard deviation) plasma concentration of cediranib 30mg on days 7 and 21. Inlaid tables show pharmacokinetic parameters for cediranib.

(a) Part A2, (b) part B2
### Table 3a. Statistical analysis of the effect of gefitinib on the pharmacokinetics of cediranib

<table>
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<th>cediranib 30mg + gefitinib 500mg : cediranib 30mg</th>
<th>cediranib 30mg + gefitinib 250 or 500mg : cediranib 30mg</th>
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<tr>
<td></td>
<td></td>
<td>n</td>
<td>11</td>
<td>8</td>
</tr>
<tr>
<td>AUC(_{ss}) (ng.h/ml)</td>
<td>Ratio</td>
<td>0.79</td>
<td>0.92</td>
<td>0.84</td>
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<tr>
<td></td>
<td>(90% CI)</td>
<td>(0.62, 1.00)</td>
<td>(0.74, 1.15)</td>
<td>(0.72, 0.98)</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>12</td>
<td>11</td>
<td>23</td>
</tr>
<tr>
<td>C(_{ss,max}) (ng/ml)</td>
<td>Ratio</td>
<td>0.96</td>
<td>1.01</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td>(90% CI)</td>
<td>(0.73, 1.26)</td>
<td>(0.84, 1.21)</td>
<td>(0.84, 1.15)</td>
</tr>
</tbody>
</table>

Ratios of >1 indicate an increase in exposure of cediranib when dosing with gefitinib AUC\(_{ss}\), area under plasma concentration–time curve at steady state; C\(_{ss,max}\), maximum plasma drug concentration at steady state; CI, confidence interval.

### Table 3b. Pharmacokinetic parameters for gefitinib

<table>
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<td>C(_{ss,max}) (ng/ml)</td>
<td>gmean (CV%)</td>
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<td>C(_{ss,min}) (ng/ml)</td>
<td>gmean (CV%)</td>
<td>112 (107)</td>
<td>305 (114)</td>
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<td>t(_{max}) (h)</td>
<td>Median (range)</td>
<td>4.0 (2.0–8.1)</td>
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<td>AUC(_{ss}) (ng.h/ml)</td>
<td>gmean (CV%)</td>
<td>3630 (84.8)</td>
<td>9520 (87.8)*</td>
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</table>

*Data were only available for 9 patients
Figure 4 - Tumor response evaluation. (a) Durable partial response of a 73-year-old male with RCC. Duration of response was 7 months; RECIST assessment halted due to surgery of metastases after which the patient became disease free. (b) Best change in target lesion size. Each bar represents one patient. Scan data were not available for eight patients. Of these four withdrew before the first scan, one due to an adverse event and three withdrew consent.
(a) Median percentage change in VEGF levels from baseline

- Median percentage change in VEGF levels from baseline.
- Doses of Cediranib (mg): 20, 30, 45.
- Doses of Gefitinib (mg): 250, 300.
- Data points for Day 8 and Day 28.

(b) Median percentage change in bFGF levels from baseline

- Median percentage change in bFGF levels from baseline.
- Doses of Cediranib (mg): 20, 30, 45.
- Doses of Gefitinib (mg): 250, 300.
- Data points for Day 8 and Day 28.
Figure 5 - Changes in (a) VEGF, (b) bFGF, (c) soluble VEGFR-2 and (d) soluble Tie-2 levels on days 8 and 28. Data are presented on a log scale.
Pharmacodynamics

Acute increases in VEGF levels were seen at all cediranib doses (Figure 5a). Acute increases in bFGF levels were observed in some patients although data were generally inconclusive (Figure 5b). Reductions in sVEGFR-2 levels were observed following treatment, although no clear dose-relationship could be detected (Figure 5c). Decreases were seen in sTie-2 levels (Figure 5d).

Discussion

This is the first clinical evaluation of cediranib in combination with gefitinib. Combination treatment was generally well tolerated with manageable AEs. The protocol-defined MTD of cediranib was 30mg/day with gefitinib 250mg/day (part A1). Cediranib 45mg/day was the maximum dose investigated with gefitinib 500mg/day (part B1) and was considered to be a tolerated dose, however as no further doses were investigated it was not possible to formally identify this dose as the MTD in part B1. Cediranib 30mg steady-state plasma pharmacokinetic parameters were not substantially different when cediranib was given alone for 7 days or in combination with gefitinib 250 or 500mg for 14 days. Encouraging evidence of antitumor activity was observed following combination treatment in this population of patients with advanced solid tumors.

The most common AEs considered to be related to cediranib were diarrhea, hypertension, anorexia and fatigue. Diarrhea, anorexia and fatigue have previously been associated with either agent alone.16,22 Hypertension is emerging as a class effect of VEGF signaling inhibitors.16,23-26 In this study hypertension was generally mild (most events were CTC grades 1 and 2) and manageable using a standardized hypertension management protocol.27 Skin toxicities were also frequently reported, such as dry skin, hand–foot syndrome and acneiform rash. Acneiform rash is most likely to result from EGFR inhibition by gefitinib.28 Hoarseness has been linked with VEGF signaling inhibition29 and was reported by 44 (49%) patients in this study. Increases in TSH were observed, particularly at cediranib 30mg and above, however no consistent reductions in total or free T4 were observed. Modulation of the function of the thyroid gland has been associated with certain inhibitors of VEGF signaling30-33 although the mechanism underlying the association is unclear.

The pharmacokinetic results did not show any increase in exposure to cediranib following the addition of gefitinib, suggesting that any augmentation in antitumor activity of cediranib and gefitinib compared with either agent alone results from additional mechanisms of action rather than an altered drug exposure. Reductions in AUC_{ss} were observed in both part A2 and B2 of the study; the reduction was of greater magnitude in part A2 (21%) when patients were receiving gefitinib 250mg compared with part B (8%)
when patients were receiving gefitinib 500mg. From the 90% confidence intervals of the combined analysis, overall reductions in AUC_{ss} in excess of 28% were ruled out. No significant change in C_{ss,max} was observed in either part A2 or B2. Taking the AUC_{ss} results into consideration, along with the lack of any clinically or statistically significant changes in C_{ss,max} it was concluded that changes seen in cediranib 30mg steady-state plasma pharmacokinetic parameters, when given for 7 days or in combination with gefitinib 250 or 500mg for 14 days, did not appear to be substantial or clinically meaningful.

In this study combination treatment with cediranib and gefitinib showed encouraging antitumor activity with eight patients (9%) achieving a partial response. In addition, 38 patients (42%) had a best response of stable disease, of which nine patients had confirmed reductions of 10–30% on consecutive visits. A recent Phase I study of sorafenib in combination with gefitinib with refractory or recurrent NSCLC patients reported that one patient (3%) achieved a partial response and 20 (65%) experienced stable disease.\(^3^4\) The most common primary tumor type was RCC (18 patients). The response rates in this subgroup were of particular interest given the positive efficacy data from two recent Phase III studies of the multitargeted tyrosine kinase inhibitors sorafenib\(^3^5\) and sunitinib.\(^3^6\) In the present study six patients (33%) with RCC achieved a partial response with a median response duration of 6 months (range 3.5–12) at the time of the analysis. Although the number of RCC patients in the present study was small, the antitumor activity may be largely due to treatment with cediranib since previous studies of EGFR inhibitors as monotherapy showed poorer response rates.\(^3^7\)-\(^3^9\) Furthermore, in a recently reported Phase II study of cediranib in patients with previously untreated metastatic RCC, 38% of patients achieved a partial response.\(^4^0\)

In early clinical trials, biomarker assays can provide supporting evidence as to whether the drug concentration achieved can inhibit the putative target(s). Consistent with findings from a previous study of cediranib monotherapy, decreases in sVEGFR-2 levels were observed following treatment.\(^1^6\) This reduction might reflect a decreased release or production of VEGFR-2 by vascular endothelial cells. An increase in VEGF levels were also observed in this study, consistent with previous studies of cediranib\(^1^6\) and other VEGFR tyrosine kinase inhibitors.\(^4^1\) This increase might partly reflect an increased production of VEGF by tumor cells due to acute stress induced by inhibition of VEGFR tyrosine kinase signaling. However, recent preclinical data suggest that the canonical changes in sVEGFR-2 and VEGF observed with VEGFR tyrosine kinase inhibitors may derive from both tumor-independent and tumor-dependent responses.\(^4^2\) Decreased tumor perfusion induced by cediranib and gefitinib treatment was established by CT perfusion in subset of patients in this study.\(^4^2\) The results of the current study show that the combination of cediranib and gefitinib is generally well tolerated with evidence of clinical activity, and supports the rationale for concurrent inhibition of the VEGFR and EGFR signaling pathways. Further
investigation is merited. This combination may be of particular interest in patients with RCC and NSCLC.

Acknowledgements

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We would like to thank Ute Zirrgiebel for assessment of soluble biomarkers.

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CHAPTER 5

Defective differentiation of myeloid and plasmacytoid dendritic cells in advanced cancer patients is not normalized by tyrosine kinase inhibition of the vascular endothelial growth factor receptor

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Abstract

Tumor-derived vascular endothelial growth factor (VEGF) has previously been identified as a causative factor in the disturbed differentiation of myeloid dendritic cells (DC) in advanced cancer patients. Here, we investigated the potential of vascular endothelial growth factor receptor (VEGFR) tyrosine kinase (TK) inhibition to overcome this defective DC differentiation. To this end peripheral blood DC (PBDC) precursor and subset frequencies were measured in 13 patients with advanced cancer before and after treatment with AZD2171, a TK inhibitor (TKI) of VEGFR, co-administered with gefitinib, an epidermal growth factor receptor (EGFR) TKI. Of note, not only myeloid DC but also plasmacytoid DC frequencies were significantly reduced in the blood of the cancer patients prior to treatment, as compared to healthy controls. Moreover, beside an accumulated population of immature myeloid cells (ImC), a population of myeloid suppressor cells (MSC) was significantly increased. Upon systemic VEGFR TK inhibition DC frequencies did not increase, whereas the rate of circulating MSC showed a slight, but not significant, decrease. In conclusion, TK inhibition of VEGFR with AZD2171 does not restore the defective PBDC differentiation observed in advanced cancer patients.
Introduction

Defective dendritic cell (DC) differentiation, maturation and functionality is one of the mechanisms underlying impaired anti-tumor immunity in cancer patients [1]. DCs play a central role in the immune system as powerful antigen-presenting cells, and are essential for the induction of tumor-specific T cell-mediated immune responses [2]. In cancer patients the frequencies of circulating DCs are significantly lower as compared to healthy individuals [3-5]. Accumulation of immature myeloid cells (ImC) and functionally impaired DCs has been documented in blood, tumors, and tumor-draining lymph nodes and found to be a poor prognostic factor [3, 4, 6]. Preclinical studies show that tumor-induced inhibition of DC differentiation is mediated by tumor-derived soluble factors such as IL-10, IL-6, M-CSF, prostaglandins, and vascular endothelial growth factor (VEGF) [7-11]. VEGF, produced by most tumors, is a strong inhibitor of myeloid DC differentiation in vitro [7] and affects the early stages of functional DC differentiation [12, 13]. High systemic VEGF levels present in most cancer patients correlate with low DC frequencies [3, 4], while abnormally elevated numbers of immature DC precursors reportedly decreased in three out of three cancer patients during treatment with the anti-VEGF antibody bevacizumab [3]. VEGF is also one of the most important pro-angiogenic molecules and induces proliferation, differentiation, and migration of endothelial cells in tumors. Over the past decades, many trials with inhibitors of angiogenesis have been conducted and have resulted in the registration of bevacizumab as anti-cancer therapy [14]. VEGF exerts its effect via binding to three tyrosine kinase (TK) receptors, VEGFR-1, -2 and -3, which are mainly, but not exclusively, present on endothelial cells (VEGFR-1, and -2) and lymphatic endothelium (VEGFR-3) [15]. Blocking VEGF signaling by inhibiting TK activity of its receptor is a promising anti-cancer strategy. AZD2171 is a novel potent inhibitor of VEGFR-2 kinase activity, with additional activity against VEGFR-1 and -3 [16]. Currently, AZD2171 is being evaluated in clinical trials as an oral anti-cancer agent with anti-angiogenic effects in a variety of solid tumors. To further investigate the relationship between VEGFR signaling and DC differentiation we evaluated the effect of administration of the VEGFR inhibitor AZD2171 on peripheral blood DC (PBDC) subsets in advanced cancer patients. To our knowledge, this is the first study to monitor DC subsets in the blood of cancer patients who are treated with a VEGFR tyrosine kinase inhibitor (TKI).
Material and methods

Patients and healthy donors
Between May 2004 and December 2004, 13 patients of the VU Medical Center, Amsterdam, were selected to participate in a phase I study combining AZD2171 (AstraZeneca, Wilmington DE), a VEGFR TKI [16], with gefitinib (AstraZeneca, Wilmington DE), a TKI of the epidermal growth factor receptor (EGFR). The dose of AZD2171 was escalated in small, consecutive cohorts of advanced cancer patients co-administered with 250 mg gefitinib in order to establish a maximum tolerated dose. Thirteen patients (3 women, 10 men) were included in one of the three AZD2171 dosing cohorts: 20 mg (n = 3), 30 mg (n = 7), and 45 mg (n = 3). Major inclusion criteria were locally advanced or disseminated disease, which was refractory to standard therapy, age over 18 years, and a performance status of 0-2. Excluded were patients with impaired renal or liver function or inadequate bone marrow reserve. The clinical trial was approved by the Medical Ethical Committee, and after obtaining informed consent, blood from the patients (mean age of 52 [range 31-66]) was drawn before treatment and after 4 or 5 weeks of daily oral dosing of both drugs (depending on the dosing schedule of the protocol). A variety of primary tumors was represented: colon cancer (n = 3), mesothelioma (n = 2), melanoma (n = 2), fibrosarcoma, osteosarcoma, renal cell cancer, cervical cancer, pancreas cancer and NSCLC (all n = 1). After 4 or 5 weeks of treatment tumor status was evaluated according to RECIST [17].

Two control groups were included. First, blood was drawn from nine age- and sex-matched healthy donors (2 women, 7 men; mean age of 47 [range 32-55]) to collect peripheral blood mononuclear cells (PBMCs). A second control group consisted of four advanced non-small cell lung cancer (NSCLC) patients (1 woman, 3 men; mean age of 61 [range 51-66]) who received gefitinib monotherapy. PBMCs were obtained at baseline and 4 weeks after daily dosing of 250 mg gefitinib.

PBDC monitoring
PBMCs were isolated by Ficoll density-gradient centrifugation (Lymphoprep™, Oslo, Norway) within 24 hours of blood sampling. FACS analysis (Becton Dickinson, Franklin Lakes, NJ) was performed to measure peripheral blood DC (PBDC) subset frequencies and their maturation status using four-color staining with antibodies directly conjugated with fluorochromes FITC, PE, PerCP-Cy5, or APC. Monoclonal antibodies against the following markers were used: CD3, CD11c, CD14, CD19, CD56, CD86, CD123, EGFR, HLA-DR (all BD Biosciences, San Jose, CA), BDCA-1, BDCA-2, BDCA-3, BDCA-4 (all Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), VEGFR-1, VEGFR-2 (R&D Systems, Minneapolis, MN) and CD33 (Immunotech, Marseille, France). A gate was set on the
lymphocyte/mononuclear populations based on the forward and sideward scatter plots to avoid erythrocytes, cell debris and neutrophil contamination.

Different PBDC precursor populations and subsets were studied, reflecting different stages of development (schematically presented for the myeloid lineage in Fig. 1). **Myeloid DC (MDC):** Immature myeloid cells (ImC), previously identified as MDC/macrophage precursors in varying stages of differentiation [18], were defined as positive for CD11c, but negative for the Lineage (Lin) markers CD3 (T cells), CD14 (monocytes), CD19 (B cells), and CD56 (NK cells), as well as for HLA-DR as previously described [3]. More mature MDC precursors (pMDC) were defined as CD11c<sup>hi</sup>Lin<sup>-</sup>HLA-DR<sup>+</sup> and the frequencies of two MDC subsets contained within this population were more specifically determined based on the expression of Blood DC Antigen (BDCA) markers: DCs belonging to the so-called myeloid DC subset-1 (MDC-1) were identified as CD11c<sup>hi</sup>, CD14<sup>+</sup>, and BDCA-1/CD1c<sup>+</sup>, and MDC-2 were detected as CD11c<sup>+</sup>, CD14<sup>-</sup>, and BDCA-3/CD141<sup>+</sup> [19]. **Plasmacytoid DC (PDC):** PDCs were detected as CD11c<sup>-</sup>, CD14<sup>-</sup>, CD123<sup>hi</sup>, and BDCA-2<sup>+</sup> or as Lin<sup>-</sup>BDCA4<sup>+</sup>. As previously described, in blood both BDCA-2 and BDCA-4 are exclusively expressed on PDCs [19].

**Figure 1** - A model of myeloid dendritic cell differentiation under cancer conditions, in which tumor-derived factors exert their inhibitory effect at the stage of immature Lin<sup>-</sup> CD11c<sup>+</sup> HLA-DR<sup>-</sup> myeloid cells (ImC), blocking their differentiation into mature myeloid DC precursors (pMDC), while simultaneously skewing their differentiation towards a population of CD14<sup>+</sup> HLA-DR<sup>hi/low</sup> myeloid suppressive cells (MSC).

HPC: Hematopoietic cells; Lin: Lineage markers (CD3, CD14, CD19 and CD56); MDC: myeloid dendritic cell.
Data were obtained from a minimum of 150,000 cells and were analyzed using CellQuest software (Macintosh). Results are shown as percentages of the total number of PBMCs. Labelled isotype-matched IgG antibodies were used to determine background fluorescence in each analysis. Median fluorescence indices were calculated by dividing the median expression of the antibody of interest by the median background fluorescence as determined by the isotype-matched IgG antibody.

**Measurement of circulating VEGF**
At the time of PBMC isolation, serum samples were also collected. After clot formation (60 minutes at room temperature) and centrifugation, serum was harvested and stored at -80°C. Circulating VEGF levels were measured in serum samples with a Quantikine ELISA kit (R&D systems, Minneapolis, MN), following the manufacturer’s instructions.

**Statistical analysis**
Since we could not assume a normal distribution of the DC subset frequencies, we applied nonparametric tests. Percentages of PBDCs and levels of circulating VEGF obtained from samples during VEGFR TK inhibition were compared with baseline values using a Wilcoxon signed ranks test and a paired t-test respectively, to determine statistical significance. In addition, the Mann-Whitney U test was used to determine significance of differences between patient and healthy donor data. Differences were considered to be statistically significant when p < 0.05.

