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REVIEW PAPER

Immune Effects of Bevacizumab: Killing Two Birds with One Stone

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Abstract Angiogenesis or new vessel formation is essential for tumour growth and progression. Therefore, targeting angiogenesis has been an attractive strategy in the treatment ofcancer. Bevacizumab is a recombinant humanized monoclonal IgG1 antibody thattargets vascular endothelial growth factor-A (VEGF-A) - a key molecular player inangiogenesis. Bevacizumumab has shown clinical efficacy in phase III clinical trials inseveral advanced solid malignancies. The clinical efficacy of bevacizumumab isprimarily due to its antiangiogenic effects; however, there are direct antitumor effectsand immunomodulatory effects. Enhancing the immune system to restore itsantitumour activity has been utilized successfully in clinical setting. In this article we willdiscuss the possible immunomodulatory effects of the most clinically usedantiangiogenic agent; bevacizumumab.

Keywords Bevacizumab · Immunomodulation · Antiangiogenesis · Tumour microenvironment

Introduction

Angiogenesis or new vessel formation is essential for tumour growth and progression. Therefore, targeting angiogenesis has been an attractive strategy in the treatment of cancer. Bevacizumab is a recombinant humanized monoclonal IgG1 antibody that targets vascular endothelial growth factor-A (VEGF-A) - a key molecular player in angiogenesis [1]. Bevacizumab has shown clinical efficacy in phase III clinical trials in several advanced solid malignancies like lung [2, 3], colorectal [4, 5], breast [6–8], renal [9, 10], ovarian [11–13], and cervical cancer [14]. Based on the results of these clinical trials bevacizumab is currently used in the treatment of metastatic colorectal, non-small cell lung, ovarian, cervical and breast cancers. VEGF-A is the main member of the angiogenic VEGF family of proteins [15, 16]. It acts by signalling through two type III receptor tyrosine kinases: VEGFR1 (also known as FLT1) and VEGFR2 (also known as KDR) [15, 16]. VEGFR2 is expressed in vasculature and it is the main mediator of VEGF-A induced angiogenesis. The clinical efficacy of bevacizumab is primarily due to its antiangiogenic effects; however, there are direct antitumor effects [16, 17] and immunomodulatory effects.

The dual role the immune system plays in cancer has drawn a lot of interest in the past few years. The immunoediting theory states that in early stages of cancer a vigilant immune system identifies and eliminates incipient neoplastic cells [18, 19]. In later stages of cancer and due to a selective pressure imposed on surviving malignant cells by the immune system, the tumour cells not only escape immune system eradication, but they recruit some of the immune system elements to aid cancer progression [20, 21]. Therefore, the blockade of tumour immunosuppressive mechanisms might be effective to overcome immunological tolerance and promote cancer progression. Cancer immunotherapy attempts to harness the power and specificity of the immune system to treat cancer [20, 21]. This concept led to the development of several immunomodulatory agents that are in clinical use nowadays [21]. In this article we will discuss the possible immunomodulatory effects of the most clinically used antiangiogenic agent; bevacizumab.

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VEGF-A Effects on Immune Cells Maturation

VEGF-A is abundant in the tumour microenvironment. It has been linked to advanced cancer stage and poor prognosis [1, 18, 20]. VEGF is produced mainly by tumour associated macrophages (TAMs), tumour cells and cancer associated fibroblasts (CAFs) [20]. The best described immune effects of VEGF are those related to dendritic cells (DCs) maturation and function. DCs are the main antigen presenting cells, they are essential for activating a T-cell response [22].

