Medical Research Archives

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OPEN ACCESS

Published: March 31, 2023

Citation: Lallemand C, Ferrando-Miguel R, et al., 2023. Analysis of Bevacizumab Activity Following Treatment of Patients with Ovarian Cancer or Glioblastoma, Medical Research Archives, [online] 11(3). https://doi.org/10.18103/mra.v11i3.3652

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DOI
https://doi.org/10.18103/mra.v11i3.3652

ISSN: 2375-1924

RESEARCH ARTICLE

Analysis of Bevacizumab Activity Following Treatment of Patients with Ovarian Cancer or Glioblastoma

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ABSTRACT
Highly sensitive reporter-gene assays have been developed that allow the precise quantification of both the direct vascular endothelial growth factor-A neutralizing activity of bevacizumab and the ability of bevacizumab to activate antibody dependent cellular cytotoxicity. The use of these assays to analyzes samples from patients with ovarian cancer following four cycle of bevacizumab treatment revealed a close correlation between bevacizumab neutralizing activity and antibody dependent cellular cytotoxicity activity, and a reasonably good correlation between both activities and circulating drug levels determined using an enzyme-linked immunosorbent assay. Analysis of longitudinal samples from a small cohort of patients with glioblastoma treated with bevacizumab revealed a lower correlation between these parameters. We report herein that reanalysis of the grouped samples from the two studies using the nonparametric Spearman rank correlation coefficient revealed a surprisingly good correlation between the two facets of bevacizumab activity, and between both activities and circulating drug levels despite the different indications and treatment regimens, revealing new insights into the action of bevacizumab in neoplastic disease.

Keywords: Ovarian cancer, glioblastoma, bevacizumab, angiogenesis, antibody dependent cellular cytotoxicity

1. INTRODUCTION

The anti-vascular endothelial growth factor-A (VEGFA) monoclonal antibody bevacizumab (Avastin®) is used extensively to treat both recurrent disease in patients with glioblastoma who have failed first line therapy[1,2] and in treatment of other solid tumors including ovarian cancer[3,4]. Although, bevacizumab treatment results in a high initial response rate the effect is transient, and most patient's tumors eventually progress[2,4-6]. The mechanisms of bevacizumab treatment failure are poorly understood and an accurate assessment of the treatment response in individual patients is key to a better understanding of the most effective means of optimizing bevacizumab treatment. Current methods for quantifying the activity of human VEGF, or antibodies that neutralize its activity, such as bevacizumab, are bioassays based on the ability of anti-VEGF antibodies to inhibit the proliferation or migration of primary human umbilical vein endothelial cells (HUVEC) or other cells expressing VEGF receptors following treatment of the cells with VEGF. Such assays can take several days to perform, are subject to a high degree of variation, and are difficult to validate. Highly sensitive i*Lite*® reporter-gene assays have been developed that allow both circulating and membrane-bound VEGF activity to be quantified rapidly and specifically, as well as the neutralizing activity of bevacizumab and the ability of bevacizumab to activate antibody dependent cellular cytotoxicity (ADCC). These assays have been used previously to monitor the action of bevacizumab in patients with ovarian cancer following bevacizumab treatment and in longitudinal samples from in a small cohort of patients with glioblastoma treated with bevacizumab[7]. The reanalysis of the pooled samples from both studies using the nonparametric Spearman rank correlation coefficient is described herein and may provide new insights into the antitumor action of bevacizumab.

2. MATERIALS & METHODS

2.1. Vascular endothelial growth factor responsive reporter cells

The establishment of the human VEGF-responsive cell line expressing the firefly luciferase (FL) reporter-gene regulated by a 5-fold tandem repeat of the upstream activation sequence (UAS), of gal-4 and expressing a chimeric transcription factor consisting of the gal4 DNA binding domain fused to the trans-activating domain of Elk-1 together with an expression vector for human VEGFR2 has been described previously[7]. The cells exhibit a high degree of VEGF responsiveness following treatment with increasing concentrations of VEGFA (PHC9391, ThermoFisher) for 18 hours at 37°C prior to quantification of VEGF-induced FL activity using the Bright-Glo® reagent (Promega, Madison WI) and a GloMax® luminometer (Promega, Madison WI).