**Results**
Thirteen patients included in the phase I study combining AZD2171 with gefitinib participated in the DC monitoring study. Preliminary efficacy of treatment was observed in 4 patients. In one patient a partial remission was observed; in three patients a decrease in tumor size was noticed, but this decrease did not reach the criteria for partial remission and was considered stable disease.

**VEGFR-1, VEGFR-2 and EGFR expression on the studied PBDC subsets**
Different PBDC precursor populations and subsets were studied, reflecting different stages of development, as described in Materials and Methods (see also Fig.1). To establish the ImC, pMDC and the MDC and PDC subsets as viable targets for the employed VEGFR and EGFR TKIs, expression levels of VEGFR-1, VEGFR-2 and EGFR were determined and expressed by their median fluorescence indices (med FI). Both VEGFR-1 and VEGFR-2 were expressed, albeit at generally low levels, on ImC and on pMDC (med FI range: 2.1 – 3.1), as well as on the MDC-1, MDC-2, and PDC subsets (med FI range: 2.0 – 3.8). In
contrast, EGFR was not expressed on any of the studied PBDC subsets or precursor stages (med FI range: 0.4 – 0.9).

**PBDC frequencies before and after VEGFR TK inhibition**

*Immature myeloid cells (ImC) and MDC precursors (pMDC)*

Almand et al. [3] reported an accumulation of immature Lin\(^-\)HLA-DR\(^-\) ImC in the blood of cancer patients, while more mature Lin\(^-\)HLA-DR\(^+\) MDC precursors (pMDC) were found to be reduced. To further characterize these populations we included the myeloid lineage-associated markers CD11c and CD33. BDCA-4 (neuropilin-1, expressed by PDC) was included in our analyses to ascertain if the previously reported accumulation of immature DC precursors contained within the Lin\(^-\)DR\(^-\) ImC fraction might actually involve PDCs. Typical results, shown in Fig. 2a, demonstrate that the Lin\(^-\)HLA-DR\(^-\) ImC were CD11c\(^+\) and did not express BDCA-4 and were therefore unlikely to include PDCs. In Fig. 2a CD33 expression is shown for the Lin\(^-\)CD11c\(^+\)HLA-DR\(^+\) pMDC population. Within this pMDC population two subpopulations were clearly discernable based on different CD33 expression levels (indicated as A and B in Fig. 2a). As CD33 is a myeloid marker known to be associated with PBDC differentiation and previously shown to be highly expressed on the MDC-1 and MDC-2 subsets but not on an immature DC progenitor subset [20], we take this CD33\(^{hi}\) population to represent a more mature MDC population. We did observe a slight accumulation of ImC in the advanced cancer patients (based on pre-treatment frequencies), but this did not reach the level of significance when compared to healthy donors (Fig. 2b). Of note, within the pMDC population, cells with high CD33 expression levels (population B in Fig. 2a) were significantly decreased in the cancer patients before treatment, as compared to the healthy donors (p = 0.02, Fig. 2c). In the cancer patients VEGFR TK inhibition through AZD2171 treatment did not significantly change the frequencies of the MDC precursors in any of these different stages of development (Fig. 2b,c). Of note, Fig 2c shows three outlying postdose pMDC frequencies well above the mean of the postdose cancer patient group. In conjunction with a restoration of pMDC frequencies to values within the range observed for healthy donors, these three patients experienced clinical benefit from AZD2171 and gefitinib treatment: two patients had a minor response and the third patient had stable disease lasting for 31 weeks.
**Figure 2** - Typical results of blood DC analysis based on the absence of lineage (Lin) markers (CD3, CD14, CD19, CD56) in a patient with mesothelioma. Events are gated for the absence of Lin marker expression and presence of CD33 expression. Lin− cells, i.e. immature myeloid cells (ImCs) and more mature MDC precursors (pMDC), were distinguished from plasmacytoid dendritic cells (PDC) by BDCA-4 expression (a). CD33 expression was determined for the CD11chi HLA-DR+ population. Within the HLA-DR positive population (pMDC) two subpopulations (A and B) were discernable based on intermediate and high CD33 expression (a). Results for immature myeloid cells (ImC, Lin− HLA-DR−) (b) and mature MDC precursors (pMDC, Lin− CD33hi HLA-DRhi) (c) are shown for healthy donors and for cancer patients before (predose) and after VEGFR inhibition (postdose) (c). Peripheral blood DC percentages are of total peripheral blood mononuclear cells. In both graphs, individual values and the means are shown.

**Myeloid suppressor cells (MSC)**

We identified a population of CD14+ HLA-DRneg/low cells in the blood of our patients (Fig. 3a), which, before treatment, was significantly increased as compared to healthy donors (p = 0.005; Fig. 3b). A recent report suggests that these so-called myeloid suppressor cells (MSC) exert immunosuppressive effects via secretion of cytokines including transforming growth factor (TGF)-β [21]. Hypothetically these MSC may derive from ImC accumulating due to disturbed DC differentiation (see Fig.1). After 4-5 weeks of treatment with AZD2171, MSC frequencies in the cancer patients went down, although not significantly (p = 0.08; Fig. 3b). Of note, normalization upon VEGFR TK inhibition of
extremely high predose MSC frequencies in two patients (clear outliers of > 2% of PBMCs in Fig. 3b) was in both cases followed by a minor clinical response.

Figure 3 - Results for myeloid suppressor cells (CD14+ HLA-DRneg/low) in healthy donors and in cancer patients before and after VEGFR inhibition. Typical results of myeloid suppressor cells in a patient and one healthy donor are shown (a). Peripheral blood DC percentages are of total peripheral blood mononuclear cells. The individual values and the means for healthy donors and cancer patients before (predose) and after VEGFR TKI inhibition (postdose) are shown (b).

Myeloid (MDC-1, MDC-2) and plasmacytoid (PDC) DC subsets
Typical pre-treatment FACS data for MDC-1 (BDCA-1/CD1c+), MDC-2 (BDCA-3/CD141+), and PDC (BDCA-2/CD303+) from one patient are shown in Fig. 4, next to comparable data from a healthy donor. Pre-treatment MDC subset frequencies in the mononuclear cell population were significantly lower in the blood of cancer patients as compared to healthy donors (MDC-1: patients 0.19 ± 0.10 % [mean ± SD] vs. controls 0.39 ± 0.11 %, p = 0.002; MDC-2: patients 0.019 ± 0.01 % vs. controls 0.04 ± 0.01 %, p = 0.001; Fig. 4g,h). Of note, PDC frequencies in the blood of the cancer patients were also significantly decreased (patients 0.19 ± 0.15 % vs. controls 0.31 ± 0.10 %; p = 0.04; Fig. 4i).

Treatment with AZD2171 did not raise the frequencies of the MDC-1, MDC-2, or PDC subsets in patients with advanced cancer (Fig. 4g-i). Absolute numbers of the measured DC subsets per ml of blood were also calculated, and these numbers were not significantly affected by the TKI therapy either (data not shown). Apart from the anecdotal observations for the pMDC and MSC discussed above, no further correlations could be established between the dose of AZD2171 or the tumor response and the measured ImC, PBDC, or MSC frequencies.

Since patients received daily dosing of both AZD2171 and 250 mg gefitinib, we also included four advanced NSCLC patients receiving 250 mg gefitinib monotherapy to
Figure 4 - Typical results of peripheral blood DC monitoring based on BDCA marker analysis of a patient with fibrosarcoma (a, c, e) and one healthy donor (b, d, f). Plots for MDC-1 (BDCA-1/CD1c⁺ [a, b]), MDC-2 (BDCA-3/CD141⁺ [c, d]), and PDC (BDCA-2/CD303⁺ [e, f]) are shown. In all plots, peripheral blood DC percentages are of total peripheral blood mononuclear cells and based on absence of CD14 expression. MDC-1 frequencies in healthy donors and in cancer patients before (predose) and after VEGFR inhibition (postdose) (g), MDC-2 frequencies in healthy donors and in cancer patients before (predose) and after VEGFR inhibition (postdose) (h), and PDC frequencies in healthy donors and in cancer patients before (predose) and after VEGFR inhibition (postdose) (i) are shown. Peripheral blood DC percentages are of total peripheral blood mononuclear cells. In all graphs, individual values and the means are shown.

distinguish between any effects of VEGFR and EGFR inhibition. Gefitinib monotherapy did not affect the PBDC nor the MSC frequencies in the blood of the studied cancer patients (data not shown), consistent with the observed lack of EGFR expression on these cell populations.
PBDC maturation status before and after VEGFR TK inhibition
To assess the maturation status of the pMDC, MDC and PDC subsets before and after VEGFR TK inhibition, expression levels of CD86 and/or HLA-DR were determined. Median fluorescence indices for healthy donors and cancer patients are listed in Table 1. No significant differences in CD86 and/or HLA-DR expression levels on the pMDC, MDC and PDC subsets were found between healthy volunteers (n = 8) and tested cancer patients (n = 13), neither before nor after treatment with AZD2171.

Circulating VEGF levels
To correlate the levels of tumor-derived VEGF to PBDC frequencies, serum levels of VEGF were measured. We measured 2-fold higher levels of serum VEGF in the cancer patients at baseline than previously reported in healthy donors [22]. Interestingly, serum VEGF levels in cancer patients tended to increase after 4-5 weeks of treatment with AZD2171 and gefitinib with a mean pre-treatment VEGF level of 626 pg/ml (range 114 - 2847 pg/ml) and a mean post-treatment level of 947 pg/ml (range 245 - 3360 pg/ml; p = 0.2). VEGFR TK inhibition is known to up-regulate circulating VEGF levels, which has previously been demonstrated for multiple VEGFR TK inhibitors [23, 24].
In our studies, no correlation was found between serum VEGF levels and ImC, PBDC, or MSC frequencies, neither before nor after treatment. In addition, no correlation was observed between serum VEGF levels and clinical outcome.

Discussion
Since VEGF has been shown to block DC differentiation and maturation in preclinical models, high levels of VEGF in cancer patients may induce an accumulation of immature and functionally impaired DC contributing to tumor escape from immunosurveillance. As indicated in Fig. 1 and based on previous reports [3], we hypothesized that tumor-derived VEGF might exert its inhibitory effect at the stage of immature HLA-DR^- MDC precursors within the ImC fraction blocking their development into pMDC, while simultaneously skewing their differentiation towards a newly identified population of myeloid CD14^+ HLA-DR^-/low suppressor cells with immunosuppressive traits [21].
We therefore evaluated the effect of administration of the VEGFR TKI AZD2171 on PBDC precursor and subset frequencies in advanced cancer patients. It is important to keep in mind that conclusions drawn from this study may be hampered by the applied phase I study design, including multiple dose levels and a heterogeneous patient population. Nevertheless, we found an increased number of immature DC precursors in cancer
<table>
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<tr>
<th>PBDC subset</th>
<th>pMDC</th>
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<th>PDC</th>
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<td>60.4 (7.3 -</td>
<td>18.4 (7.2 -</td>
<td>802 (403.3 -</td>
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<td></td>
<td>274.8)</td>
<td>31.6)</td>
<td>1471.6)</td>
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<td>63.9 (19.9 -</td>
<td>17.5 (7.7 -</td>
<td>650.4 (166.2 -</td>
<td>10.4 (2.3 - 41.4</td>
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<td>212.4)</td>
<td>55.5)</td>
<td>1028.1)</td>
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<td>Cancer patients, postdose****</td>
<td>51.9 (26.2 -</td>
<td>13.1 (7.9 -</td>
<td>495.3 (264.2 -</td>
<td>7.5 (2.2 - 11.0</td>
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<td></td>
<td>134.1)</td>
<td>33.7)</td>
<td>842.6)</td>
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</table>

* Mean and range of median fluorescence index are listed.
** Healthy donors, n = 8
*** Cancer patients, n = 13
**** Postdose, after 4-5 weeks of VEGFR inhibition
patients, although this difference did not reach the level of significance. We also identified a significantly increased number of CD14+HLA-DRneg/low MSC in the blood of cancer patients as compared to healthy donors (p = 0.005), which tended to be lower after 4 or 5 weeks of VEGFR inhibition. Furthermore, we found that the frequencies of pMDCs, including MDC-1 and MDC-2, were significantly reduced in advanced cancer patients as compared to healthy individuals. In addition, PDC frequencies were significantly reduced in cancer patients compared to healthy donors as previously reported for patients with Kaposi sarcoma or advanced prostate cancer [25, 26]. These results point to a generalized defective DC differentiation, involving multiple DC lineages, across a variety of different tumor types. Thus, the effect of advanced tumors on DC differentiation is systemic and results in a profound reduction of mature DC precursors in the circulation and a simultaneous accumulation of immature myeloid DC precursors with a potentially immunosuppressive role (Fig. 1), in line with previous reports [3, 4, 6]. After a period of 4 to 5 weeks of AZD2171 administration, we did not observe an overall significant increase in pMDC (or indeed PDC) frequencies. Nor did we observe a difference between CD86 and/or HLA-DR expression levels on pMDCs and PBDCs in cancer patients versus healthy donors. This is in contrast to earlier findings by Almand et al. [3], who reported a lower expression of co-stimulatory molecules on immature myeloid cells.

Might the duration and dosing of AZD2171 administration have been insufficient to effect a reversal of the observed systemic DC differentiation? A period of 4 or 5 weeks of AZD2171 administration should be sufficient to affect PBDC frequencies, since frequencies of DC precursors were reported to improve already 3 to 4 weeks after tumor resection [3, 5]. Although it remains to be formally proven that AZD2171 actually inhibits the phosphorylation of VEGFR on PBMCs, pharmacokinetic data show that after multiple daily dosages of 20, 30 or 45 mg of AZD2171, biologically active plasma concentrations are reached, sufficient for sustained VEGFR-1, -2, and -3 inhibition with subsequent effects on clinical parameters, e.g. a clear rise in blood pressure [23]. Furthermore, the pharmacokinetics of AZD2171 were not affected by co-administration of gefitinib (van Cruijsen et al, Proceedings of the 41st annual meeting of the American Society of Clinical Oncology, 2005). Rather than by these pharmacodynamic or -kinetic considerations, the lack of effect on PBDC frequencies of VEGFR TK inhibition in our study may be explained by the advanced disease state of the participating patients, which likely resulted in a redundancy of DC suppressive factors. Beside VEGF, other cytokines secreted by tumor cells are involved in the inhibition of DC differentiation and maturation, a.o. IL-6, IL-10 and M-CSF [27-29]. These other soluble tumor-derived factors may thus overrule the potentially beneficial effect of VEGFR signaling blockade, particularly in late stages of cancer development, which are associated with relatively high systemic levels of these suppressive factors. VEGF may also exert its effect on DC differentiation via another mechanism than phosphorylation of VEGFR on DC [30, 31]. A small trial using
bevacizumab (a monoclonal antibody binding and neutralizing VEGF) in combination with chemotherapy did report improved DC frequencies after treatment [3]. These results, which are in contrast to our findings using AZD2171, a TKI of VEGFR, might indicate an indirect, TK-independent effect of VEGF on DC differentiation.

Although we did not observe an effect of VEGFR TK inhibition on PBDC precursors and subsets in cancer patients, the frequencies of accumulated MSCs tended to decrease after AZD2171 treatment. This CD14+HLA-DRneg/low MSC population may be the human equivalent of CD11b+ and Gr-1 myeloid suppressive cells identified in mice [32, 33] and we hypothesized that tumor-derived VEGF might have skewed the hematopoiesis towards an expansion of these myeloid cells with immunosuppressive traits. In mice this scarce population of immunosuppressive cells could be increased by tumor-derived factors, while neutralizing VEGF-antibodies inhibited expansion of this myeloid subset [34]. Reduction of MSC in murine models has been shown to facilitate the rejection of established metastatic disease [35]. It is notable in this regard that the two patients with high pre-treatment MSC frequencies, which normalised upon VEGFR TKI administration, both had a minor clinical response. One of these also showed a simultaneous increase in pMDC to normal levels. However, due to the nature and size of this Phase I trial these clinical observations remained anecdotal.

Ideally, phenotypic analyses of MSC and PBDC precursors and subsets should be accompanied by functional assays. Large volumes of blood would have been needed to evaluate the effect of AZD2171 on PBDC function, which made this an unfeasible approach in the current setting. Additional in vitro studies are therefore ongoing to assess the effect of VEGFR TK inhibition on DC and MSC differentiation and functionality.

In conclusion, our results indicate that advanced cancer patients harbor increased immature myeloid DC precursor and MSC frequencies, both with potential immunosuppressive effects, as well as reduced MDC and PDC frequencies in their circulation. VEGFR TK inhibition by AZD2171 with anti-angiogenic and preliminary anti-cancer effects did not appear to change any of these DC (precursor) frequencies, although a trend was observed towards reduced MSC frequencies. Our results support the idea that tumor-induced inhibition of DC differentiation is systemic and most likely caused by multiple factors. Clinical approaches to reverse this process should therefore encompass systemic blockade of additional tumor-derived immunosuppressive cytokines beside VEGF.

Acknowledgements
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References


CHAPTER 6

Sunitinib-induced myeloid lineage redistribution in renal cell cancer patients: \( CD1c^+ \) dendritic cell frequency predicts progression-free survival

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Abstract

A disturbed myeloid lineage development with abnormally abundant neutrophils and impaired dendritic cell (DC) differentiation may contribute to tumor immune escape. We investigated the effect of sunitinib, a tyrosine kinase inhibitor of FLT3, KIT, and VEGF receptors, on myeloid differentiation in renal cell cancer (RCC) patients. Twenty-six advanced RCC patients were treated with sunitinib in a 4-week on / 2-week off schedule. Enumeration and extensive phenotyping of myeloid subsets in the blood was performed at baseline and at weeks 4 and 6 of the first treatment cycle. Baseline patient data were compared with sex- and age-matched healthy donor data. Baseline frequencies of DC subsets were lower in RCC patients than in healthy donors. After 4 weeks of sunitinib treatment a generalized decrease in myeloid frequencies was observed. Whereas neutrophils and monocytes, which were both abnormally high at baseline, remained low during the 2-week off period, DC rates recovered, resulting in a normalized myeloid lineage distribution. Subsequent to sunitinib treatment, an increase to high levels of myeloid DC (MDC) subset frequencies relative to other myeloid subsets, was specifically observed in patients experiencing tumor regression. Moreover, high CD1c/BDCA-1+ MDC frequencies were predictive for tumor regression and improved progression-free survival.