VEGF-A inhibits maturation of DCs into functional cells; it phosphorylates transcription factors STAT3 and ERK [23]. Hyperactivated STAT3 in turn inhibits NFκB leading to failure of DCs maturation [23–26]. In a study of six multiple myeloma patients, the levels of phosphorylated STAT3 and ERK were increased when DCs derived from patients were pulsed with myeloma cell lysates. Blocking VEGF-A led to reduced phosphorylation of STAT3 and ERK with resultant significant increase of NFκB signalling leading to DCs maturation [23]. Several studies have shown that the inhibitory effect of VEGF-A on NFκB is mediated via VEGFR1 receptor. The latter is also expressed on CD34+ hematopoetoc progenitor cells (HPCs), Hence VEGF acts as a chemoattractant to those cells. CD34+ HPCs numbers were found to be increased in within tumour tissue and cancer patients' peripheral blood [25, 27]. These cells are precursor cells capable of differentiation into mature immune cells like neutrophils, dendritic cells and macrophages. Not that only but CD34+ HPCs can also differentiate into endothelial cells and contribute to vascularization. CD34+ HPCs supress T-cell functions including T-cell antitumor function, several reports have shown that VEGF leads to accumulation of CD34+ cells within tumour tissue. Also by inhibiting NFκB, VEGF prevents HPCs differentiation into mature immune cells. Using murine models, Young et al. showed that Lewis lung carcinoma cells chemoattract CD34+ HPCs through the production of VEGF [25]. Accumulation of HPCs had immune suppression effects. It is possible that VEGF shifts the differentiation of HPCs towards endothelial cells to enhance angiogenesis. HPCs express both VEGR1 and VEGFR2 receptors, Oyama and his colleagues examined which of these receptors is the mediator of VEGF effects on HPCs maturation [26]. Authors isolated HPCs form umbilical cord blood and firstly showed that VEGF binds specifically to these cells, and then to identify the functional receptor of VEGF on CD34+ HPCs they used PIGF, an angiogenic factor that binds to VEGFR1 but not to VEGFR2. They found that PIGF was able to block VEGF binding to HPCs suggesting that VEGF binds to these cells through VEGFR1 receptor. It is important to note that authors were able to isolate mRNA for VEGFR1 but not for VEGFR2. However other groups have detected mRNA for VEGFR2 in HPCs [28, 29]. The differential roles of VEGFR1 and VEGFR2 receptors in dendritic cell differentiation were examined in vitro [30]. Using myeloid DC from embryonic stem cells, Dikov and colleagues showed VEGFR1 is the main mediator VEGF-inhibition of HPCs and DCs maturation. Authors used fluorescent-labelled VEGF-A to find that most specific binding of VEGF-A to HPCs id due to VEGFR1. In agreement with other studies, authors reported that VEGFR1 mediated the inhibition of NFκB by VEGF.

VEGF-A and Mature DCs

VEGF impairs the antigen presenting function of mature DCs in several ways: as shown in several reports VEGF treated DC have low expression levels of major histocompatibility complex II (MHC II) and B7-2, not that only but VEGF reduces DCs ability to uptake antigens [31–33]. In an elegantly written study, Mimura and colleagues examined the effects of VEGF on mature DCs [32]. They generated DCs from monocytes taken from peripheral blood from healthy donors; lipopolysaccharide was used to induce DCs maturation. They subsequently treated mature DCs with VEGF and assessed its effect on DCs apoptosis, IL-12 production and antigen presenting. Interestingly, authors found no phenotypical changes in treated DCs, there were no changes in maturation markers of DCs (CD86, CD80, CD83, MHC I & II). Moreover, VEGF did not affect DCs production of IL-12, an important interleukin in T-cell activation. Another interesting finding from this study is that VEGF-A did not induce DCs apoptosis in contrast to other previous reports. The immune suppression effects of VEGF-A were demonstrated in this study by the failure of DCs to stimulate allogeneic T cells in a dose dependant manner, authors showed that this effect is mediated through VEGFR2 (unlike in immature DCs, in which main VEGF-A receptor is VEGFR1 as discussed earlier in this essay), the VEGF-induced inability of DCs to stimulate allogeneic T-cells was completely inhibited by anti-VEGFR2 blocking antibody, while blocking VEGFR1 did not have any effect. It is important to note that researchers in this study did not report the molecular mechanisms of VEGF-A inhibitory effects on mature DCs, and that was the focus of a study by another group [33]. That group used mouse models to show that inhibitory effect of VEGF-A on mature DCs is mediated via Id protein family, a group of proteins involved in regulation of cell cycle and differentiation.