2.2 Quantification of bevacizumab activity

A fixed dilution or serial dilutions of the sample, or bevacizumab standard were tested in triplicate. Samples were incubated for 30 minutes at 37°C with 25 ng/mL of VEGFA. The samples are then incubated with the VEGF responsive reporter-gene cells for 18 hours at 37°C prior to quantification of VEGF-induced firefly luciferase (FL) activity.

2.3 Quantification of antibody dependent cellular cytotoxicity activity

The novel engineered Jurkat ADCC effector cells expressing the FcγRIIIA receptor (CD16a) and the FL reporter-gene under the control of the principal transcription factors involved in FcγRIIIA signal transduction has been described previously[8]. VEGF (-) target cells expressing the costimulatory molecules CD80 and CD86 were found not express detectable levels of VEGF or ADCC activity in the presence of bevacizumab and the ADCC effector cells. The VEGF (+) target cells that express membrane-bound non-cleavable VEGF2A were established by transfecting VEGF (-) target cells expressing the costimulatory molecules CD80 and CD86 with the gene encoding codon-optimized VEGF2A fused to the coding sequence of the transmembrane region of TNFα bearing a mutation in the protease cleavage site as described previously[8].

2.4 Quantification of bevacizumab serum concentrations by capture enzyme-linked immunosorbent assay

The capture enzyme-linked immunosorbent assay (ELISA) for the quantification of bevacizumab using microtiter plates were coated overnight with human recombinant $VEGF_{165}$ (R&D systems) has been described previously[7]

2.5 Patient population

A cohort of 46 patients with ovarian cancer and a second cohort of 6 patients diagnosed with glioblastoma according to the World Health Organization classification scheme[9] were both hospitalized at the Innsbruck University Hospital. Patients with glioblastoma were classified according to clinical and/or radiological outcomes as responders, who presented no tumor progression,

primary non-responders presenting no response at all, and secondary non-responders, who exhibited an objective response followed by tumor progression. Patients were included in the study following written informed consent in accordance with the institution guidelines. This study was approved by the ethics committee of the University of Innsbruck: Approval number EKNR 1054/2017 and has been described in detail previously[7].

3. RESULTS

A reporter-gene cell line expressing firefly luciferase under the control of an Elk-1-responsive chimeric promoter, one of the principal transcription factors involved in the VEGF signal transduction pathway[10], was developed that responds specifically to treatment of cells with VEGF[7]. The reporter-gene cell line was used to quantify bevacizumab activity based on the ability of bevacizumab to neutralize soluble VEGFA as reflected by an inhibition of VEGF-induced firefly luciferase activity[7]. The assay is sensitive, precise, and accurate with the ratio of the measured to the expected values for concentrations of bevacizumab ranging from 50 to 150 % of the expected values yielded a linear curve with a R^2 of 0.963. The presence of normal human serum was without effect on assay results tested at final concentrations of human serum ranging from 2.5 to 10.0 % within the range of the dilutions of samples tested[7].

The ability of the VEGF-responsive reporter-gene cell line to quantify the activity of membrane-bound non-cleavable VEGF2A led to the observation that bevacizumab was able to neutralize membrane bound VEGF and provides a means of quantifying this activity using the mVEGF (+) target cells that express membrane-bound non-cleavable VEGF2A[7]. The ability of bevacizumab to activate ADCC was also quantified using an effector cell line expressing the FcγRIIIa receptor (CD16) that responds specifically to binding of the Fc moiety of an antibody to the FcγRIIIa receptor by activation of the FL reporter-gene[7] in the presence of target cells that express non-cleavable membrane bound VEGFA[7]. No ADCC activity was detected when ranibizumab (Lucentis®), a derivative of bevacizumab lacking a Fc moiety[11], was tested in the presence of target cells that express non-cleavable membrane bound VEGFA[7].