The sunitinib-induced myeloid lineage redistribution observed in advanced RCC patients is consistent with an improved immune status. Immunological recovery may contribute to clinical efficacy as suggested by the finding of highly increased MDC frequencies relative to other myeloid subsets in patients with tumor regression.

Statement of Clinical Relevance

Studying the in vivo effects of sunitinib on the myeloid lineage profile in the blood (including dendritic cell (DC) subsets) of patients with advanced renal cell cancer (RCC), we were able to confirm a disturbed myeloid cell blood profile as compared to healthy donors. Specifically, abnormally high frequencies of potentially immunosuppressive neutrophils and abnormally low frequencies of immunostimulatory DC subsets were observed. Beside a predictive value of high CD1c+ DC frequencies for improved progression-free survival, our results show that sunitinib, at least in part, normalizes disturbed myeloid differentiation pathways, resulting in a potentially more favorable immunocompetent state. This suggests that sunitinib may be a promising therapeutic agent to combine clinically with DC-based immunotherapeutic approaches.
**Introduction**

One of the major mechanisms of tumors to escape from immunosurveillance is hampered dendritic cell (DC) differentiation (1). In cancer patients, frequencies of circulating DCs are significantly lower as compared to those in healthy individuals (2;3). Accumulation of immature myeloid cells (ImC) and functionally impaired DCs has been documented in blood, tumors, and tumor-draining lymph nodes of cancer patients and found to be a poor prognostic factor (3-5).

Vascular endothelial growth factor (VEGF) can contribute to tumor-induced DC defects *in vitro* and *in vivo* (6-8). In cancer patients an association has been found between high levels of circulating VEGF and increased numbers of circulating ImCs (4) and decreased numbers of more mature DC precursors (9). In gastric and non-small cell lung cancer biopsies DC density was inversely correlated with intratumoral VEGF levels (10;11). In a heterogeneous population of advanced cancer patients inhibition of VEGF signaling with VEGF-Trap, a fusion protein neutralizing VEGF and placental growth factor, was shown to increase the frequency of mature DC precursors in the blood, but did not alter frequencies of immature myeloid cells (12). Another study examining the DC immune potentiating effects of bevacizumab, a monoclonal antibody against VEGF, showed a decrease in immature myeloid cells, but no significant increase in mature DC precursors in the blood of cancer patients (13). We previously failed to observe normalizing effects of cediranib (AZD2171), a VEGF receptor tyrosine kinase inhibitor, on mature DC precursors in a heterogeneous group of advanced cancer patients. We did, however, observe a slight decrease in myeloid suppressor cells (MSCs), an immature myeloid cell population, which has recently been reported to exert an immunosuppressive effect (3;14).

Sunitinib, a tyrosine kinase inhibitor of the VEGFRs, has been registered for first and second-line treatment in advanced renal cell cancer (RCC) patients based on the results of a phase III study and two phase II studies, respectively (15-17). Sunitinib targets not only the tyrosine kinase activity of all three VEGFRs (VEGFR-1, -2, and -3), but also inhibits the tyrosine kinase activity of the platelet derived growth factor receptor (PDGFR)-α and -β, fms-like tyrosine kinase-3 (FLT3), and KIT. In particular, FLT3 and KIT have been implicated in myeloid differentiation, including the differentiation of neutrophils and DCs (18).

As a consequence of the common Von Hippel-Lindau tumor suppressor gene defect, the majority of RCC of the clear cell type is characterized by high levels of VEGF (19-21), which could affect DC differentiation. Furthermore, RCC patients can have abnormally high frequencies of myeloid cells, especially neutrophilic granulocytes, as a result of an interleukin (IL)-6 induced paraneoplastic syndrome (22). Interestingly, neutrophils have been implicated as a major cause of T cell suppression in advanced cancer patients and were associated with poor prognosis (23;24). On the other hand, mature DC precursors
 Peripheral blood dendritic cells (PBDCs) have the potential to develop into functional DCs with T-cell stimulatory activity and the capacity to induce an anti-tumor T cell response (25;26).

In light of the abnormal myeloid lineage differentiation profile in RCC patients and its possible adverse consequences in terms of immune competence, we studied the influence of sunitinib’s broad inhibitory activity on the myeloid blood profile, including PBDCs. Our data show that daily administration of sunitinib for 4 weeks followed by a 2-week rest period results in a normalization of circulating neutrophils and PBDC frequencies relative to other myeloid subsets. In addition, we report an association between a sunitinib-induced tumor regression and an increased frequency of one PBDC subset in particular, the CD1c/BDCA-1\(^+\) myeloid DC (MDC)-1 subset.

**Patients and methods**

**Patients**

Patients with advanced RCC were treated with sunitinib in an expanded access program. Between January 2006 and March 2007, 26 patients were enrolled in the DC monitoring studies.

Patients received sunitinib 50 mg per day orally during the first 4 weeks, followed by 2 weeks of rest. This 6-week time period was defined as one treatment cycle. Sunitinib has a half-life ranging from 41 to 68 hours (27). Leukocytes and their differential were quantitated by the use of CELL-DYN\(^\text{®}\) (Abbott, Abbott Park, IL). Hematological adverse events were graded using the Common Terminology Criteria for Adverse Events of the National Cancer Institute, version 3.0. Efficacy of sunitinib was determined after every two or three cycles using one-dimensional measurements on computed tomography (CT) scans. At baseline the sum of the largest diameters of appointed target tumor lesions was calculated and was compared to the sum calculated in follow-up scans. A 20% decrease of the sum of the target lesions was considered regression, and 20% increase of the sum or clear clinical evidence of progressive disease was considered progression. When these criteria were not met, responses were considered stable. Patients continued on sunitinib treatment for the duration of clinical benefit. Progression-free survival (PFS) was the time between the first day of sunitinib and the date of evidence for progression. For PFS analysis, data collection was closed on January 1\(^{st}\), 2008.

The DC monitoring study was approved by the Medical Ethical Committee of the participating institutes. After obtaining written informed consent patients were enrolled.
**DC monitoring**

Blood for DC monitoring was drawn at baseline, and 4 and 6 weeks after the start of sunitinib treatment. Peripheral blood mononuclear cells (PBMC) were isolated after a density gradient separation by the use of a Vacutainer® CPT™ Cell Preparation Tube (BD, Franklin Lakes, NJ), following the manufacturer’s instructions. After isolation, PBMCs were washed twice, and viable cells were counted based on trypan blue exclusion.

Analysis of the ImCs, MSCs, and PBDC subset frequencies (Table 1) and their maturation status was performed using four-color staining with antibodies directly conjugated with fluorochromes FITC, PE, PerCP-Cy5, or APC, followed by flowcytometric analysis of 50-150,000 events, using a FACS Calibur and Cellquest FACS analysis software (BD Biosciences, San Jose, CA). Monoclonal antibodies against the following markers were used: CD3, CD11c, CD14, CD19, CD56, CD86, CD123, HLA-DR (all BD Biosciences, San Jose, CA), CD40 (Immunotech, Marseille, France), and blood DC antigen (BDCA)-1 (i.e. CD1c), BDCA-2, BDCA-3, (all Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). A gate was set at the lymphocyte/mononuclear populations based on the forward and sideward scatter plots to avoid erythrocytes, cell debris and neutrophil contamination.

ImCs, previously identified as MDC/macrophage precursors in varying stages of differentiation (28), were defined as positive for CD11c, but negative for the lineage (Lin) markers CD3 (T cells), CD14 (monocytes), CD19 (B cells), and CD56 (NK cells), as well as for HLA-DR as previously described (Table 1) (3;4). Myeloid suppressor cells (MSC) were defined as CD14^+HLA-DR^{neg/low} cells (Table 1) (3;14). PBDC frequencies were determined on the basis of expression of BDCA markers (Table 1): DCs belonging to the so-called myeloid DC subset-1 (MDC-1) were identified as CD11c^{high}, CD19^-, CD14^-, and BDCA-1/CD1c^+, MDC-2 were detected as CD11c^-, CD14^-, and BDCA-3^+ (29), and plasmacytoid DC (PDC) were detected as CD11c^-, CD14^-, CD123^{high}, and BDCA-2^+ (3).

To assess the maturation status of the MDC-1, MDC-2 and PDC subsets before and after sunitinib administration, median fluorescence indices (MFI) of HLA-DR, CD40 and/or CD86 were calculated by dividing the median fluorescence of the test antibody by the median fluorescence of the isotype-matched control antibody (BD Biosciences, San Jose, CA).

**Plasma levels of IL-6, IL-10 and VEGF**

After the first centrifugation step of the Vacutainer® CPT™ Cell Preparation Tube and before harvesting the PBMCs, citrate plasma was collected to measure circulating IL-6 and IL-10 levels by the use of commercially available ELISA kits, following the manufacturers’ instructions (both from Sanquin, Amsterdam, The Netherlands). VEGF levels were determined in EDTA plasma samples by ELISA following the manufacturers’ instructions (R&D Systems, Minneapolis, MN).
**Statistical analysis**

The Mann-Whitney U test was used to determine significance of differences between patient and healthy donor data. Patient data of multiple time-points or groups were compared using analysis of variance. Post hoc analysis of these differences between the time-points or response groups was performed by the Bonferroni correction for multiple comparisons. Correlations between continuous datasets were analyzed using Spearman correlation coefficient. Kaplan-Meier plots and log rank analysis were applied to determine the significance of differences in PFS. Values of $P \leq 0.05$ (two-tailed) were considered statistically significant.

**Results**

**Patients and healthy donors**

Twenty-six advanced RCC patients treated with sunitinib were enrolled in the DC monitoring study. Patient characteristics were as follows: in 20 patients (77%) tumors were of clear-cell histology; mean age was 62 years (range 41-82); 6 patients (23%) were female and 14 patients (54%) had received prior systemic cytokine therapy (IFN-α). Two patients were pre-treated with an experimental VEGFR tyrosine kinase inhibitor. Seventeen patients (65%) had undergone a nephrectomy for their disease. Two patients had a good prognosis, while 16 patients had an intermediate and 8 patients had a poor prognosis according to the Memorial Sloan Kettering Cancer Center (MSKCC) prognostic factors (30). There was a minimum period of 4 weeks between previous treatment and the start of sunitinib administration.

During the period of DC monitoring in the first cycle, 33% of the patients experienced any grade of leucopenia, including one patient with grade 3, and 13% of the patients experienced any grade of neutropenia, including one patient with grade 3. Due to treatment-related side-effects, sunitinib treatment was stopped in one patient before tumor evaluation. This patient was not included in the efficacy analysis. As best response, tumor regression was observed in 8 patients (32%) and 11 patients (44%) had stable disease. Six patients (24%) had progression. Median PFS was 5.8 months.

The control group consisted of 10 age- and gender-matched healthy donors (3 women, 7 men; mean age: 60 years, range 55-68, $P = 0.6$ compared with RCC patients).
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<th>Subset</th>
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</tr>
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DC, Dendritic cell
Lin, Lineage-markers: CD3, CD14, CD19, CD56
BDCA, Blood dendritic cell antigen
Sunitinib-induced changes in Immature myeloid Cell (ImC) and Myeloid Suppressor Cell (MSC) frequencies

Although not significant, ImC frequencies as percentage of PBMCs were slightly lower in RCC patients than in healthy donors (0.50% vs. 0.72%, respectively; \( P = 0.2 \), Fig. 1A). Upon sunitinib treatment, ImC frequencies rose, but went down again to baseline levels after the 2 weeks of rest (Fig. 1A). Opposite trends were observed for MSCs, which were defined as positive for CD14 with low or negative HLA-DR expression. As recently reported for a heterogeneous group of advanced cancer patients (3;14), we also observed an increased frequency of MSCs as percentage of PBMCs in the RCC patients (patients 0.92% vs controls 0.42%, \( P = 0.09 \), Fig. 1A). In contrast to ImCs, MSC frequencies decreased on sunitinib treatment but went back up to baseline levels after the 2-week off period.

Sunitinib-induced changes in classic Myeloid DC (MDC) and Plasmacytoid DC (PDC) frequencies

Mean frequencies in the mononuclear cell population of all three PBDC subsets (Table 1) were significantly lower at baseline in RCC patients as compared to frequencies in healthy donors (Fig. 1B). Absolute numbers of MDC-1, MDC-2 and PDC per mL of blood were also significantly lower at baseline in RCC patients as compared to healthy donors (data not shown).

During sunitinib treatment, MDC-1 and MDC-2 frequencies decreased, but both MDC-1 and -2 rates went up again after the 2-week off period (Bonferroni-correction: MDC-1, \( P = 0.008 \); MDC-2, \( P < 0.001 \)). Of note, average MDC-1 frequencies even reached levels comparable to those observed in healthy donors (Fig. 1B). PDC frequencies showed similar trends, but changes were not significantly different (Fig. 1B).

In order to assess the maturation status of the MDC-1, MDC-2 and PDC subsets before and after the first cycle of sunitinib treatment, expression levels of HLA-DR, CD40 and/or CD86 were determined.

CD86 and HLA-DR expression levels on the MDC-1 subset in RCC patients were significantly lower than in healthy donors, but did not change upon sunitinib treatment (Fig. 1C). Comparing RCC patients with healthy donors, no significant differences were found for HLA-DR, CD86 and/or CD40 expression on both MDC-2 and PDCs, nor did sunitinib change these expression levels (Fig. 1C, or data not shown).

Abnormal distribution of myeloid lineage subsets normalizes upon sunitinib treatment

RCC patients often have a disturbed blood profile of myeloid leukocytes, possibly associated with a tumor-induced paraneoplastic syndrome (22).
Effect of sunitinib on myeloid subsets

Figure 1 - (A) Immature myeloid cell and myeloid suppressor cell frequencies as percentage of peripheral blood mononuclear cells (PBMCs) in RCC patients before, and 4 and 6 weeks after start of sunitinib treatment. Of note, week 6 represents the time-point after the 2-week period of rest. Frequencies of these populations in healthy donors (HD) are also shown. (B) Myeloid DC-1, myeloid DC-2, and plasmacytoid DC frequencies (as % PBMC) in HD and RCC patients before, and 4 and 6 weeks after start of sunitinib. (C) CD86 and HLA-DR expression levels on MDC-1 and MDC-2 for the same time-points. In Fig. 1A and 1B, individual data and the mean values are shown, while in Fig. 1C, mean values and standard deviations are shown. MFI, median fluorescence index
Figure 2 - (A) Differential leukocyte analysis in RCC patients during the first cycle of sunitinib treatment. Gray bars indicate normal ranges for the indicated leukocyte populations. (B) MDC-1 and MDC-2 frequencies as a percentage of myeloid leukocytes (i.e. granulocytes and monocytes) in healthy donors (HD) and RCC patients before, and 4 and 6 weeks after start of sunitinib. Week 6 represents the time-point after the 2-week period of rest. Individual data and the mean values are shown.
Our patient group showed mean baseline levels of neutrophils and monocytes as high as the upper limit of normal values, with levels exceeding the normal range in a considerable number of patients (Fig. 2A).

Phase III studies of sunitinib have clearly demonstrated a drug-induced decrease in neutrophil counts (17;31). This finding was confirmed by our study: the high neutrophil and monocyte counts normalized upon sunitinib treatment and remained low after the 2-week off period (Fig. 2A). To assess the overall myeloid normalization upon sunitinib treatment, we calculated the PBDC frequencies as percentage of myeloid leukocytes. Baseline frequencies of MDC-1, MDC-2 or PDC as percentage of myeloid leukocytes were significantly decreased in RCC patients as compared to those in healthy donors (Fig. 2B). Upon sunitinib treatment, including the 2 weeks of rest, the frequencies of MDC-1 and MDC-2 as percentage of myeloid leukocytes increased significantly (Fig. 2B), while PDC frequencies did not (data not shown). Importantly, for the MDC-1 subset, relative frequencies measured at week 6 did not significantly differ from those in healthy donors (Fig. 2B).

**Abnormal myeloid distribution is related to IL-6**

IL-6 levels decreased upon sunitinib and increased again after the 2 weeks of rest (mean values [SD] in pg/mL at baseline, 60.1 [93.9]; week 4, 27.1 [28.1]; and week 6, 45.2 [64.0]); these changes were not significant by analysis of variance. In keeping with previous reports (27;32), we observed a trend, albeit not significant, towards increased VEGF levels after sunitinib administration, with a subsequent decrease in the 2-week off period (mean values [SD] in pg/mL at baseline, 156.5 [156.6]; week 4, 304.8 [382.1]; and week 6, 117.6 [87.0]). Of note, plasma VEGF levels at baseline were abnormally high compared to reported data (32). IL-10 levels showed small, non-significant increases throughout the whole first cycle of sunitinib (mean values [SD] in pg/mL at baseline, 69.2 [77.1]; week 4, 98.7 [83.7]; and week 6, 118.9 [54.8]).

As mentioned, RCC-related abnormal peripheral distribution of myeloid subsets may be caused by tumor-derived IL-6 (22). Indeed, a significant negative correlation was observed between baseline IL-6 levels and baseline MDC-1 frequencies (Spearman’s rho -0.58, \( P = 0.005 \); Fig. 3) as well as a significant positive correlation between IL-6 levels and neutrophil counts at baseline (Spearman’s rho -0.63, \( P = 0.004 \); Fig. 3). In addition, significant correlations were found between baseline IL-6 levels and baseline MDC-2 or monocytes (Spearman’s rho -0.49, \( P = 0.03 \); rho 0.60, \( P = 0.007 \), respectively; data not shown). In contrast, we did not observe a correlation between baseline IL-6 levels and baseline lymphocyte counts. No correlations were found between levels of VEGF or IL-10, and MDC-1 frequencies or frequencies of any other myeloid subsets (data not shown).
Myeloid leukocyte profiles in relation to tumor response upon sunitinib: predictive value of MDC-1 frequencies

The relationship between myeloid lineage subset distribution and tumor response was determined in those patients for whom both parameters were available. High baseline counts of neutrophils and monocytes were associated with a lack of tumor response to sunitinib (Fig. 4A), whereas baseline lymphocyte counts were not associated with response (data not shown). In contrast, baseline MDC-1 frequencies as percentage of PBMCs were significantly higher in patients experiencing a regression as compared to MDC-1 frequencies in patients with stable disease ($P = 0.02$) or progression ($P = 0.004$) as best response (Fig. 4B). This association could not be established for MDC-2 frequencies and tumor response (Fig. 4B).