VEGF-A and T Cells

We have discussed how VEGF-A can affect T-cells function via defective DCs maturation and function. In addition to this effect, VEGF-A inhibits T-cell development and contributes to tumour-induced immune suppression [34]. Ohm and his colleagues exposed mice to recombinant VEGF-A at

concentrations similar to those observed in late stages of cancer [34]. That resulted in defective seeding of the thymus by T-cells precursors, those precursors failed to replace mature T-cells emigrating from thymus to peripheral blood, which resulted in depletion of total T-cells. The inhibition of T-cell development is likely to be contributing in tumour-induced immune suppression. Moreover, VEGF-A reduces the cytotoxic activity of T cells [35]. In an elegant study, T-cells isolated from the ascites of ovarian cancer patients were cultured with anti-CD3 and IL-2, with or without VEGF-A for 14 days and the number of viable T cells was counted. Cytotoxic activity of cultured T cells and expression of VEGF receptor-2 (VEGFR-2) was assayed [35]. The addition of VEGF-A in cultures significantly reduced the number and proliferation rate of T cells in a dose-dependent manner and CD3+ T cells expressed VEGFR-2 on their surface upon activation. Experiments with specific anti-VEGFR-2 antibodies revealed that the direct suppressive effect of VEGF on T-cell proliferation is mediated by VEGFR-2. Useful mechanistic insights on how VEGF affects T-Cells function come from a study that found that CD45RO + memory populations of CD4+ T lymphocytes express VEGFR1 and VEGFR2 at both the mRNA and protein levels [36]. Furthermore, by Western blot analysis, it was found that VEGF increases the phosphorylation and activation of ERK and Akt within CD4 + CD45RO + T cells. These VEGF-mediated signalling responses were inhibited by a VEGFR2-specific small interfering RNA in a VEGF receptor-expressing Jurkat T cell line and by SU5416, a pharmacological VEGFR2 inhibitor, in CD4 + CD45RO + T cells. VEGF also augmented mitogen-induced production of IFN-γ in a dose-dependent manner and increased directed chemotaxis of this T cell subset.

VEGF-A directly enhances the recruitment of T-cells [37–39]. This recruitment can be attributed to the expression of VEGFR1 on a subpopulation of CD3+ T-cells. VEGF blockade using bevacizumab inhibits lymphocyte recruitment and ameliorates immune-mediated vascular remodelling [37]. Moreover, VEGFR2 is selectively expressed by FoxP3+ T-regs, and VEGF-A recruits T-regs to the tumour microenvironment [40]. Thus, VEGFR2 might be a target for elimination of T-regs from the tumour microenvironment.

A recently described mechanism of regulating the tumour-endothelial barrier and T-cell infiltration into tumours involves the selective expression of the death mediator Fas ligand (FasL, also called CD95L) in the vasculature of human and mouse solid tumours but not in normal vasculature [41]. In these tumours, FasL expression was associated with scarce CD8+ infiltration and a predominance of FoxP3+ T Treg cells. Tumour-derived vascular VEGF, interleukin 10 (IL-10) and prostaglandin E2 (PGE2) cooperatively induced FasL expression in endothelial cells, which acquired the ability to kill effector CD8+ T cells but not Treg cells because of higher levels of c-FLIP expression in Treg cells. Pharmacologic inhibition of VEGF and PGE2 produced a marked increase in the influx of tumour-rejecting CD8+ over FoxP3+ T cells that was dependent on attenuation of FasL expression and led to CD8-dependent tumour growth suppression [41]. These findings strongly affirm the critical role the VEGF plays in the intimate relationship between angiogenesis and immunosuppression in cancer.