Analysis of serum samples from a cohort of 46 patients with ovarian cancer following four cycles of bevacizumab treatment revealed a close correlation between the VEGF2A neutralizing activity of bevacizumab and the ability of bevacizumab to activate ADCC[7] The ability of bevacizumab to activate ADCC correlated less well with circulating drug levels determined by ELISA than with the VEGF2A neutralizing activity of bevacizumab[7]. Analysis of longitudinal samples from a small cohort of patients diagnosed with glioblastoma according to the World Health Organization classification scheme[9] and presenting different types of response to treatment with bevacizumab, revealed overall a less good correlation between the neutralizing activity of bevacizumab and ADCC activity, and bevacizumab neutralizing activity with circulating protein levels in most samples from patients irrespective of whether they were classified as responders, primary non-responders, or secondary non-responders[7]. Indeed, the host mediated ADCC activity of bevacizumab did not appear to be correlated solely with the level of circulating bevacizumab determined by ELISA and may reflect the presence of neutralizing anti-bevacizumab antibodies[7]. To determine whether common aspects of the action of bevacizumab across individual indications, could be identified, notwithstanding different treatment regimens, serum samples from a cohort of 46 patients with ovarian cancer following four cycles of bevacizumab treatment were pooled with longitudinal samples from a small cohort of patients diagnosed with glioblastoma and analyzed using the nonparametric Spearman rank correlation coefficient (Figure 1).

Serum samples from 46 patients with ovarian cancer following four cycles of bevacizumab treatment, at a dose of 15 mg/kg body weight administered as monotherapy, were pooled with longitudinal serum samples from a small cohort of six patients with glioblastoma treated with the same dose of bevacizumab but treated concomitantly with corticosteroids, and treated in addition with temozolomide and radiotherapy[7]. The pooled samples were tested for the presence of circulating levels of bevacizumab using an ELISA, and for the ability of bevacizumab to neutralize VEGF2A, and to activate ADCC in the presence of target cells expressing membrane-bound non-cleavable VEGF2A as described previously[7]. The results were analyzed using the nonparametric Spearman rank correlation coefficient. A good correlation was observed between bevacizumab VEGF2A neutralizing activity and the ability of bevacizumab to activate ADCC with a Pearson r value of 0.9 (Figure 1A). The neutralizing activity of bevacizumab also correlated reasonably well with circulating levels of bevacizumab determined by ELISA with Pearson r value of 0.63 (Figure 1B). Similarly, the ability of bevacizumab to activate ADCC also correlated to a similar extent with

circulating levels of bevacizumab determined by ELISA with a Pearson r value of 0.63 (Figure 1C).