Following treatment, MDC-1 and MDC-2 frequencies as percentage of myeloid leukocytes significantly increased to high levels, even exceeding those observed in healthy donors, specifically in the patients experiencing tumor regression (Fig. 4C). Unlike the “classic” MDC subsets, neither baseline PDC frequencies, nor subsequent therapy-induced changes in PDC frequencies were associated with tumor response (data not shown).

We observed a significant correlation between the decrease in tumor size and high MDC-1 frequencies as percentage of PBMCs both at baseline (Spearman’s rho $-0.56$, $p = 0.007$) and post-treatment (Spearman’s rho $-0.62$, $P = 0.006$; Fig. 5A). Of note, no such correlation was observed for the MDC-2 subset (Fig. 5B). This correlation between MDC-1 frequencies and therapy-induced changes in tumor size also translated into prolonged PFS: on univariate analysis, a high (i.e., above median) baseline MDC-1 frequency as a percentage of PBMCs was prognostic for a better PFS (log rank, $P = 0.04$; Fig. 5C). In contrast, high baseline frequencies of MDC-2 were not associated with prolonged PFS (Fig. 5D).
Figure 4 - (A) Baseline and post-treatment (week 6) counts of neutrophils and monocytes in sunitinib-treated RCC patients in relation to tumor response. (B) Baseline MDC-1 and MDC-2 frequencies as percentage of mononuclear cells in relation to tumor response. (C) Baseline and post-treatment (week 6) counts of MDC-1 and MDC-2 as percentage of myeloid leukocytes in sunitinib-treated RCC patients in relation to tumor response.

Individual data and the mean values are shown. HD, healthy donors
Figure 5 - (A) Correlation between baseline and week 6 MDC-1 frequencies as a percentage of mononuclear cells and the best observed change in the sum of tumor diameters as a percentage of baseline value. (B) Neither baseline nor post treatment MDC-2 frequencies correlated with the best observed change in the sum of tumor diameters as a percentage of baseline value. The circles and solid lines represent baseline levels, squares and dotted lines represent week 6 (post treatment) levels. Kaplan-Meier survival analyses for progression-free survival (PFS) based on (C) MDC-1 and (D) MDC-2 frequencies as a percentage of mononuclear cells in sunitinib-treated RCC patients. High (i.e., above median) baseline MDC-1 frequencies (bold lines) were prognostic for better PFS (log rank, $P = 0.04$) (C), but high baseline MDC-2 frequencies were not (D).
Discussion

In the present study we evaluated different PBDC subsets and their precursor populations in advanced RCC patients treated with sunitinib. As compared to healthy donors we found abnormally high levels of neutrophils and monocytes, and low levels of PBDC frequencies reflecting more mature DC precursors. Our finding of low frequencies of both ImCs, which contrasts with previous reports (1;4), and PBDCs is indicative of a very early block in myeloid DC differentiation in patients with advanced RCC that appears to be accompanied by increased levels of MSCs. Monocytes, macrophages, and neutrophils have been implicated in T cell suppression through their production of effector molecules such as arginase and hydrogen peroxide (33-36), while decreased PBDC frequencies may further aggravate these immune suppressive effects by a generalized reduction in antigen presentation and T cell stimulatory capacity. Altogether, this abnormal distribution of myeloid lineage subsets observed in advanced RCC patients is consistent with an immunosuppressed state and may contribute to tumor progression.

After four weeks of treatment of the RCC patients, sunitinib induced significant decreases in peripheral blood frequencies of all studied myeloid populations, except for the ImCs, which actually increased in frequency. The latter observation might reflect a temporary relief of a block in early myeloid DC differentiation. However, since total leukocyte counts decreased upon sunitinib, absolute numbers of ImCs did not significantly change in the first treatment cycle (data not shown). We also observed a decrease in MDC frequencies as a percentage of mononuclear cells after four weeks of sunitinib. After the subsequent 2-week period of rest, neutrophils and monocytes remained low and were within the range of normal levels as observed in healthy donors, while MDC frequencies significantly increased again. This increase was particularly apparent for MDC-1 frequencies, which on average reached levels observed in healthy donors. The combined lowering of abnormally high levels of immunosuppressive myeloid leukocytes and a relative increase of immunostimulatory MDC-1 frequencies, the latter specifically in patients with tumor regression, suggests that the observed myeloid lineage redistribution in the sunitinib-treated RCC patients results in an overall improved immune status. Whether the myeloid redistribution might actually contribute to, rather than merely result from, the sunitinib-induced anti-tumor response, remains unresolved. Also, our results are limited to the first cycle of sunitinib treatment. It would be very interesting to investigate whether long-term treatment with sunitinib induces an even more profound and sustained myeloid lineage redistribution in advanced RCC patients.

Whereas high MDC-1 frequencies were associated with decreases in tumor size and prolonged PFS, this was not the case for the MDC-2 subset suggesting that MDC-1 plays a more important role in the immunocompetence of RCC patients. In keeping with this, we only found a decrease in phenotypic activation status, as determined by HLA-DR and CD86
expression levels, in the MDC-1 subset. One cycle of sunitinib treatment could not correct for this: HLA-DR and CD86 expression levels remained at the same reduced level as baseline. It remains to be investigated whether sunitinib has actually improved antigen-presenting functions of MDCs on a “per cell basis”, e.g. through \textit{ex vivo} T cell stimulations. Due to limited amounts of blood available to us we were unable to do so. Nevertheless, baseline MDC-1 frequencies were positively associated with prolonged PFS, suggesting that increased MDC-1 frequencies with reduced activation status may still contribute to the immunocompetence of RCC patients. Possibly, upon recruitment to peripheral tissues, immature MDC-1 precursors from the blood can rapidly mature to competent DC with upregulated levels of costimulatory and MHC molecules.

Since sunitinib is a promiscuous agent inhibiting multiple receptor tyrosine kinases, it is hard to establish which receptor or pathway was targeted to achieve the effects on myeloid differentiation as observed in our study. The sunitinib-induced generalized decrease in frequencies of myeloid subsets are likely to result from its FLT3 or KIT inhibitory activity (37;38), since both pathways have shown their importance in early stages of myeloid cell differentiation (18). The mechanism underlying the observed increase in MDC-1 frequencies is harder to unravel. Although interference with VEGFR signaling might be an obvious explanation, we could not establish a correlation between VEGF levels and baseline MDC-1 frequencies in our study population. We did, however, find a negative correlation between baseline IL-6 levels and baseline MDC-1 and -2 frequencies. The relevance of this association has previously been confirmed in a preclinical RCC study, in which tumor-derived IL-6 was found to cause defective DC differentiation (39). Sunitinib may alter the responsiveness of MDCs, or possibly of an earlier DC precursor, to IL-6, as MDC frequencies rise during the 2-week rest period despite a simultaneous rise in IL-6 levels. Regarding this possibly altered IL-6 responsiveness, it is of particular interest that sunitinib can inhibit phosphorylation of signaling transduction and activator of transcription (STAT)-3, a signaling molecule downstream of the IL-6 receptor (40;41). Modulating STAT3 activation in DCs might result in a decreased responsiveness to the inhibitory activity of IL-6 (42), which subsequently might lead to increased MDC frequencies. We performed a pilot study to determine the STAT3 inhibitory effects of sunitinib in PBMCs of five RCC patients treated with this agent. We were able to detect pSTAT3 expression in PBMCs by Western blot analysis, but a modulatory effect of sunitinib was not conclusively established and will require further investigation in larger numbers of patients (Supplementary Fig. 1).

Until recently, interferon-α immunotherapy was the standard of care in locally advanced or metastatic RCC. The efficacy of sunitinib has changed the first- and second-line treatment for this disease (15-17;17). Patients with RCC, however, eventually suffer from tumor progression after initial tumor regression upon VEGFR-interfering therapy. Hence, oncologists have a growing interest in regimens combining immunotherapy with agents
interfering with VEGFR signaling. Our observation that sunitinib treatment, at least in part, normalizes disturbed myeloid differentiation pathways, resulting in a potentially more favorable immunocompetent state, certainly supports this notion. A sustained reduction in abnormally high numbers of potentially immunosuppressive neutrophils, combined with increased rates of otherwise down-regulated immunostimulatory MDC-1, for up to at least 2 weeks after the 4 weeks of sunitinib administration, may provide a window, in which patients are particularly amenable to immunotherapeutic approaches. Such regimens should be explored in future clinical trials.

Acknowledgements

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Supplementary Figure 1 - Western blot analysis of (p)STAT3 expression in PBMCs of two renal cell cancer patients on three time-points: at baseline (BL), and 4 (Wk 4) and 6 (Wk 6) weeks after start of sunitinib. Results from two out of five patients are shown. Two patients showed a decreased expression of both pSTAT3 and STAT3 over the course of the first treatment cycle (as shown for Patient 1), two showed a simultaneous increase in expression of both pSTAT3 and STAT3 (as shown for Patient 2), while one patient showed STAT3 expression, but no pSTAT3 expression, neither of which changed in the course of treatment (not shown). Clinical response data did not correlate in any way with the changes in (p)STAT3 expression. In short, PBMC lysates were loaded on the gel and separated by SDS-PAGE, after which they were transferred to a membrane (Bio-Rad, Hercules, CA, USA) by electrophoresis. Membranes were blocked and probed overnight either with anti-STAT3 antibody or anti-pSTAT3 antibody (both obtained from Cell Signaling Technology Inc., Danvers, MA, USA). After washing, the membranes were incubated with horseradish peroxidase-conjugated antibody (Dako, Glostrup, Denmark) for 1 hour at room temperature. Immunoreactive bands were visualized in film using the enhanced chemiluminescence (ECL) detection kit (Amersham Biosciences, Buckinghamshire, UK). As loading control, membranes were also probed with a monoclonal anti-β-actin antibody (Sigma-Aldrich, Steinheim, Germany).
References


CHAPTER 7

Tissue micro array analysis of ganglioside N-glycolyl GM3 expression and signal transducer and activator of transcription (STAT)-3 activation in relation to dendritic cell infiltration and microvessel density in non-small cell lung cancer

Hester van Cruijsen, Mariëlle Gallegos Ruiz, Paul van der Valk, Tanja D de Gruijl and Giuseppe Giaccone

Submitted for publication
Abstract

Tumor immune escape and angiogenesis contribute to tumor progression, and gangliosides and activation of signal transducer and activator of transcription (STAT)-3 are implicated in these processes. As both are considered as novel therapeutic targets, we assessed the possible association of ganglioside GM3 expression and STAT3 activation with suppression of dendritic cell (DC) activation and angiogenesis in non-small cell lung cancer (NSCLC).

Immunohistochemistry was performed on a tissue array to determine N-glycolyl GM3 (GM3) and phosphorylated STAT3 (pSTAT3) expression in 176 primary NSCLC resections. Median values of GM3 and pSTAT3 expression were used as cut off. Microvessel density (MVD) was determined by CD34 staining and morphology. CD1a and CD83 were used to determine infiltrating immature and mature dendritic cells, respectively.

94% and 71% of the NSCLC samples expressed GM3 and nuclear pSTAT3, respectively. Median overall survival was 40.0 months. Both low GM3 expression and high pSTAT3 expression were associated with a worse survival, which reached near significance for GM3 ($P = 0.08$). Microvessel density (MVD), determined by CD34 staining and morphology, was lower in NSCLC samples with high GM3 expression. CD1a$^+$ cells (immature DCs) were more frequent in NSCLC tissues as compared to peritumoral lung tissue, while CD83$^+$ cells (mature DCs) were more frequent in peritumoral lung tissue. CD83$^+$ DCs were less frequent in NSCLC tissues with high GM3 expression.

GM3 and pSTAT3 are widely expressed in NSCLC. Based on CD83 expression, GM3, but not pSTAT3, appeared to be involved in tumor-induced DC suppression. pSTAT3 expression was not associated with MVD, while GM3 might play an anti-angiogenic role.
Background

Lung cancer is the leading cause of cancer-related deaths worldwide. Non-small cell lung cancer (NSCLC), consisting mainly of adenocarcinoma, squamous cell and large-cell carcinoma, accounts for almost 80% of lung cancer cases. Five-year survival rate for NSCLC patients, irrespective of histological subtype and stage at diagnosis, approximates 15% [1]. Of the 25% who are candidates for curative surgery at diagnosis (stage I-IIIA), 65% will relapse within two years. Most patients present with advanced disease, and despite recent improvements in systemic combination regimens, advanced NSCLC patients still have a poor prognosis [2].

To design new and effective therapies in order to improve the outcome for NSCLC patients, understanding the tumor biology and the interplay between tumor cells and their micro-environment is of utmost importance. Some characteristics of tumor biology, like deregulated expression of gangliosides and constitutive activation of signal transducer and activator of transcription (STAT)-3, have been implicated in tumor-host interactions, i.e. tumor immune escape and angiogenesis. Tumor immune escape is established through a wide variety of active mechanisms employed by tumors to escape or to frustrate immune responses [3] and angiogenesis is the formation of new blood vessels from the existing vasculature [4]. Both processes are employed by virtually all solid tumors to initiate and facilitate tumor progression.

Gangliosides are ubiquitous membrane-associated glycosphingolipids containing at least one sialic acid. Besides regulatory roles in normal physiological processes, gangliosides have been implicated in tumor development and progression [5]. The composition and production of gangliosides is altered in many tumour types. N-glycolyl GM3, a monosialic ganglioside, is not expressed in normal human tissues [6], but increased levels of N-glycolyl GM3 were detected in human breast tumors [7]. Gangliosides are also shed in the tumor microenvironment and eventually circulate in patients’ blood [8,9]. These circulating gangliosides are thought to be involved in tumor-host interactions facilitating metastasis through tumor immune escape and tumor-associated angiogenesis. GM3 has been described to impair differentiation and function of both CD34+ and CD14+-precursor derived dendritic cells (DC) [10,11], which are the most potent antigen presenting cells essential for elicitation of an anti-tumor immune response, but are often hampered in their development and function by tumors [12]. In addition, maturation and function of Langerhans cells (i.e. DCs from the epidermis) were also shown to be inhibited by GM3 [13]. Not all studies, however, could establish such a DC immune suppressive role of GM3 [14]. Gangliosides can also modulate tumor-associated angiogenesis. Low GM3 levels relative to another ganglioside GD3 have been demonstrated to stimulate angiogenesis [15,16] and GM3 was the only investigated ganglioside found not to increase endothelial cell responsiveness to the pro-angiogenic factor, vascular endothelial growth factor.
Although these observations suggest an anti-angiogenic role for GM3, the opposite has also been demonstrated: GM3 synergistically increased basic fibroblast growth factor (bFGF, another important pro-angiogenic factor)-induced proliferation of bovine aortic endothelial cells [18].

STAT-proteins are important in oncogenic signaling. This family comprises seven members: STAT1 to 4, STAT5a and STAT5b, and STAT6 [19]. Normally, STAT proteins transmit cytoplasmic signals from polypeptide cytokines or growth factors that have receptors with intrinsic or associated tyrosine-kinase activity, and consequently modulate the expression of target genes. Constitutive activation of STAT3 has been implicated in lung cancer development [20,21]. In addition, STAT3 activation through phosphorylation in tumor cells has been demonstrated to negatively regulate the adaptive immune responses both by reducing pro-inflammatory cytokine production, and by production of soluble factors inhibiting DC maturation [22]. Inhibiting STAT3 in tumor cells resulted in increased production of pro-inflammatory factors and reversed DC suppressive effects [22]. STAT3 activation has also been implicated in angiogenesis: STAT3 is a direct transcription activator of the VEGF gene and activation of STAT3 leads to tumor-associated angiogenesis in vivo [23].

More and more evidence is emerging for a relationship between angiogenesis and tumor-associated immune suppression. Many tumor-derived factors, such as interleukin-6, prostaglandins and VEGF, are implicated in both processes. In the present study we further set out to establish a possible role of GM3 expression and STAT3 activation in tumor immune escape and angiogenesis in NSCLC, since both are considered as novel therapeutic targets for this tumor type. To this end, we examined through immunohistochemistry the GM3 and phosphorylated STAT3 (pSTAT3) status of 176 primary NSCLC sections in a tissue micro array (TMA) and correlated this to DC infiltration, microvessel density and overall survival. We found that both GM3 and pSTAT3 are ubiquitously expressed in NSCLC. pSTAT3 expression could not be associated with either tumor immune escape or angiogenesis. In addition, we did not find evidence for a pro-angiogenic role of GM3, but we did observe an association of high GM3 expression levels with a decrease in the number of activated tumor-infiltrating DC in NSCLC patients.

**Methods**

**Patients and NSCLC tissue micro array**

We studied 176 NSCLC patients, who underwent a primary tumor resection at the VU University Medical Center from 1988 until 2005. From the resected NSCLC material, tissue micro arrays (TMA) were created (Figure 1A) [24]. In short, paraffin-embedded tumor material was cut into 4 μm-thick sections and placed onto glass slides. Slides were
stained with hematoxylin and eosin and an experienced pathologist verified the presence of tumor cells and marked the tumor area. 0.6 mm diameter biopsies were taken from the donor block, two from the tumor and one from the normal tissue area surrounding the tumor. Biopsies from the donor blocks were included in recipient tissue array blocks using a precision tissue array instrument (Beecher Instruments, Sun Prairie, WI, USA). From this tissue array block sections were made for immunohistochemistry. Normal lung tissue, referred to as peritumoral lung tissue, was only available in 126 cases. As few biopsies mounted on the glass slide got lost during the procedure of creation and staining, in some analyses we did not obtain the maximum NSCLC samples of 176.

The study was approved by the Medical Ethical Committee and carried out in accordance with the ethical guidelines of our institution concerning informed consent about the use of patient’s material after surgical procedures.

**Figure 1** - (A) Overview of multiple NSCLC sections stained with 14F7 and examples of cytoplasmic, nuclear and negative staining of 14F7 in NSCLC. (B) Positive or negative nuclear pSTAT3 expression in NSCLC. Open arrows indicate example of nuclear staining; magnifications are indicated. (C) Kaplan-Meier survival analyses for overall survival based on GM3 expression or nuclear pSTAT3 expression in early-stage NSCLC patients. Median expression values were used to generate dichotomous variables.

NSCLC, non-small cell lung cancer
Immunohistochemistry

TMA sections were deparaffinized and endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide/methanol for 30 minutes. TMAs were subjected to none, 1 mM Tris/EDTA- or 10 mM citrate-based antigen-retrieval, depending on which primary antibody was used. Subsequently, slides were incubated with the appropriately diluted primary antibody at 4°C in a moist chamber. Bound primary antibodies were visualized using EnVision-reagents and diaminobenzidine (DAB+) chromogen (DakoCytomation, Glostrup, Denmark). Slides were counterstained with haematoxylin. Scoring and quantitation for all stainings were based on the whole biopsy surface (0.28 mm²) and on consensus between two independent observers.