VEGF-A and Innate Immunity

There is substantial evidence that VEGF-A induces neutrophils chemotaxis with several suggested mechanisms. VEGF-A upregulates intercellular adhesion molecule-1 (ICAM-1), the receptor for neutrophil adhesion molecules CD11/CD18 (MAC-1) [42]. Furthermore, VEGF leads to the upregulation of IL-8, a strong neutrophils chemoattractant, in endothelial cells [43]. Other neutrophils adhesive molecules upregulated by VEGF include β1 and β2 integrins [44]. VEGF can also be released by neutrophils, pointing to an autocrine regulatory loop and further confirm the link between inflammation and angiogenesis, via VEGF and neutrophils [44]. Finally, VEGF recruits a proangiogenic circulating subset of MMP-9 producing neutrophils. MMP-9 is requires for neovascularisation [45].

Beside its effects on neutrophils, VEGF induces migration and adhesion of macrophages, monocytes, and natural killers [46–49]. Blockade of the VEGF-VEGFR2 axis in breast cancer xenografts reduces tumour-associated macrophages infiltration [50].

VEGF-A increases the levels of myeloid-derived suppressor cell (MDSC) in cancer patients' peripheral blood [51]. MDSC are characterized by their potent ability to suppress T and NK cell function via increased expression of arginase I and inducible nitric oxide synthase, and increased production of reactive oxygen species [52]. Additionally, MDSC promote the development of FoxP3+ regulatory T cells and modulate cytokine production by macrophages [52]. Surprisingly anti VEGF antibody treatment in patients with renal cell carcinoma did not appear to reduce the levels of peripheral blood MDSC [53]. In contrast, a phase I clinical trial of a VEGF trap demonstrated no effect on peripheral blood levels of MDSC [54]. However, these early phase clinical studies are often not powered to provide definitive conclusions based on correlative markers [51].

Therapeutic Implications

VEGF-induced defects in DCs function need to be tested clinically. A phase I trial that was reported in 2007 enrolled 15 patients of refractory solid malignancies to examine the clinical implication of targeting VEGF-A on DCs function

[55]. Authors used VEGF-trap which is a fusion protein consisting of human VEGF receptor extracellular domains fused to the Fc portion of human IgG1. The VEGF trap is a specific antagonist that binds and inactivates VEGF-A. Patients enrolled were treated with VEGF-trap every 2 weeks, both bound and free VEGF-trap were measured in plasma samples at days 15, 22 and 43. Treatment with VEGF-trap did not affect the total population of DCs, but significantly increased the proportion of mature DCs. Disappointingly; the increase in mature DCs proportion did not translate into a stronger T-cell response or improved anti-tumour activity. It is possible that as these patients had significant tumour burden, targeting VEGF alone was not enough to induce an antigen-specific T-cell response. It is important to interpret the results of this study cautiously given the small number of patients enrolled.

Another Phase I trial took a different therapeutic approach by targeting VEGFR1 and VEGFR2 receptors using a tyrosine kinase inhibitor [56]. AZD2171 is a novel potent inhibitor of VEGFR2 kinase activity, with additional activity against VEGFR1 and VEGFR3. AZD2171 was used in combination with gefitinib, a tyrosine kinase inhibitor that targets EGFR, the combination was used in 13 patients with advanced stage refractory cancer. Levels of immature DCs and mature DCs were measured in the study cohort before and after the treatment and compared to a control group of healthy donors. It was found that the experimental group had lower levels of mature DCs and higher levels of immature DCs precursors in comparison to the control group. The treatment combination did not affect the proportion of immature DCs in the cancer patients group. Moreover, authors found no correlation between circulating VEGF and clinical outcome. Interestingly, they found that VEGF circulating levels increased following treatment with AZD2171 and gefitinib.

A combination of VEGF-A blockade and granulocyte macrophage colony stimulating factor (GM-CSF) secreting tumour cell immunotherapy was used in murine tumour models [57]. Authors reported that this combination significantly prolonged the survival of tumour-bearing mice. Enhanced anti-tumour protection correlated with an increased number of activated CD4+ and CD8+ tumour-infiltrating T-cells and a pronounced decrease in the number of suppressive regulatory T cells (T-regs) residing in the tumour. Conversely, overexpression of VEGF from tumours resulted in elevated numbers of T-regs in the tumour, suggesting that Tregs recruitment is another mechanism of VEGF-mediated immune suppression at the tumour site. This preclinical study clearly strengthens the concept of combining agents targeting angiogenesis and immunotherapy in cancer treatment.