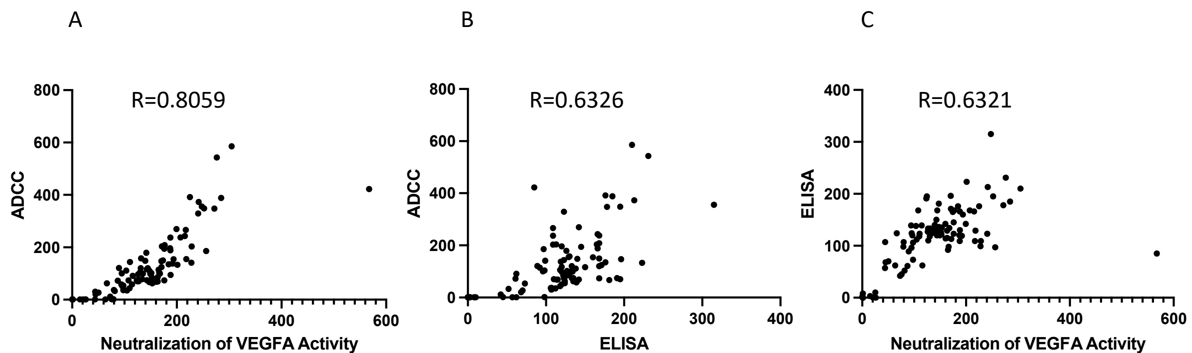

Figure 1 : Serum samples from 46 patients with ovarian cancer after four cycles of bevacizumab treatment and 48 longitudinal serum samples from a small cohort of patients with glioblastoma treated with bevacizumab were pooled, and a total of 94 samples were tested in triplicate for the presence of circulating levels of bevacizumab using an ELISA, and for the ability of bevacizumab to neutralize VEGF, and to activate ADCC activity as described in the Materials & Methods. To eliminate possible non-specific effects, the ADCC activity of each serum sample was first determined using both mVEGF (+) and mVEGF(-) target cells and the values obtained in the presence of the mVEGF(-) target cells were subtracted from those obtained using the mVEGF (+) and the final results were determined by interpolation of the standard curve of bevacizumab activity using the Prism software (GraphPad, France). The VEGF2A neutralizing activity of bevacizumab was compared with the ability of bevacizumab to activate ADCC (**Panel A**), the ability of bevacizumab to activate ADCC was also compared with circulating drug levels determined by ELISA (**Panel B**) and the VEGF2A neutralizing activity of bevacizumab was compared with the ability of bevacizumab to activate ADCC (**Panel C**), using the nonparametric Spearman rank correlation coefficient.

4. DISCUSSION

Solid tumors often induce neovascularization, that stimulates tumor growth, by promoting angiogenesis through the secretion of VEGFs and in particular VEGFA[4]. VEGFA exists in multiple isoforms, arising from both alternative splicing of exons 6 and 7 and proteolysis [12], VEGFA165 is the most common isoform and is present in a soluble form and as part of the extracellular matrix[12]. The humanized monoclonal antibody bevacizumab (Avastin®) binds to all circulating, soluble VEGFA isoforms[5], is used to target VEGF-dependent angiogenesis in patients with platinum-sensitive recurrent ovarian cancer[3,4] and is also used extensively to treat recurrent disease in patients with glioblastoma who have failed first line therapy[1,2,5]. Although bevacizumab treatment is without clinically significant interactions with concomitant standard chemotherapy and results in a high initial response rate and improves both progression-free survival and overall survival, the results are transient and most patient's tumors eventually progress[1,3]. High grade glioblastomas produce large quantities of VEGF, in particular VEGFA, that stimulates the proliferation of endothelial cells by binding to the VEGF receptor tyrosine kinases, VEGFR1 to 3, and activating STAT3 signaling via the VEGFR2 receptor, leading to the development of an abnormal vasculature[6]. The abnormal vascular network can lead to a reduction in oxygen delivery and regions of hypoxia within the tumor[13] and has been implicated in decreased drug uptake and resistance to radiation therapy[13, 14]. Elevated intra-tumoral levels of VEGF expression is associated with a poor prognosis or more aggressive disease in several solid tumors including ovarian cancer[15] and glioblastoma[16].

Bevacizumab is approved for both first line and second line treatment of a wide range of solid tumor[5] and is of particular benefit in glioblastoma where the number of chemotherapeutic agents that can cross the blood-brain barrier is limited[5]. Bevacizumab treatment has also been shown to be associated with improved quality of life[17] and a reduced requirement for glucocorticoids compared with radiotherapy and temozolomide treatment alone[5]. Bevacizumab also constitutes an important standard of care and is the only approved anti-angiogenic agent for the treatment of ovarian cancer[5,18]. In both indications, however, the benefits of bevacizumab treatment are transitory[5], and the

patient's disease eventually progresses underlining the need for a better understanding of the mechanisms of the antitumor activity of bevacizumab and the reasons for treatment failure[19,20].

Bevacizumab is thought to act in part by reducing tumor-induced vascularization, preventing new vessel formation and reducing microvascular permeability, thereby limiting tumor growth[1]. The ability to quantify the activity of membrane bound VEGFA in addition to soluble VEGF as described previously[7] may facilitate a better understanding of the action of bevacizumab on tumor-induced vascularization in both gynecologic and neurologic tumors. In addition to reducing tumor-induced vascularization, bevacizumab is also thought to exert direct anti-tumor activity against gliomas that express VEGF on their cell surface[5,16], and to increase the sensitization of tumor cells to cytotoxic agents[21]. Again, the ability to quantify the activity of membrane bound VEGFA may also shed light on this process.