To determine N-glycolyl GM3 expression in NSCLC tissue, the murine IgG1 anti-GM3 (N-glycolyl) monoclonal antibody 14F7 was used (kindly provided by Daniel Alonso, Buenos Aires, Argentina) [7]. Immunostaining with 14F7 did not require antigen retrieval, and 14F7 (1:1000) was incubated for 18 hours at 4°C. Mean percentage 14F7 positive cells of total number of tumor cells were determined per NSCLC case. In most statistical analyses, median value was used to create dichotomous variables.

STAT3 activation was determined by staining with a monoclonal antibody against STAT3 phosphorylated at tyrosine residue 705 (Clone D3A7; Cell Signaling, Boston, MA). Pretreatment involved heating the slides in Tris/EDTA for 30 minutes. The primary antibody (1:50) was then incubated overnight at 4°C. NSCLC samples with nuclear expression of pSTAT3 were designated positive. Number of positive tumor cells was multiplied by intensity of staining (0, 1, 2, or 3). Dichotomous variables were generated using median values as cut off.

Immature and mature DC infiltration was determined by monoclonal antibodies against CD1a (Clone MTB1; Monosan, Uden, The Netherlands) and CD83 (Clone 1H4b; Monosan, Uden, The Netherlands), respectively [25,25]. Both CD1a and CD83 immunostaining required citrate-based antigen retrieval. Both CD1a (1:5) and CD83 antibodies (1:25) were incubated for 1 hour at 4°C. Mean number of CD1a or CD83 positive cells per NSCLC case or per corresponding peritumoral lung tissue was calculated. If any infiltrating immature or mature DC were present, NSCLC cases were designated positive for immature or mature DC infiltration.

Microvessel density (MVD) per section was measured using immunostaining with a CD34-monoclonal antibody (Clone QBEnd10; DakoCytomation, Glostrup, Denmark), which required citrate-based antigen retrieval and an one-hour incubation of the primary antibody (1:50) at 4°C. MVD of each NSCLC case was defined as the mean number of CD34⁺ vessels per section (0.28 mm²). Since the sections were small, we did not identify a hot spot to count the CD34⁺ vessels, but counted vessels in the entire section.
Statistics
The statistical analysis between categorical data was done using the Pearson’s Chi-Square test. Student’s t-tests were performed to compare categorical data with continuous data. Kaplan-Meier plots and log rank analysis were applied to determine the significance of differences in overall survival. Values of $P \leq 0.05$ (two-tailed) were considered statistically significant.
Overall survival was the time between diagnosis and the date of death or the date at which patients were last known to be alive. For survival analysis, data collection was locked on 21st of February, 2007.

Results

Patient characteristics
NSCLC samples of 176 patients, who underwent resection of their primary tumor, were included on the TMAs. A summary of patient characteristics is listed in Table 1. Median overall survival of the population was 40.0 months. Low tumor stage and complete resection were predictive of favourable prognosis (log rank, $P = 0.02$ and $P = 0.0009$, respectively). Age, gender and histological subtype were not correlated with overall survival.

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*According to American Joint Committee on Cancer
GM3 and pSTAT3 expression in NSCLC

GM3 was expressed in almost all NSCLC samples: 94% of the 165 evaluable NSCLC samples expressed GM3, i.e. more than 5% of the tumor cells were positive. We defined positive cells as having cytoplasmic staining. Furthermore, GM3 expression was high: in 52% of all NSCLC samples, over 90% of the tumor cells were positive for 14F7. In 15% of the cases additional nuclear staining of 14F7 could be detected (Figure 1A). No association could be established between the different histological subtypes of NSCLC and GM3 expression, nor between tumor stage and GM3 expression. Of note, the GM3

**Figure 2** - (A) CD1a and CD83 immunostaining in both NSCLC tissues and peritumoral lung tissues. Magnifications are indicated. (B) Mature (i.e. CD83⁺) DC infiltration in NSCLC with low (below median) or high (above median) GM3 or pSTAT3 expression. (C) Compartmentalization of immature (CD1a⁺) and mature (CD83⁺) DCs as determined by their infiltration in peritumoral lung tissue or NSCLC tissue.

NSCLC, non-small cell lung cancer
expression rate in peritumoral lung tissue samples was also high, i.e. of 97% of the peritumoral samples, more than 5% of the lung epithelial cells were positive. However, expression levels were significantly lower than in NSCLC samples: in only 19% of all peritumoral lung samples, over 90% of the tumor cells were positive for 14F7 (as compared to 52% in NSCLC, peritumoral vs NSCLC, \( P < 0.001 \)).

pSTAT3 expression in the nucleus was present in 71% of the 164 evaluable NSCLC samples. Typical nuclear pSTAT3 immunostaining in NSCLC is shown in Figure 1B. Although previously reported to be associated with smaller tumors, limited smoking history and adenocarcinoma [20], we could detect no such associations between nuclear pSTAT3 expression and the clinical parameters tumor stage, histology, and smoking history. Peritumoral pSTAT3 expression did not differ significantly from pSTAT3 expression in NSCLC.

Using the median value as cut off, NSCLC patients with low GM3 expression (median percentage of GM3-positive cells, 91%) or high pSTAT3 expression (median number of pSTAT3 positive cells multiplied by intensity of staining [see Materials and Methods], 110) tended to have a worse overall survival, although this did not reach statistical significance (Figure 1C).

**Figure 3** - (A) CD34 immunostaining of vasculature in NSCLC to determine microvessel density. Magnification is indicated. (B) Microvessel density (MVD) according to low (i.e. below median) or high (i.e. above median) GM3 expression or pSTAT3 expression in NSCLC samples. Mean MVD values and SD are shown.

NSCLC, non-small cell lung cancer
DC infiltration and GM3 or pSTAT3 expression in NSCLC tissue

Typical immunostainings of CD1a and CD83 in NSCLC and peritumoral lung tissue are shown in Figure 2A. CD1a+ DC infiltration and GM3 expression status in NSCLC were not found to be associated (data not shown). Mature CD83+ DCs, however, were significantly more often present in NSCLC samples with low GM3 expression as compared to NSCLC samples with high GM3 expression (Figure 2B). Presence of either immature or mature DCs was not associated with nuclear pSTAT3 expression in NSCLC. In addition, DC numbers were not associated with tumor stage, histology, or smoking history and we could not establish any association between the number of infiltrating CD1a+ or CD83+ DCs and overall survival of NSCLC patients.

The compartmentalization of DCs in tumoral versus peritumoral tissues was previously associated with differential DC maturation status in breast and colorectal carcinomas [25,26,27]. Since TMAs contain small sections (0.28 mm²), exact architecture of the NSCLC tissue and the tumor-associated stroma is often lost. To investigate whether the (peri-)tumoral localization of DC is correlated with its maturation status, we therefore compared the presence of immature (CD1a+) and mature (CD83+) DC in the NSCLC samples with their incidence in peritumoral lung sections. Although overall frequencies of both immature and mature DCs were low, we found an increased number of immature CD1a+ DCs in NSCLC tissue as compared to peritumoral lung tissue (Figure 2C). Consistently, we observed less mature CD83+ DCs in NSCLC tissue as compared to peritumoral lung tissue (Figure 2C), which was in keeping with a previously observed compartmentalization according to DC maturation status [25,26,27].

Microvessel density and GM3 or pSTAT3 expression in NSCLC tissue

MVD was defined as the mean number of CD34+ vessels per section of one NSCLC sample (Figure 3A). MVD in NSCLC was not associated with tumor stage and smoking history. Conflicting reports have been made about the association between MVD and histology [28,29,30]. We found a significantly higher MVD in adenocarcinoma as compared to squamous cell carcinoma (mean values per NSCLC case [SD]: adenocarcinoma 32.2 [26.2]; squamous cell carcinoma 22.6 [11.4], $P = 0.02$). MVD was not found to be associated with overall survival of NSCLC patients.

NSCLC samples with low GM3 expression had a slightly, but significantly, higher MVD as compared to high GM3-expressing NSCLC samples (Figure 3B), while MVD was not significantly different between NSCLC with low or high nuclear pSTAT3 expression (Figure 3B).
Discussion

Our studies show that both GM3 and pSTAT3 are widely expressed in NSCLC and that GM3 expression is associated with a favourable patient survival ($P = 0.08$). To date, no reports have been made about the prognostic value of GM3 expression, while pSTAT3 has previously been demonstrated not to be associated with survival in NSCLC patients [20,31]. In our patient population, we found a trend for an association between high pSTAT3 expression in NSCLC and worse prognosis, although this did not reach significance ($P = 0.29$).

In literature contradictory reports have been made about the anti-angiogenic or pro-angiogenic potential of GM3 [15-17]. We found no evidence for a pro-angiogenic role, but rather the opposite, since GM3 expressing tumors were observed to have a slightly lower MVD. Gangliosides are biosynthesized by sequential enzymatic modifications by sialyltransferases; GM3 can be converted into GD3 by alpha2,8-sialyltransferase [5]. As low GM3 levels relative to GD3 has been implicated in angiogenesis [15], GD3 rather than GM3 may be the active pro-angiogenic factor. The next issue to be addressed should therefore be whether alpha2,8-sialyltransferase is expressed in the NSCLC cases with low GM3 expression and whether this coincides with high GD3 expression. GD3 in turn may then be correlated to high MVD in contrast to GM3. Further functional studies are clearly needed to confirm the distinct roles of GM3 and GD3 in angiogenesis.

pSTAT3 expression has been implicated in tumoral VEGF production and angiogenesis [23]. We could not establish a correlation between MVD and nuclear pSTAT3 expression in NSCLC. However, since the correlation between VEGF and MVD in NSCLC remains controversial [30], the lack of the correlation between pSTAT3 expression and MVD may not be unexpected. We did not observe a difference in nuclear pSTAT3 expression between peritumoral and NSCLC tissue. The lack of this difference might result from the production of cytokines and growth factors (like interleukin-6 and VEGF) by tumor cells. These factors might condition the surrounding cells, resulting in translocation of activated pSTAT3 to the nucleus in peritumoral non-malignant tissues.

Although the frequencies of both CD1a- and CD83-positive cells were low, we observed an inverse correlation between GM3 expression and infiltrating mature DCs, in keeping with previous reports, which showed that GM3 inhibited DC differentiation, maturation and migration [10,11,13]. GM3-induced inhibition of DC maturation and migration may have led to less mature DCs infiltrating the tumoral tissue, and consequently, may have contributed to the observed compartmentalization of DCs according to their maturation status: consistently, we observed cells expressing CD1a, which is generally believed to be a marker of immature DCs and to be down-regulated upon maturation, more frequently in NSCLC tissue as compared to the corresponding peritumoral lung tissue. In addition, we
observed cells positive for CD83, a DC maturation marker, more frequently in the peritumoral lung tissue as compared to the NSCLC sections.

Although pSTAT3 expression in tumor cells was reported to be associated with production of DC suppressive cytokines [22], DCs of any maturation status were not differentially infiltrated in NSCLC samples with high pSTAT3 expression as compared to NSCLC samples with low pSTAT3 expression.

Although some reports have suggested that infiltrating mature DCs predict a favourable prognosis in NSCLC [32,33], we could not establish a correlation between high numbers of mature tumor-infiltrating DCs and patient survival. The general paucity of infiltrating DCs in the relatively small NSCLC sections examined on the TMAs might explain the observed lack of correlation between DC infiltration and patient survival. More data have been published about the prognostic significance of MVD in NSCLC, and although many reports suggest that higher MVD predicts an adverse prognosis, the exact prognostic value of MVD remains unclear [30,32] We could not establish any correlation between MVD in NSCLC tissue and patient survival. Differences in technical procedures might make our results on MVD hard to compare with published data. Since only small NSCLC sections (0.28 mm²) were mounted on the TMA glass slides, structure of the tumor-microenvironment might have been lost. Scoring microvessels might be underestimated in these small sections, especially when considered that MVD is normally evaluated in the most intense vascularization areas of the tumor stroma [32,34]. Although TMAs are suitable for studying tumor cell characteristics, they may not be applicable when studying tumor-associated angiogenesis. On the other hand, one could argue that TMAs with a fixed size might be more appropriate to score MVD, since scoring MVD in the most intense vascularization area of the tumor stroma is susceptible to investigator’s bias.

Our observations of comparable levels of pSTAT3 between healthy and tumor tissues and a lack of correlation with MVD or mature DC infiltration do not support the implementation of STAT3 inhibitors as anti-angiogenic or immunostimulatory therapeutics in NSCLC. In contrast, GM3 was expressed at significantly higher levels in NSCLC than peritumoral lung tissues and was associated with tumor-related DC suppression, making this an attractive target for anti-cancer therapies. Approaches to target gangliosides are currently in early clinical development. Many studies focus on monoclonal antibodies including antibodies against N-glycolyl-containing gangliosides (i.e., 1E10) and on vaccine-based strategies [35,36,37,38]. Although induced immune responses have been reported, data on clinical efficacy are still lacking. The more specific murine monoclonal antibody 14F7, which we used in this study and recognizes N-glycolyl GM3, has entered clinical development [39]. Further studies are awaited to establish the clinical efficacy of humanized 14F7. Our present data suggest that side studies monitoring the immune status could be of additional value to define the role of GM3 in tumor immune escape, e.g. through DC suppression.
Conclusion

Our immunohistochemical TMA studies show that GM3 and pSTAT3 are widely expressed in NSCLC. pSTAT3 expression could not be associated with either DC-based tumor immune escape or angiogenesis, negating its suggested role as a 'master-switch' in these tumorigenic processes, at least for stage I-III NSCLC. In addition, we did not find evidence for a pro-angiogenic role of GM3. We could, however, confirm a possible involvement of GM3 in tumor-induced DC suppression in NSCLC patients. Its high tumor-specific expression makes GM3 a possible candidate for tumor targeting. Our findings suggest that it might be worthwhile to also monitor any angiogenic and immune effects of these therapeutic strategies that are currently under clinical development.

Acknowledgements

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References


CHAPTER 8

Glioblastoma-induced inhibition of dendritic cell differentiation from CD34+ precursors is mediated by interleukin-6 and unaffected by JAK2/STAT3 inhibition

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Abstract

Through immune suppressive mechanisms the glioblastoma microenvironment may be conditioned to resist anti-tumor immune responses. Glioblastoma can even systemically affect both T cell and monocyte functions, but little is known about its direct effect on dendritic cell (DC) differentiation. CD34+ precursor cells of the human acute myeloid leukemia-derived cell line MUTZ-3 were differentiated into interstitial DCs or Langerhans Cells (LCs) in the presence of conditioned media of the human glioblastoma cell lines U251 or U373. Glioblastoma-conditioned media inhibited DC and LC differentiation, resulting in maintained CD14 and reduced CD1a expression as well as in reduced CD86 and HLA-DR expression levels. This phenotypic inhibition coincided with functional DC impairment as determined by allogeneic mixed leukocyte reactivity and cytokine release. Furthermore, western blot analysis showed that glioblastoma-conditioned medium induced phosphorylation of STAT3 in both DCs and LCs. IL-6 blockade completely abrogated these glioblastoma-induced immunosuppressive effects and reduced STAT3 phosphorylation. However, neither addition of JSI-124 (cucurbitacin-I), a JAK2/STAT3 phosphorylation inhibitor, nor of GW5074, a Raf1-inhibitor, both of which interfere with signaling pathways reported to act downstream of the IL-6 receptor, prevented the observed inhibitory effects on DC differentiation. In conclusion, glioblastoma-derived IL-6 is responsible for the observed suppression of DC and LC differentiation from CD34+ precursors and appears to exert this effect in a STAT3 and Raf-1 independent fashion.
Introduction

Glioblastoma is the most common primary brain tumor and despite recent improvements in neurosurgical resection techniques, radiation therapy and chemotherapy (1) the median survival of glioblastoma patients is still only about one year (2). Glioblastoma is a highly vascularized, aggressive tumor mainly restricted to the intracranial compartment. Several immunotherapeutic approaches have been applied in patients with glioblastoma (3). Although some promising results were obtained, i.e. induction of antitumor cytotoxicity and some radiologic responses, the success of these anti-cancer strategies appeared to be hampered by the capability of glioblastoma to induce systemic immune suppression (4). Defective T-cell function has been reported in glioblastoma patients and included low peripheral lymphocyte counts and decreased response to T-cell mitogens or antigens. Moreover, an increased fraction of T-cell suppressive regulatory T-cells (T-regs) among an already reduced number of CD4+ T-cells has been described in glioblastoma patients (5). Although the effects on T-cell function have been well-documented, little is known about the effects of glioblastoma on dendritic cell (DC) differentiation and function.

DCs play a central role in the immune system as powerful antigen-presenting cells, and are essential for the induction of tumor-specific T cell-mediated immune responses. Hampered DC differentiation and activation has been reported in many tumors and decreased tumor infiltration by DC has been identified as a poor prognostic factor (6;7). It has been reported that circulating monocytes in glioblastoma patients have a reduced capacity to differentiate ex vivo into mature DCs (8). Various tumor-derived soluble factors, such as interleukin (IL)-6, IL-10, macrophage colony stimulating factor (M-CSF), transforming growth factor (TGF)-β, prostaglandins, and vascular endothelial growth factor (VEGF), have been identified (9-11) as causative agents for the disturbed systemic DC differentiation observed in a variety of solid tumor types (12;13). As all these factors bind to different cellular receptors, but exert similar inhibitory effects on DC differentiation, they might converge at a common intracellular signaling level. One such a possible common intracellular pathway has been suggested to be the Janus-activated kinase-(JAK)2/signal transducer and activator of transcription (STAT)3 pathway. Normal hematopoietic cell differentiation requires JAK2/STAT3 activity because this is one of the main pathways used by cytokines that support myeloid cell differentiation (14). During normal DC differentiation, however, it has been demonstrated that STAT3 activity is decreased (15). This decrease in STAT3 activity of DCs was prevented and the differentiation was suppressed when precursor cells were differentiated in the presence of tumor-conditioned medium. Inhibition of STAT3 activation abrogated the suppressive effects of factors derived from colon or pancreas carcinoma or melanoma cell lines on DC differentiation, both in human and in murine models (9;15;16).
Both epithelium-associated Langerhans cells (LCs) and connective tissue-associated DCs may play vital roles in the generation of anti-tumor immune responses. We therefore studied the effects of glioblastoma-derived soluble immune suppressive factors on the differentiation of both subsets employing the MUTZ-3 DC cell line model. We previously showed the CD34+ acute myeloid leukemia-derived cell line MUTZ-3 to provide a very relevant and representative model for the differentiation of both LC and DC subsets. Both MUTZ-3 DC and LC display typical phenotypic traits (both at the transcriptional and at the protein level (17-19) as well as functional characteristics (20) of their physiological counterparts.