Manzoni and his colleagues examined the effects of bevacizumab on T-cells and B-cells levels in a group of 51 patients with metastatic colorectal cancer [58]. Patients received bevacizumab based treatment as first line, their T- cells and B-cells levels were determined at baseline, following 3 cycles and following 6 cycles of treatment. The baseline levels of T-cells and B-cells were lower than those of a group of healthy participants. Treatment with bevacizumab led to a significant increase in T-cells and B-cells levels, perhaps due to neutralization of VEGF inhibitory effect of DCs maturation. However the correlation between increased levels of T-cells and B-cells and a better clinical outcome did not reach statistical significance. A recently published report showed that there were increased numbers of DCs in tumour tissue and adjacent healthy tissues of colorectal cancer patients compared to age matched healthy control tissue [59]. There was no correlation between the presence of DCs and survival in colorectal patients who received bevacizumab. However, authors did not report if treatment with bevacizumab had any effect on DCs maturation and level, nether peripherally nor in the colon.

Bevacizumab was shown to enhance DCs maturation and numbers in peripheral blood from patients with lung, breast, and colorectal carcinoma [60]. Compared with healthy volunteers, cancer patients in a study by Osada and colleagues had a bias towards the immunoregulatory DCs, had deficits in DC maturation after overnight in vitro culture, and had a significant increase in immature myeloid cell progenitors of DC [60]. Bevacizumab administration to the 41 cancer patients included in the study was associated with a decrease in the accumulation of immature progenitor cells and induced a modest increase in the DC population in peripheral blood. This data adds to the accumulating evidence that DCs maturation is negatively associated with VEGF levels and may be one explanation for impaired anti-cancer immunity. These findings are consistent with those of a study carried out in a mouse model of breast cancer [61]. Blockade of VEGF led to increase in the maturation of DCs and to the inhibition of tumour infiltration with immune-regulatory cells like T-regs and myeloid-derived suppressor cells (MDSC). These changes in the immune cells population in the tumour microenvironment were correlated with changes in the serum levels of cytokines like IL-6 and IL-1β.

The effects of bevacizumab on immune-regulatory cells are not limited to MDSCs and regulatory DCs. A recent study found that bevacizumab reduces T-regs proportion in the blood of metastatic colorectal cancer patients [62]. VEGF directly induces T-regs proliferation, therefore the blockade of VEGF-VEGFR2 axis leads to the reduction of T-regs levels. Interestingly, authors found that VEGF blockade leads to the reduction of T-regs levels without affecting their function. They found that Tregs from both sunitinib- and anti-VEGF-A–treated mice maintained their capacity to inhibit conventional T-cell proliferation and IFN-γ secretion [62].

Conclusion

There is compelling evidence that VEGF plays immune suppressive role. It inhibits that DCs precursors differentiation and maturation by inhibiting NFκB, this is mediated through VEGFR1 receptor. The effect of VEGF on mature DCs is mediated through VEGFR2 possibly by interacting with Id proteins family. Mature DCs exposed to supraphysiological levels of VEGF have low expression of MHC II and less ability to uptake antigens with resulting impaired T-cells priming.

Available data suggest that supressing VEGF alone is not sufficient to translate into clinically meaningful immune antitumor response. This probably reflects the complexity and the multifactorial nature of mechanisms that tumours use to escape and supress host immune system.

Targeting more than one hallmark of cancer is an appealing therapeutic approach that has been increasingly implemented over the past few years. Of particular interest is combining antiangiogenic therapy and immunotherapy [63, 64]. A phase III trial of the combination of bevacizumab and interferon α-2a given as first line for metastatic renal cell carcinoma showed improved progression free survival compared with interferon α-2a alone [65]. This approach is promising and clinically feasible.

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