Bevacizumab is also thought to have angiogenesis-independent effects on solid tumors including reversing the suppressive effect of VEGF on adaptive immunity[22]. In glioblastoma, alterations in the blood-brain barrier can lead to the presence of numerous immune cells including microglia-macrophage in the tumor microenvironment[23]. Studies in animal models suggest that VEGF blockage can led to an increased recruitment of monocytes as well as changes in dendritic cell sub-sets that may alter the adaptive immune response to the tumor[22,24]. The anti-tumor activity of numerous monoclonal antibodies is mediated in part by the stimulation of cellular immunity including antibody dependent cellular cytotoxicity and antibody dependent cellular phagocytosis (ADCP)[25,26] that in the case of the monoclonal antibodies rituximab and trastuzumab is considered to be the principal mechanism of their antitumor action[27=29]. In this context we have shown for the first time that bevacizumab stimulates ADCC against target cells that express non cleavable membrane bound VEGFA, while ranibizumab (Lucentis®), a derivative of bevacizumab lacking a Fc moiety[11], is devoid of ADCC activity attesting to the validity of the results[7]. As shown herein, there is close correlation between the stimulation of ADCC activity and the VEGFA neutralizing activity of bevacizumab across indications not withstanding different types of solid tumor and different treatment regimens. Both the ADCC activity and VEGFA neutralizing activities of bevacizumab also correlated with circulating levels of bevacizumab determined by ELISA across indications although less closely than between the ADCC and VEGFA neutralizing activities of bevacizumab. Although it is well established that the neutralizing antibody response to the variable region of therapeutic antibodies can limit their efficacy[30,31] our results suggest that for antibodies that act in part by the activation of cellular immunity an immune response the Fc moiety of the antibody may also limit their efficacy[7]. Although it is difficult to draw any firm conclusions from these results due to the restricted number of samples tested from the two small cohorts of patients included in the pilot study, they do show that it is indeed possible to quantify both the direct VEGF neutralizing activity of bevacizumab and the host mediated ADCC of activity bevacizumab in samples from patients with ovarian cancer or glioblastoma treated with bevacizumab and that there appear to be similarities in the action of bevacizumab in two different solid tumors. An accurate assessment of the treatment response in individual patients is key to a better understand of the most effective means of optimizing bevacizumab treatment.

5. CONCLUSION

Analysis using the nonparametric Spearman rank correlation coefficient of grouped samples, from a clinical study in which patients with ovarian cancer were treated with four cycle of bevacizumab, and longitudinal samples from a small cohort of patients with glioblastoma treated with bevacizumab, revealed a close correlation between bevacizumab neutralizing activity and the ability of bevacizumab to activate antibody dependent cellular cytotoxicity. Furthermore, a surprisingly good correlation was also observed between circulating drug levels in the two studies and the anti-angiogenic activity of bevacizumab, using an assay capable of quantifying the effect of bevacizumab on both soluble and membrane bound VEGF, and the ability of bevacizumab to activate cellular immunity. These results suggest that both the anti-angiogenic activity of bevacizumab and its ability to activate cellular immunity play a role in the anti-tumor activity of bevacizumab in two quite different solid tumors in patients with different treatment regimens.

Conflict of Interest
CL, LH and MT are employees of Svar life Science France but have no financial conflict of interest. FP and FD have no conflict of interest.

Author Contributions
CL & RFM performed the VEGF reporter-gene and ADCC assay and analysis of the data under the direction of MT who also wrote the manuscript. FP

and FD supervised the clinical studies that served as the basis for this analysis.

Funding
The work at Svar life Science France was funded in full by Svar life Science France. The research leading to these results has received support from the Innovative Medicines Initiative Joint Undertaking under grant agreement n° [115303], resources of which are composed of financial contribution from the European Union's Seventh Framework Program (FP7/2007-2013) and EFPIA companies' in kind contribution.

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