In the current studies, we identified glioblastoma-derived IL-6 as the major contributor to the inhibition of DC and LC differentiation and function. Tumor-induced defective DC and LC differentiation resulted in STAT3 activation as part of a transduction pathway downstream of the IL-6 receptor. However, inhibition of STAT3 and Raf-1, both components of reported pathways signaling downstream of the IL-6 receptor, could not overcome the glioblastoma-mediated suppressive effects on DC and LC differentiation.

Materials and Methods

**Dendritic cell and Langerhans cell differentiation**

**MUTZ-3 DC and MUTZ-3 LC**

The human acute myeloid leukemia-derived cell line MUTZ-3 (DSMZ, Braunschweig, Germany) was maintained in MEM-α with ribonucleosides and deoxyribonucleosides (Gibco, Grand Island, NY) supplemented with 20% FCS, penicillin, streptomycin, 50 μM β-mercaptoethanol and 10% conditioned medium from the human renal cell carcinoma cell line 5637. In a cytokine-dependent manner, MUTZ-3 cells can be differentiated into DCs or LCs, as previously described (19). In short, for the generation of immature MUTZ-3 DCs (iMUTZ-3 DC), 5637-conditioned medium was replaced by GM-CSF (100 ng/mL), TNF-α (2.5 ng/mL) and IL-4 (20 ng/mL) and MUTZ-3 cells were cultured for 7 days. For the generation of immature MUTZ-3 LCs (iMUTZ-3 LC), MUTZ-3 cells were cultured for 10 days in the presence of GM-CSF (100 ng/mL), TNF-α (2.5 ng/mL) and TGF-β (10 ng/mL) without 5637-conditioned medium. Cytokines were added every 3-4 days. Maturation of immature MUTZ-3 DCs or LCs (mMUTZ-3 DCs and LCs) was accomplished by adding 20% monocyte-conditioned medium for an additional 2-3 days.

**Monocyte-derived DC (MoDC)**

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors via density-gradient centrifugation (Lymphoprep™, Oslo, Norway). Monocytes were isolated via adherence and after vigorously washing to remove non-adherent cells, the adherent cell fraction was cultured in IMDM (10% FCS, penicillin, streptomycin), GM-CSF (100 ng/mL)
and IL-4 (10 ng/mL) during 6 days to generate immature MoDCs (iMoDC). Maturation of iMoDCs was accomplished by adding 20% monocyte-conditioned medium and TNF-α for an additional 2-3 days.

**Cancer cell lines**

Glioblastoma cell lines U251 and U373 and colon cancer cell lines Colo205 and HT29 were cultured in DMEM supplemented with 10% FCS, penicillin, streptomycin and L-glutamin. Tumor-derived supernatants were harvested from 10^7 cells after 72 hours of culture. Supernatants were centrifuged to remove cell debris and 20x concentrated by use of an Amicon Ultra-15 filter (Millipore Corporation, Billerica, MA, USA). 5% of 20x concentrated tumor-derived supernatant was added to the differentiation assays every 3-4 days, unless stated otherwise. 20x concentrated unconditioned control medium and 20x concentrated conditioned medium of primary fibroblasts were used as controls.

**Flowcytometric analysis**

For flowcytometric analysis, cells were incubated for 30 minutes at 4°C in PBS with 0.1% BSA and 0.01% NaN₃, in the presence of mouse isotype-matched control monoclonal antibodies (mAbs) or PE-labeled mAbs against CD34 (Sanquin, Amsterdam, The Netherlands), CD83, CD207 (Coulter Immunotech, Marseilles, France), CD1a (PharMingen, San Diego, CA), CD86, HLA-DR (BD Biosciences, San Jose, CA), or FITC-labeled mAbs against CD1a, CD14, CD40, CD80 (BD Biosciences), and DC-specific ICAM-grabbing nonintegrin (DC-SIGN; PharMingen). The cells were subsequently measured using a FACS Calibur and analyzed with CellQuest software (BD Biosciences).

**Western blot**

After harvesting, DCs and LCs were resuspended in lysisbuffer containing 100 μl phosphatase inhibitor cocktail 1, 100 μl phosphatase inhibitor cocktail 2 (both Sigma-Aldrich, Steinheim, Germany) and 1 Complete Mini Protease inhibitor tablet (Roche Diagnostics, Indianapolis, IN, USA) in TBS-NP40 (1%). The total protein content of the cell lysates was quantified in duplicate with the BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Samples containing 15 μg protein were denatured before loading on the gel and were separated by SDS-PAGE, after which they were transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA) by electrophoresis. Membranes were blocked with milk powder and probed overnight either with anti-STAT3 antibody or anti-pSTAT3 antibody (both obtained from Cell Signaling Technology Inc., Danvers, MA, USA). After washing, the membranes were incubated with horseradish peroxidase-conjugated antibody (Dako, Glostrup, Denmark) for 1 hour at room temperature. Immunoreactive bands were visualized on film using the enhanced chemiluminescence (ECL) detection kit (Amersham Biosciences, Buckinghamshire, UK).
loading control, membranes were also probed with a monoclonal anti-β-actin antibody (Sigma-Aldrich, Steinheim, Germany).

**Cytokine production by MoDCs**

Immature MoDCs were stimulated for 18 hours with interferon (IFN)-γ and irradiated J558 cells (myeloma cell line) transfected with CD40L (a kind gift of Dr. M. Kapsenberg, Amsterdam, The Netherlands). IL-8, IL-10 and IL-12p70 produced by MoDCs were determined by ELISA, following manufacturers’s instructions (Sanquin, Amsterdam, The Netherlands).

**Allogeneic mixed leukocyte reaction**

Mixed leukocyte reaction (MLR) was performed with mature, irradiated mMUTZ-3 LCs, which were added as stimulator cells to round-bottom, 96-well, tissue-culture plates (Costar, Corning, NY) at graded doses. As responder cells, $10^5$ peripheral blood lymphocytes (PBLs) per well were used, which were obtained from PBMCs after removal of monocytes by MACS using anti-human CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Stimulation of PBLs was performed in triplicate. Cells were cultured in MEM-α medium (10% human serum, penicillin, streptomycin, β-mercaptoethanol) for 5 days. Cells were pulsed for the last 18 hours of culture with $[^3H]TdR$ (Amersham, Aylesbury, UK), after which the cells were harvested onto fiberglass filters, and $[^3H]TdR$ incorporation was determined using a flatbed scintillation counter.

**Cytokine measurement in tumor-derived supernatants**

Concentrations of IL-6 and IL-10 in the various concentrated supernatants of tumor cell lines and primary fibroblasts were determined by Cytometric Bead Array (CBA) (human inflammation kit; BD Biosciences, San Diego CA). TGF-β1 and TGF-β2 were determined by ELISA (Promega, Madison WI) following manufacturer’s instructions.

**Modulation and simulation of glioblastoma-induced effects on DC and LC differentiation**

To study the inhibitory effects of separate immune suppressive factors present in the supernatants of glioblastoma cell lines, U373 supernatant was pre-incubated for 30 minutes with neutralizing antibodies against IL-6, IL-10, TGF-β, M-CSF or VEGF (R&D, Minneapolis MN), at sufficiently neutralizing concentrations as determined previously with recombinant human cytokines (21), before addition to DC and LC differentiation assays. Supernatant, pre-incubated with the neutralizing antibodies, or rhIL-6 (R&D) was replenished every 3-4 days. Moreover, interference with signal transduction in DCs and LCs was studied by the use of JSI-124 (cucurbitacin-I; Calbiochem, Darmstadt, Germany), an inhibitor of JAK2/STAT3, and GW5074 (Sigma Aldrich, St. Louis MO), an inhibitor of
Raf1. The maximum non-toxic dose was 0.03 μM and 5 μM, respectively, and these concentrations were added to the differentiation cultures and were replenished every 3 days similar to the cytokines and supernatants. For both inhibitors, DMSO served as vehicle control.

Statistics
Student’s t-tests were performed to determine significance of differences. Values of $P \leq 0.05$ (two-tailed) were considered statistically significant.

Results

Glioblastoma-conditioned medium suppresses phenotypic differentiation of dendritic and Langerhans cells

iMUTZ-3 DC and iMUTZ-3 LC were differentiated with or without tumor cell line supernatants or fibroblast supernatant for 7 and 10 days, respectively. In iMUTZ-3 DC and LC cultures, addition of glioblastoma supernatant resulted in disturbed differentiation with significantly increased mean numbers of CD14+ cells and decreased numbers of fully differentiated CD1a+DC-SIGN+ DCs and CD1a+Lang++ LCs, respectively (Fig. 1A and 1B). Of note, addition of fibroblast supernatant had no detrimental effect on DC or LC differentiation, while the particularly powerful effects of the glioblastoma supernatants in this respect were further accentuated by the inability of colon tumor cell line supernatants to reach similar levels of DC or LC inhibition (Fig. 1A and 1B). As further evidenced by the data presented in Fig. 1A and 1B, the inhibitory effects of the glioblastoma supernatants were more pronounced in LC than in DC differentiation with high numbers of CD14+ precursor cells accumulating in the MUTZ-3 LC cultures. MoDC cultures were taken along as “gold standard” and similar inhibitory effects of the glioblastoma supernatants were observed, with resulting low rates of CD1a+ and high rates of CD14+ cells in the MoDC (data not shown). The more pronounced inhibitory effects of the glioblastoma supernatants also became clear from the reduced HLA-DR expression levels after DC, and the reduced CD86 and HLA-DR expression after LC differentiation (Fig. 1C).

Comparable results to Colo205 and U373 supernatant were obtained with the tested supernatants of the other colon carcinoma (HT29) and glioblastoma cell line (U251), respectively (data not shown).
Figure 1 - Phenotypic analysis of the effects of 5% 20x concentrated conditioned media (sup) of colon carcinoma and glioblastoma cell lines on (A) MUTZ-3 derived immature DC (iMUTZ-3 DC) and (B) MUTZ-3 derived immature LC (iMUTZ-3 LC) differentiation (all n = 3). (C) Phenotypic analysis of the effects of 5% 20x concentrated conditioned media (cm) of Colo205 or U373 cell lines on the activation status of iMUTZ-3 DC and iMUTZ-3 LC determined by CD86 and HLA-DR expression levels. Results are representative for three experiments. Percentage of positive cells and mean fluorescence index (MFI) are shown.

*, P < 0.05 compared to the control

Glioblastoma-conditioned medium suppresses functional activity of MoDCs and MUTZ-3 LCs

Since both LC in general and MUTZ-3-derived LC in particular are known to be relatively poor producers of cytokines (18;20), we used glioblastoma-conditioned MoDC to
determine the release of immunostimulatory IL12p70 versus immunosuppressive IL-10. Upon CD40L stimulation, iMoDCs, which were differentiated in the presence of glioblastoma-conditioned medium, produced less IL12p70, resulting in increased IL-10/IL12p70 ratios, suggestive of a less T cell stimulatory and potentially more Th2-skewing phenotype (Fig. 2A). In addition, we determined the concentration of IL-8, a chemo-attractant with pro-angiogenic activities produced by (a.o.) macrophages. When glioblastoma supernatant was added to iMoDCs differentiation, iMoDCs produced significantly more IL-8 as compared to control iMoDCs (Fig. 2A).

In addition, mMUTZ-3 LC were tested for their ability to stimulate allogeneic T-cell proliferation. mMUTZ-3 LC differentiated in glioblastoma-conditioned medium were significantly reduced in their ability to stimulate T cells as compared to normally differentiated and matured mMUTZ-3 LC (Fig. 2B).

**Figure 2** - (A) After a 24-hour CD40L-stimulation of iMoDCs (differentiated with or without 5% 20x concentrated conditioned media [cm] of glioblastoma cell lines), concentrations of IL-8, IL-10 and IL-12p70 were determined in the supernatant by ELISA. (B) [³H] thymidine incorporation in peripheral blood lymphocytes (PBLs) stimulated with different numbers of irradiated mature MUTZ-3 LCs (mMUTZ-3 LC), which were differentiated and matured in the presence or absence of 5% 20x concentrated conditioned media (cm) of glioblastoma cell lines. One experiment of two is shown.

*, P < 0.01 control versus addition of U251 or U373 conditioned medium.
Glioblastoma-induced phenotypic inhibition of DC and LC differentiation coincides with pSTAT3 expression

Tumor-induced DC suppression was previously associated with activation of and signaling through the JAK2/STAT3 pathway. To ascertain possible involvement of this pathway in the observed DC-inhibitory effects of the glioblastoma supernatants, iMUTZ-3 DC and iMUTZ-3 LC were differentiated with or without 5% 20x conditioned medium of Colo205, HT29, U251, or U373 cells. After 6 days (for iMUTZ-3 DCs) or 10 days (for iMUTZ-3 LCs) cells were harvested and STAT3 activation was determined by Western blot. As shown in Fig. 3 for iMUTZ-3 LCs, the expression of phosphorylated STAT3 (pSTAT3) was indeed strongly increased when cells were differentiated in the presence of the glioblastoma tumor cell line supernatants. Equivalent data were obtained for iMUTZ-3 DCs (data not shown).

![Western blot analysis of (p)STAT3 expression in iMUTZ-3 LCs differentiated with or without 5% 20x concentrated conditioned media (cm) of colon carcinoma or glioblastoma cell lines.](image)

**Figure 3** - Western blot analysis of (p)STAT3 expression in iMUTZ-3 LCs differentiated with or without 5% 20x concentrated conditioned media (cm) of colon carcinoma or glioblastoma cell lines.

High levels of the immunosuppressive cytokines IL-6 and TGFβ in glioblastoma-conditioned media

The concentration of a number of immunosuppressive cytokines known to inhibit DC and LC differentiation, and with the potential to signal through STAT3, was determined in the supernatant of glioblastoma and colon carcinoma cell lines. Table 1 shows the concentrations in ng/ml of IL-6, IL-10, TGF-β1 and TGF-β2 in the 20x concentrated conditioned media. High levels of IL-6 were detected in the conditioned media from glioblastoma cell lines, in contrast to the media conditioned by the colon carcinoma cell lines. Concentrations of TGF-β1 and TGF-β2 were also more than 2-fold higher in the U251 and U373 glioblastoma supernatants as compared to Colo205 and HT29 supernatant. IL-10 was not detectable in any of the tumor cell line supernatants. Fibroblast supernatant, in which moderate levels of the tested cytokines were present, served as control and did not have an inhibitory effect on DC phenotype and function.
Table 1 - Concentrations in ng/ml of immunosuppressive factors in 20x concentrated conditioned medium of glioblastoma (U373 and U251), colon carcinoma (Colo205 and HT29) cell lines and control cells (primary fibroblasts) determined by cytokine bead array (IL-6 and IL-10) or enzyme-linked immunoassay analysis (TGFβ1 and TGFβ2).

<table>
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<th></th>
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<td>34</td>
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<td>0.68</td>
<td>26</td>
<td>&lt; 0.03</td>
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</tbody>
</table>

IL-6 blockade overcomes glioblastoma-induced inhibition of iMUTZ-3 DC and LC differentiation

The relative contribution of suppressive cytokines to the observed DC-inhibitory effects was assessed in the U373 supernatant, as this supernatant proved to be most effective in suppressing iMUTZ-3 DC and LC differentiation. Via blocking the activity of several cytokines and growth factors using neutralizing antibodies, we identified IL-6 as the responsible cytokine for the observed glioblastoma-induced suppression in MUTZ-3 LC differentiation cultures (Fig. 4A). Pre-incubation of U373 supernatant with anti-IL-6 antibody ablated the suppressive effects on iMUTZ-3 LC differentiation as compared to pre-incubation of U373 with an IgG1 isotype control antibody, with virtually normalized relative percentages CD14⁺ and CD1a⁺/Langerin⁺ cells in relation to the control cultures. Indeed, blockade of IL-6 in the supernatant significantly increased the expression levels of Langerin as compared to the isotype antibody control (P = 0.02). IL-6 blockade interfered with the inhibitory effects of glioma supernatants in MUTZ-3 iDC cultures to an equivalent extent (data not shown). Although TGFβ was secreted in high amounts by the glioblastoma lines, we were unable to test its contribution to LC inhibition through neutralization, since TGFβ is an essential cytokine for the proper differentiation of LC. However, we tested the effect of TGFβ neutralization in the U373 supernatant on DC differentiation and we found no mitigation of the suppressive effects, indicating that IL-6 is the major cause of the observed glioma-mediated DC suppression. Indeed, addition of recombinant hIL-6 to DC or LC differentiation cultures accurately mimicked the effects of the glioma-derived supernatants (data not shown).

We next determined the (p)STAT3 status in iMUTZ-3 DCs and LCs differentiated in the presence of U373-conditioned medium with neutralizing antibodies against known immunosuppressive factors and found that anti-IL-6 was capable of decreasing the
pSTAT3 levels induced by the U373 supernatant, both in iMUTZ-3 DC and in iMUTZ-3 LC (shown for LCs in Fig. 4B).

**Figure 4** - (A) Phenotypic analysis of the effects of U373-conditioned medium, pre-incubated with neutralizing antibodies against known immunosuppressive factors, on LC differentiation. Typical flowcytometric results (upper panel) and mean relative values and SD (lower panel) of three experiments are shown. (B) Western blot analysis of (p)STAT3 expression in iMUTZ-3 DC and LC.
differentiated in the presence of 5% 20x concentrated U373-conditioned medium pre-incubated with neutralizing antibodies against known immunosuppressive factors. MFI, mean fluorescence index; cm, conditioned medium

**JAK2/STAT3 inhibition does not prevent glioblastoma-induced inhibition of iMUTZ-3 DC or LC differentiation**

To assess a possible causal link between the observed differentiation inhibition and STAT3 activation down-stream from the IL-6 receptor, iMUTZ-3 DCs or LCs were differentiated in U251- or U373-conditioned medium with or without the small-molecule inhibitor of JAK2/STAT3, JSI-124, at 0.03 μM (identified as the most effective non-toxic concentration upon testing in a range of 0.0003-3 μM). Western blot analysis demonstrated that addition of JSI-124 at 0.03 μM decreased the expression of U373-induced pSTAT3 in iMUTZ-3 DCs (data not shown) and LCs (Fig. 5A). Of note, STAT3 expression as a whole was down-regulated, rather than just pSTAT3. However, phenotypic analysis of iMUTZ-3 DCs and LCs showed that U373-mediated inhibition of iMUTZ-3 DC and LC differentiation was not prevented by addition of JSI-124 to the cultures (shown for LC in Fig. 5B).

**Inhibition of Raf1 does not prevent U373-induced defective DC and LC differentiation**

Other pathways downstream of the IL-6 receptor may bypass STAT3 and could be responsible for the suppressive effects of IL-6 on DC and LC differentiation. By blocking a component of the pathway downstream of the IL-6-R leading to activation of mitogen-activated protein kinase (MAPK), i.e. Raf kinase, we tried to identify such an alternative pathway as being possibly responsible for mediating the observed inhibition of DC and LC differentiation. For this purpose, GW5074, a benzylidine oxindole derivative inhibiting the kinase activity of c-Raf1, was used. iMUTZ-3 DCs or LCs were differentiated in U373-conditioned medium with or without a 60-minute pre-incubation of the MUTZ-3 progenitors with GW5074. Phenotypic analysis of iMUTZ-3 DCs and LCs upon subsequent differentiation showed that Raf1 inhibition could not prevent the U373-mediated inhibition of DC and LC differentiation (shown for LCs in Fig. 5B).

**Discussion**

Our findings demonstrate that inhibition of DC and LC differentiation by the glioblastoma cell lines U251 and U373 is mediated primarily through IL-6. In our model, glioblastoma-conditioned medium prevented monocyte and CD34+ precursors to acquire DC and LC characteristics. In keeping with this, CD34+ precursors acquired CD14 expression, but
failed to develop into fully differentiated DC or LC, when DC or LC differentiation was induced in glioblastoma-conditioned medium. In addition, glioblastoma-derived soluble factors inhibited the up-regulation of (co-)stimulatory molecules like CD86 and HLA-DR necessary for T cell activation and antigen presentation, respectively. Of note, the suppressive effects of the tumor supernatant on CD86 expression were not evident in MUTZ-3 DCs (Fig 1C). This lack might result from the modulating effects of IL-4 used in the DC differentiation assay. Such protective effect of IL-4 from suppression by tumor-derived IL-6 has been previously described (22). The phenotypic inhibition of DCs and LCs was shown to be almost completely IL-6-dependent and was consistent with the observed glioblastoma-induced reduction in functional activity of DCs and LCs. Our functional assays showed that DCs or LCs differentiated in glioblastoma-conditioned medium, displayed a reduced T cell stimulatory capacity, while MoDCs generated in glioblastoma-conditioned medium displayed a decreased ability to release IL-12p70 and acquired a potential

**Figure 5** (A) Western blot analysis of (p)STAT3 expression in iMUTZ-3 LCs differentiated with or without JSI-124 (0.03 μM) and in the presence of U251- or U373-conditioned medium. (B) Phenotypic analysis of the effect of JSI-124 (left graph) or GW5074 (right graph) on iMUTZ-3 LCs differentiated in the presence of U373-conditioned medium. Mean relative values and SD of three experiments are shown. cm, conditioned medium
Glioblastoma-induced defective DC differentiation

macrophage-like angiogenic activity, as suggested by the increased production of the pro-angiogenic factor IL-8.

Glioblastoma-conditioned medium, like rhIL-6 (data not shown), induced STAT3 activation in both DCs and LCs, and IL-6 neutralization could overcome this STAT3 activation. These observations are in keeping with the current hypothesis that suppression of DCs by IL-6 is mediated through STAT3 (23;24). Indeed, many reports have surfaced, claiming STAT3 to be a tumorigenic "master switch", controlling multiple pro-tumorigenic mechanisms such as angiogenesis and immune escape (25). However, inhibition of STAT3 phosphorylation by JSI-124 did not reverse the glioblastoma-induced phenotypic inhibition of DC and LC differentiation. Thus, whereas the JAK2/STAT3 pathway was previously implicated in glioblastoma growth, invasion and apoptosis (26;27) and in the suppression of T cell activation in the glioblastoma microenvironment (28), our data could not confirm a role for STAT3 in glioblastoma-induced DC suppression. Beside the JAK2/STAT3 pathway, other pathways can be activated upon agonistic engagement of the IL-6 receptor, e.g. the Raf1/ERK pathway, which leads to activation of MAPK. However, blocking Raf1 could not reverse the inhibitory effects of glioblastoma-conditioned medium on DC and LC differentiation either. These results suggest that another pathway downstream of the IL-6 receptor accounts for the observed effects. One such pathway may be the PI3-K/Akt pathway which can also be activated in response to IL-6 (29). Thus, although we could clearly establish IL-6 as a major contributor to the immunosuppressive effects by glioblastoma cells, we did not yet identify the responsible downstream signaling pathway.

IL-6 is one of the most ubiquitously deregulated cytokines in cancer (30). High circulating IL-6 levels in cancer patients have been associated with a poor prognosis and are believed to contribute to paraneoplastic symptoms, like fever and weight loss (30;31). Beside a possible role as an autocrine tumor cell growth factor, IL-6 may inhibit anti-tumor immune response by hampering DC differentiation as demonstrated in renal cell cancer and multiple myeloma (10;12;32;33). In a recent study in glioblastoma patients, IL-6 gene amplification was associated with tumor grade and shortened survival (34). Remarkably, studies on the effects of IL-6 on DC differentiation in glioblastoma have so far been lacking. Based on the results of our studies, glioblastoma-derived IL-6 might interfere with proper DC and LC differentiation and thus contribute to tumor immune escape. The brain has been recognized as an immune privileged site as a consequence of the absence of a lymphatic system and the existence of the blood-brain barrier. However, activated lymphocytes do cross the blood-brain barrier and glioblastoma patients can develop specific endogenous immune responses against their tumors (35;36). Indeed, in recent years glioblastoma has become a major focus of attention as a potential target for DC-based immunotherapeutic approaches with reported T-cell responsiveness, correlating with clinical outcome (36-38). Nevertheless, many patients do not show an immunological response, calling for improvement of vaccine strategies. As DCs play a vital role in the
induction as well as the effector phase of anti-tumor immune responses, our results suggest that targeting tumor-derived IL-6 might add to the efficacy of immunotherapy. Several strategies to block IL-6 activity have been investigated, of which monoclonal antibodies directed against IL-6 are the most widely studied. Early-stage clinical trials with these antibodies for the treatment of B-lymphoproliferative disorders, multiple myeloma, and renal cell carcinoma have shown promising results (30). In addition, the therapeutic impact of antibodies against IL-6 on paraneoplastic syndromes and cancer-related anorexia and cachexia may also be of clinical benefit in cancer patients. No clinical studies in glioblastoma patients with anti-IL-6 antibodies have yet been conducted or reported, but based on the current study this strategy might be an effective adjuvant in vaccination strategies in this disease.

In conclusion, we found clear evidence for glioblastoma-derived IL-6 as the major contributor to immunosuppressive effects on both DC and LC differentiation and function, although the responsible signaling pathway downstream of the IL-6 receptor remains to be identified. Our data suggest that IL-6 is an important target in glioblastoma for immunomodulation, which may help to design more effective immunotherapeutic strategies for this lethal disease.

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**References**


CHAPTER 9

Summarizing discussion and future perspectives
Over the past two decades, new anti-cancer therapeutics have been developed based on an increased understanding of tumor biology. For tumor types with a limited number of unique alterations, a treatment specifically targeting those alterations should be developed, e.g. imatinib targeting bcr-abl or cKIT in chronic myeloid leukemia and gastrointestinal stroma cell tumors, respectively. In patients, who have tumor types with alterations in multiple pathways, such as breast cancer and the vast majority of NSCLC, we should try to design anti-cancer strategies that target multiple pathways driving the biology of these tumors in order to reduce acquired resistance and improve outcome. In the introductory chapter 1 of this thesis, we describe two general features of virtually all solid tumors that contribute to tumor progression, i.e. angiogenesis and immune escape. The cross-talk and inverse co-regulation between angiogenesis and the immune response are reminiscent of the physiological mechanisms underlying wound healing. The continuous production of growth signals by tumors leading to ongoing angiogenesis have contributed to the notion that tumors are wounds that never heal (1). Ongoing angiogenesis interferes with infiltration of immune cells into the tumor via decreased expression of adhesion molecules on endothelial cells, a process called tumor endothelial cell anergy (2). The contribution of infiltrating immune cells to tumor progression has been a matter of discussion (3). Tumors have the ability to alter normal hematopoiesis and DC differentiation via tumor-derived factors, leading to accumulation of immature myeloid-monocytic cells, which may aid tumor development by release of pro-angiogenic factors or transdifferentiation to endothelial-like cells, both in support of neovascularization (4). These so-called myeloid suppressor cells (MSC) also exert a profound inhibitory activity on the anti-tumor immune response (5). Therefore, MSCs may be a target for modulation of both angiogenesis and the immune response. All-trans retinoic acid (ATRA) and 1,25-hydroxyvitamin D3 have been shown to reduce MSC frequencies in preclinical models directly and indirectly, respectively (6-8). In addition, STAT3 inhibition may allow differentiation of hematopoietic cells into fully mature DCs in the presence of tumor-derived suppressive factors, and thus prevent induction of MSC (9). We also observed a reduction in MSC frequencies in advanced cancer patients treated with cediranib, a specific VEGFR inhibitor (chapter 5). This is consistent with the notion that some tumor-derived factors, such as VEGF and gangliosides, interfere with a proper hematopoiesis and DC differentiation, while simultaneously stimulating angiogenesis (figure 1). This inverse co-regulation between tumor-induced angiogenesis and immune suppression was the main subject of study in this thesis with the express outlook that an increased understanding of the factors and mechanisms underlying this co-regulation might translate into more effective anti-cancer strategies.
Figure 1 - The balance between DC maturation and suppression and possible consequences for immunity and angiogenesis. Figure represents situation in cancer-conditioned environment.

In part I of this thesis we describe the current clinical experience with VEGFR TKIs. VEGF is widely regarded as an important tumor-derived modulator of both angiogenesis and DC differentiation. Interference with VEGFR signaling might therefore affect both processes. Although anti-angiogenic treatment via TKIs is widely applied in the clinic, there are still issues and questions with respect to toxicity and efficacy that remain to be elucidated. Since angiogenesis is supposed to be quiescent during adult life and only activated in tumoral areas, it has been suggested that targeting angiogenesis as a therapeutic anti-cancer strategy would cause little toxicity. Nevertheless, clinical administration of VEGFR TKI has uncovered very particular side effects. Chapter 2 outlines the specific toxicities of VEGFR TKIs and their management as well as issues regarding acquired resistance and tumor evaluation. Moreover, the need for a better understanding of long-term effects of VEGFR TKIs on tumor biology and normal human cell and tissue physiology is discussed. In phase I clinical studies, in which pharmacodynamics of new agents are explored, patients are monitored for a relatively short period. Since the consequences of anti-angiogenic treatment such as with VEGFR TKI change over time (10;11) and the treatment will be administered over long periods (preferably chronically), patients should be monitored beyond the first months of treatment. Pharmacodynamics should therefore also be included in phase II or even phase III studies. As repetitive tumor biopsies are not feasible in the clinic, identification of systemic biomarkers, such as serum VEGF levels or soluble VEGFR-2 levels, is important. These can help to monitor, non-invasively and on a large-scale, long-term effects of anti-angiogenic agents. Understanding the long-term effects of these TKIs may lead to optimized administration schedules with a reduced chance of inducing acquired resistance and may help to design better combination regimens that finally will improve efficacy. One such possible regimen is the combination of anti-angiogenic TKI therapy with inhibition of the EGFR pathway. Chapter 3 describes
the rationale to combine these two strategies: preclinical evidence shows an important role of the EGFR pathway in angiogenesis, either directly by stimulating endothelial cells or indirectly by tumoral production of angiogenic molecules upon EGF stimulation. Additionally, acquired resistance to EGFR inhibitors can be circumvented by angiogenesis inhibitors. Results of a phase I study combining cediranib, a VEGFR TKI, and gefitinib, an EGFR TKI, are reported in chapter 4. This study showed that combination treatment was generally well tolerated with manageable adverse events. The most common adverse events were diarrhea, hypertension, anorexia and fatigue. The protocol-defined maximum-tolerated dose (MTD) of cediranib was 30 mg/day with gefitinib 250 mg/day. In a parallel expansion cohort, 45 mg/day cediranib was the maximum investigated dose combined with gefitinib 500 mg/day and found to be tolerated well. Since 45mg/day was previously found to be the MTD for cediranib monotherapy, no further expansions beyond this dose were investigated. Pharmacokinetics of both cediranib and gefitinib were not substantially affected when administered in combination. Encouraging evidence of anti-tumor activity was observed as 9% of the patients experienced a partial response. Of special interest were patients with advanced RCC; 33% of advanced RCC patients achieved a partial response. The results of this phase I study support the rationale as discussed in chapter 3 for concurrent inhibition of the VEGFR and EGFR signaling pathways.

As mentioned above and discussed in chapter 1, there is increasing evidence of co-regulation of angiogenesis and defective DC differentiation in cancer patients. In part II we explored whether targeting angiogenesis via VEGFR TKI could affect tumor-induced DC suppression, providing a window for combined immunotherapy. In chapter 5 we investigated whether clinical VEGFR inhibition by cediranib could overcome tumor-induced suppression of DC differentiation. Cediranib also inhibits to a lesser extent TK activity of PDGFRs and cKIT. We observed decreased frequencies of myeloid and plasmacytoid DCs and increased frequencies of MSCs in the peripheral blood of advanced cancer patients as compared to healthy donors. The effect of advanced tumors on DC differentiation is therefore systemic and results in a profound reduction of more mature DC in the circulation and a simultaneous accumulation of immature myeloid DCs with a potentially immunosuppressive role. Although tumor-derived VEGF is thought to be a major factor involved in DC suppression (12;13), one-month treatment with cediranib could not restore the aberrant DC (precursor) frequencies. It did, however, result in a trend towards reduced MSC frequencies. In advanced cancer patients DC suppression is most likely caused by multiple and/or tumor-specific factors and cediranib may have been too selective for VEGFR inhibition in this regard. In chapter 6 we investigated whether sunitinib, targeting not only the tyrosine kinase activity of all three VEGFRs, but also the tyrosine kinase activity of the PDGFRs, fms-like tyrosine kinase-3 (FLT3), and cKIT, could normalize the aberrant myeloid lineage differentiation profile in advanced RCC patients. Our data showed that daily administration of sunitinib 50 mg for 4 weeks resulted in a
combined lowering of abnormally high levels of immunosuppressive myeloid leukocytes and a relative increase of immunostimulatory CD1c+ MDC-1 frequencies, the latter specifically in patients with tumor regression. Apart from the use of different agents with their own inhibitory profile with the more promiscuous sunitinib targeting a wider range of cytokine and growth factor receptors, the discrepancy between the results described in chapters 5 and 6 could also be explained by the inclusion of multiple cancer types, with their own specific set of tumor-derived factors, in the study described in chapter 5 as opposed to just RCC in chapter 6.

In these studies we investigated the frequencies and phenotype of three major DC subsets recognized in human peripheral blood: the conventional myeloid MDC-1 (CD1c/BDCA-1+) and MDC-2 (CD141/BDCA-3+) subsets and the CD303/BDCA-2+ plasmacytoid DC (PDC) subset. While the relative contribution and importance of these subsets to the generation of an anti-tumor immune response is still largely unknown, important clues are starting to surface. While PDC can contribute directly to the priming of anti-tumor effector cytotoxic T lymphocytes, either through type-I IFN production or through antigen presentation, MDC are generally found to be more powerful in this regard. In addition, tumor-conditioned PDC have been shown to have pro-angiogenic features (14). Differential expression of TLR and C-type lectins on MDC-1 and MDC-2 suggests distinct functions of these two subsets (15-17). Intriguingly, transcriptional profiling data indicated BDCA-3/CD141+ DC to be akin to the murine CD8α+ DC subset and suggested that they might be involved in cross-tolerance or cross-priming, similarly to this specialized murine subset (17). Recently, a role for the MDC-2 subset has been suggested in Th2-skewing as the MDC-2 population is increased in atopic patients and CD141/BDCA-3+ MoDC preferentially induce T cells with a type-2 cytokine profile (18). In contrast, MDC-1 have been shown to secrete high levels of the type-1 T cell-skewing IL-12 upon appropriate stimulation (19). The latter would thus clearly favor the generation of effective anti-tumor immunity. Accordingly, we showed that MDC-1 frequencies, but not MDC-2 frequencies, were positively associated with prolonged PFS in sunitinib-treated RCC patients (chapter 6). Indeed, in stark contrast to MDC-1, we observed a trend for high (i.e. above median) MDC-2 frequencies to be associated with a reduced PFS in the same patients (see chapter 6, figure 5d). This would be in keeping with a tolerizing or Th2-skewing capacity of MDC-2. In this regard, it is of particular interest that an IL-10 induced subset of monocyte-derived DC with an immune-suppressive phenotype was previously shown to express the MDC-2 associated marker thrombomodulin (i.e. CD141/BDCA-3) (21). CD141 as a marker of tumor-conditioned immunosuppressive DC is currently under further investigation in our lab.

Based on the results reported in chapter 6, we hypothesize that decreasing immune suppressive factors and/or the responsiveness of DC precursors (in particular MDC-1) to these factors, might improve the efficacy of immunotherapeutic approaches. To our knowledge only one clinical study employing the combination of anti-angiogenesis and
vaccination has been reported so far: in hormone-refractory prostate cancer patients treated with DC-based vaccines and bevacizumab (i.e. anti-VEGF monoclonal antibody), PSA responses and immune responses were observed (22). Based on our findings sunitinib is also a promising candidate in this regard. Indeed, the beneficial pro-immune effects of this TKI are not limited to DC differentiation, but have also been demonstrated in regard to regulation of Tregs, the frequencies of which were shown to decline in RCC patients upon sunitinib treatment, concurrent with increased type-1 T cell reactivity (23). In a very small pilot experiment, we found decreased Treg frequencies in only one out of four patients receiving sunitinib (figure 2). Interestingly, the one patient in who this reduction in Treg rates was observed underwent a partial response (PR). We thus obtained anecdotal evidence that Treg rates might decline upon sunitinib administration in relation to an apparent clinical benefit from the treatment. Certainly this promising link between sunitinib treatment and a decrease in Treg numbers warrants further investigation, both phenotypically and functionally, in expanded patient groups.

![Figure 2](image)

**Figure 2** - CD25^hiFoxP3^ T-regulatory cells (Tregs) as percentage of CD3^+CD4^+ T cells in four renal cell cancer patients over the course of treatment with sunitinib for four weeks, followed by a 2-week off period (between weeks 4 and 6). Gray line represents patient experiencing a partial tumor response.

In **part III** we further examined tumor-induced DC suppression in preclinical studies to obtain clues for viable targets to develop more effective anti-cancer strategies. In **chapter 7** we studied the newly recognized therapeutic targets ganglioside GM3 and STAT3 as modulators of both angiogenesis and immunity. We investigated immunohistochemically in a tissue micro array whether these markers were associated with inhibition of DC activation (number of CD1a^+ vs CD83^+ DC) and angiogenesis (microvessel density by CD34^+ staining) in NSCLC (n=176). Our studies showed that both GM3 and phosphorylated STAT3 (pSTAT3) were widely expressed in NSCLC and that GM3 expression was associated with a favorable patient survival, although this did not reach statistical significance. pSTAT3 expression could not be associated with either suppressed DC activation or
angiogenesis. In addition, we did not find evidence for a pro-angiogenic role of GM3. We could, however, confirm a possible involvement of GM3 in tumor-induced DC suppression in NSCLC patients as decreased numbers of tumor-infiltrating mature DC were observed in tumors with high GM3 expression. Its high tumor-specific expression makes GM3 a possible candidate for tumor targeting. Approaches to target gangliosides are currently in early clinical development. Many studies focus on monoclonal antibodies including antibodies against N-glycolyl-containing gangliosides (i.e., 1E10) and on vaccine-based strategies (24-27). The more specific murine monoclonal antibody 14F7, which we used in our study, recognizes N-glycolyl GM3 and has entered clinical development (28). Although induced immune responses have been reported, data on clinical efficacy are still lacking. Our findings in chapter 7 suggest that, beside angiogenic effects, it might also be worthwhile to monitor immune effects of therapeutic strategies targeting GM3.

Chapter 8 concerns an additional immunotherapeutic target, IL-6, in the biology of glioblastoma. In an in vitro DC differentiation model, using the human acute myeloid leukemia cell line MUTZ-3, glioblastoma-conditioned medium prevented CD34+ precursors to acquire DC and LC characteristics and functions, and concomitantly induced STAT3 activation in DCs and LCs. These effects were mediated primarily through IL-6. The responsible signaling pathway downstream of the IL-6 receptor remains to be identified, since neither inhibition of pSTAT3 nor Raf1 could overcome the glioblastoma-induced phenotypic DC or LC inhibition. In recent years, glioblastoma has become a potential target for DC-based immunotherapeutic approaches. The majority of patients, however, did not show an immunological response, calling for improvement of vaccine strategies. Chapter 8 provides evidence that targeting tumor-derived IL-6 might add to the efficacy of immunotherapy. Of note, the glioma-induced disturbed DC differentiation also affected cytokine release by the stunted DC. Glioma-conditioned and poorly differentiated monocyte-derived DC released lower levels of the cytokine IL-12p70, which, beside its strong immune stimulatory effects, is also recognized for its anti-angiogenic effects. Combined with an observed rise in the production of the pro-angiogenic factor IL-8, our data suggest that blocking IL-6 might prevent the accumulation of immature DC in the glioma microenvironment that would otherwise not only inhibit anti-tumor immunity but also effectively stimulate angiogenesis. Another example of co-regulation of angiogenesis and immune suppression.

Both chapter 7 and 8 investigated the role of pSTAT3, either expressed in tumor cells or in DCs, in tumor immune escape and angiogenesis. In NSCLC we could not find an association between pSTAT3 expression and angiogenesis and the activation status or numbers of tumor-infiltrating DC. In addition, inhibition of pSTAT3 in DC could not overcome the suppressive effects of glioblastoma-derived IL-6. The proposed role of STAT3 as a key regulator in angiogenesis and DC suppression (mostly based on numerous murine studies (29)) could thus not be confirmed in our human studies. Of note, we did
find evidence for an important role of tumor-derived IL-6 in the suppression of DC differentiation in two separate studies: in advanced RCC patients treated with sunitinib, we observed a significant inverse correlation between serum IL-6 levels and MDC-1 frequencies (chapter 6), while glioblastoma-derived IL-6 was identified as the major culprit in the inhibition of LC and DC from CD34⁺ precursors in vitro (chapter 8). These findings are in line with several previous studies on various tumor types, implicating IL-6 as a key suppressive factor in this regard (30). We therefore postulate that systemic IL-6 blockade might be a beneficial addition to any (DC-based) immunotherapeutic approach. Perhaps surprisingly, beside neutralizing anti-IL-6 mAbs, sunitinib might also be a useful tool in this regard, as we observed that the significant inverse correlation between serum IL-6 levels and MDC-1 rates was abrogated upon sunitinib treatment (week 4), but reasserted itself after a subsequent 2-week off-period (week 6, figure 3). This observation is suggestive of a reduced IL-6 responsiveness of the MDC-1 subset due to down-stream TKI activity of sunitinib, although the possibly involved TK targets remain to be identified.

Figure 3 - Correlation between IL-6 plasma concentrations and myeloid DC-1 frequencies as percentage PBMCs over follow-up in renal cell carcinoma patients treated with sunitinib in a 4-week on/2-week off schedule.

Besides identifying the specific factors involved in tumor-induced DC suppression and angiogenesis in the particular tumor type to be treated, other considerations should be taken into account when developing a regimen combining anti-angiogenic treatment and immunotherapy. Firstly, anti-angiogenic agents should be evaluated for their potency to overcome tumor-induced defective DC differentiation in vitro. Ideally, these anti-angiogenic agents should also be combined with immunotherapeutic approaches and tested in preclinical in vivo models for proof of principle. Subsequently, the most effective combined strategy should be tested in a clinical setting. Special attention should be given to the schedule and frequency of the regimen. Added immunotherapeutic approaches may have the greatest potential to increase therapeutic efficacy when given to patients upon simultaneous lifting of tumor-induced immune suppression and normalization of tumor blood flow through the use of angiogenesis/immune modulators, like VEGFR TKI.
Normalized tumor blood flow and abrogated tumor endothelial cell anergy may facilitate tumor infiltration by recruited immune effector cells and thus further contribute to an effective anti-tumor response (2). Secondly, response evaluation in clinical studies combining anti-angiogenic treatment with immunotherapy should focus on survival. As discussed in chapter 2, response evaluation in studies using anti-angiogenic agents is under debate. Uni- or bi-dimensional tumor measurements have been the standard for response evaluation in studies using conventional agents; however, for novel targeted therapies functional imaging may be an alternative. Beside radiologic or morphologic responses, immunomonitoring, especially of in vivo primed cytotoxic T-cells, is widely applied to evaluate response in immunotherapeutic strategies. All these evaluations can serve as surrogate markers for response, but survival should nevertheless remain the endpoint of clinical studies.

In conclusion, in this thesis the co-regulation of tumor-induced angiogenesis and immune escape has been explored. Although more (translational) research is needed to fully understand this complicated relationship and to identify additional co-regulators, this thesis provides evidence for the rationale that anti-angiogenic treatment can be a useful addition to immunotherapy. Cediranib has the potential to reduce MSCs and sunitinib increases frequencies of the immune-stimulatory MDC-1 subset. Both might thus provide a window in which increased myeloid immune competence could add to the efficacy of cancer immunotherapy. In the initial clinical evaluation of the efficacy of such combination regimens, one should aim to select a tumor type which has been shown to be responsive to immunotherapeutic approaches, such as glioblastoma, RCC, melanoma or prostate (31-33). An anti-angiogenic agent with proven efficacy for the tumor type of choice, such as sunitinib in RCC (34) or cediranib in glioblastoma (10), could then be combined with a vaccination strategy. Similarly, the vaccination strategy should be selected based on previously reported efficacy data, for instance autologous tumor lysate-pulsed DCs in glioblastoma patients (33) or GVAX in prostate cancer patients (31). Eventually, prospective clinical trials should establish whether immunotherapy can indeed synergize with combined anti-angiogenic treatment.

References


CHAPTER 10

Nederlandse samenvatting
Om uit te groeien tot een kwaadaardige tumor met uitzettingen moeten kankercellen een aantal karakteristieken verwerven. Eén daarvan is het vermogen om angiogenese, dat wil zeggen bloedvat-nieuwvorming uit bestaande bloedvaten om de tumorcellen te voorzien van voeding en zuurstof, te induceren. Een andere eigenschap is de inductie van tumor-immuunsuppressie, dat wil zeggen het onderdrukken van het immuunsysteem van de patiënt, zodat deze de tumor niet meer aan kan vallen. Een belangrijke cel van het immuunsysteem die onderdrukt wordt in zijn ontwikkeling en activatie, is de dendritische cel (DC), waarvan er twee soorten beschreven zijn: myeloïde en plasmacytoïde DC. DC kunnen tumoreiwitten opnemen en presenteren aan T-cellen, die uiteindelijk specifiek de kankercellen zouden moeten vernietigen. In het inleidende hoofdstuk 1 beschrijven we de twee processen van angiogenese en tumor-immuunsuppressie afzonderlijk en hoe ze samenhangen. Kankercellen produceren groeifactoren die tegelijkertijd de angiogenese stimuleren en de ontwikkeling van DC remmen. Een belangrijk voorbeeld hiervan is vascular endothelial growth factor (VEGF). Daarnaast is bekend dat voorlopercellen van DC onder invloed van de tumor kunnen bijdragen aan angiogenese door het produceren van stimulerende groeifactoren of door zich te gaan gedragen als een bloedvatcel. Tevens is bekend dat afweercellen minder makkelijk vanuit de circulatie de tumorweefsen kunnen binnendringen, omdat de bloedvaten in en rond de tumoren minder eiwitten op het oppervlak hebben waaraan afweercellen zich kunnen binden. Dit zou de afweerreactie tegen de tumor kunnen hinderen. De omgekeerde correlatie tussen de afweerreactie van het lichaam tegen de tumor en angiogenese is het onderwerp van dit proefschrift. Een beter begrip van deze correlatie zou de ontwikkeling van meer effectieve anti-kanker behandelwijzen kunnen bevorderen.

Nadat het belang van angiogenese voor de tumorgroei was onderkend, zijn klinische behandelingen gericht tegen angiogenese ontwikkeld. Hiertoe behoren ook de tyrosine kinase (TK) remmers van VEGF receptoren (VEGFR). VEGF receptoren zijn de belangrijkste receptoren op de bloedvatcellen die uiteindelijk zorgen voor deling en uitgroei van deze cellen. Door het activerende eiwit (tyrosine kinase) van deze receptoren te remmen wordt beoogd de angiogenese te onderdrukken. Bij volwassenen is angiogenese niet meer geactiveerd, behalve in tumorweefsen. De gedachte was dat TK remmers van VEGFR zonder veel bijwerkingen zouden zijn, omdat het specifiek op tumorweefsel zou aangrijpen. Nu TK remmers van VEGFR veel in de kliniek gebruikt worden, wordt duidelijk dat er toch specifieke en eigenaardige bijwerkingen van deze behandeling kunnen optreden. In hoofdstuk 2 worden deze bijwerkingen en hun behandeling beschreven. Tevens bespreken we hoe de effectiviteit van de TK remmers op de tumoractiviteit het beste gemeten kan worden. Een mogelijkheid om de effectiviteit te vergroten van TK remmers van VEGFR, is om deze te combineren met andere anti-kanker behandelingen. Hoofdstuk 3 beschrijft de achterliggende gedachte om TK remmers van de VEGFR te
combineren met TK remmers van de epidermale groeifactor receptor (EGFR). Deze laatste receptor bevindt zich onder andere op kankercellen zelf en is daar verantwoordelijk voor celdeling, overleving en angiogenese. Resistentie van kankercellen tegen TK remmers van EGFR kan worden doorbroken door deze te combineren met TK remmers van VEGFR.

**Hoofdstuk 4** beschrijft een klinische studie waarin een TK remmer van VEGFR, cediranib, gecombineerd wordt met een TK remmer van EGFR, gefitinib. Deze studie had als hoofddoel om de veiligheid van deze combinatie te onderzoeken. De resultaten laten zien dat deze combinatie over het algemeen goed werd verdragen. De meest frequente bijwerkingen waren diarree, hoge bloeddruk, verminderde eetlust en moeheid. De maximale dosering van cediranib die nog veilig gecombineerd kan worden met gefitinib (250 of 500 mg), is 30 mg per dag. Ook de resultaten met betrekking tot de anti-tumor effectiviteit van deze combinatie waren veelbelovend, in het bijzonder in de patiënten met nierkanker. In 33% van de patiënten met nierkanker was er sprake van een significante afname van de tumorgrootte.

Omdat er een belangrijke wisselwerking bestaat tussen angiogenese en tumor-immuunsuppressie, hebben we in het tweede deel van het proefschrift onderzocht of anti-angiogene behandeling middels TK remmers van VEGFR de onderdrukte ontwikkeling van DC kon tegengaan. In **hoofdstuk 5** hebben we onderzocht of cediranib, een TK remmer van zowel VEGFR als twee andere receptoren, *platelet-derived growth factor receptor* (PDGFR) en cKIT, de onderdrukte DC ontwikkeling kon bevorderen in patiënten met kanker. We bevestigen dat de ontwikkeling van DC in patiënten met kanker gestoord is, aangezien er minder myeloïde en plasmacytoïde DC en meer onrijpe voorloper cellen en myeloïde suppressor cellen (MSC) in de circulatie aanwezig waren dan bij gezonde vrijwilligers. Hoewel wordt aangenomen dat VEGF hierin een belangrijke, onderdrukkende rol speelt, konden we niet aantonen dat myeloïde of plasmacytoïde DC in frequentie toenamen wanneer de patiënten werden behandeld met cediranib. Wel zagen we een lichte, maar niet significante, daling van potentiële suppressieve voorlopercellen, de MSC.

**Hoofdstuk 6** beschrijft een studie waarin we onderzochten of een andere TK remmer van VEGFR, sunitinib, die ook PDGFR, cKIT en FLT-3 remt, de DC ontwikkeling in patiënten met nierkanker kan bevorderen. Behandeling met sunitinib resulteerde in een verlaging van immuunsuppressieve witte bloedcellen en een relatieve stijging van immuunstimulerende myeloïde DC. De tegengestelde uikomsten van hoofdstuk 5 en 6 kunnen verklaard worden door de gebruikte medicijnen (cediranib versus sunitinib) of door de onderzochte patiënten populatie. In hoofdstuk 5 wordt een patiënten populatie onderzocht met verschillende tumoren, welke hun eigen typische productie van verschillende immuunsuppressieve groeifactoren kunnen hebben, en in hoofdstuk 6 wordt slechts één type kanker onderzocht, te weten nierkanker, met wellicht een meer uniform profiel van uitgescheiden suppressieve factoren.
In het volgende deel van het proefschrift wordt onderzocht of er meerdere aangrijpingspunten in kankercellen te vinden zijn waartegen een anti-angiogenese en/of immuuntherapie ontwikkeld kan worden. In hoofdstuk 7 onderzochten we of ganglioside GM3 of signaling transducer and activator of transcription-3 (STAT3), beide betrokken bij zowel angiogenese als tumor-immuunsuppressie, een doelwit zou kunnen vormen in de behandeling van longkanker. Gangliosiden zijn celmembraan-gebonden glycosphingolipiden die betrokken zijn bij zowel fysiologische cellulaire processen als bij de ontwikkeling en groei van tumoren. STAT3 reguleert de omzetting van extra-cellulaire signalen naar het intracellulair aanzetten van bepaalde (immuunsuppressieve) genen in de celkern. Voortdurend actief STAT3 heeft een belangrijke rol in de vorming en groei van kankercellen. Het blijkt dat zowel GM3 als actief STAT3 veelvuldig en in grote hoeveelheden aanwezig zijn in longkanker. Daarnaast hebben we gekeken of beide ook geassocieerd zijn met angiogenese of infiltratie van DC in longkanker. Actief STAT3 had geen associatie met angiogenese of DC infiltratie. GM3 was ook niet geassocieerd met angiogenese, maar was wel geassocieerd met DC infiltratie: in longkanker met een hoge expressie van GM3 waren minder uitgerijpte DC aanwezig. Een aanwijzing dat GM3 betrokken is bij de onderdrukking van het immuunsysteem door longkanker. In de literatuur worden de eerste resultaten van de behandelingen gericht tegen gangliosiden, waaronder GM3, reeds beschreven. Onze bevindingen lijken aan te tonen dat interferentie met GM3 mogelijk kan leiden tot betere infiltratie van longtumoren met rijpe DC. Dit zou de anti-tumor immunititeit kunnen bevorderen. In hoofdstuk 8 wordt onderzocht of er een plaats is voor een therapie gericht op betere DC ontwikkeling bij patiënten met glioblastoom, een agressieve en letale vorm van hersenkanker. Immuuntherapie is een veelbelovende therapie bij deze vreselijke ziekte. Echter, bij een aantal glioblastoompatiënten die met immuuntherapie behandeld worden, wordt geen afweerrespons tegen de tumor gevonden. Een verklaring hiervoor zou kunnen zijn dat de tumor remmende factoren produceert die het succes van immuuntherapie in de weg staan. Hoofdstuk 8 laat zien dat interleukine-6 afkomstig van glioblastoom een belangrijke remmende factor voor DC ontwikkeling is. Immuuntherapie gecombineerd met therapie tegen interleukine-6 zou daarom de effectiviteit van deze behandeling kunnen vergroten.

In dit proefschrift is de wisselwerking tussen en de co-regulatie van angiogenese en tumor-immuunsuppressie onderzocht. Hoewel meer onderzoek nodig is om de achterliggende processen volledig te begrijpen, hebben we bewijs aangevoerd dat anti-angiogene therapie een belangrijke aanvulling kan zijn op immuuntherapie. Cediranib heeft de potentie om immuunsuppressieve MSC te verlagen en sunitinib verhoogt de aantallen van immuunstimulerende myeloïde DC. Beide middelen verschaffen zo randvoorwaarden waarbinnen immuuntherapie mogelijk meer effectief zou kunnen zijn. Uiteindelijk zullen grote en gerandomiseerde klinische studies die immuuntherapie
combineren met anti-angiogenese behandelingen, moeten uitwijzen of de overleving van patiënten met kanker inderdaad verbeterd kan worden.
Publications
and curriculum vitae


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