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The influence of bevacizumab on platelet function

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Summary

Systemic treatment with bevacizumab is associated with increased rates of arterial and venous thromboembolism and haemorrhage. In order to investigate the pathophysiological mechanism involved, platelet adhesive and aggregatory functions were tested with a platelet function analyser (PFA-100®) in an in vitro study and in a longitudinal clinical observation study. For the in vitro study, blood from ten healthy volunteers was incubated with different concentrations of bevacizumab (0–1000 µg/ml plasma) and vascular endothelial growth factor (0–500 µg/ml). In the clinical observation study, PFA-100® closure times (CTs) and soluble P-selectin (sP-selectin) serum levels as a serological marker of platelet activation were assessed in 20 patients with metastatic cancer who were treated with bevacizumab in addition to cytotoxic chemotherapy. No significant changes of PFA-100® CTs were observed in the in vitro study. In the clinical observation study, mean PFA-100® CTs after treatment with bevacizumab were unchanged. sP-selectin was decreased after bevacizumab infusion by 18% ($p = 0.045$), which could suggest an inhibitory action on platelets. Our data do not support the view that increased platelet activation or increased platelet adhesiveness and aggregation by bevacizumab are relevant mechanisms for thrombus formation in clinical practice.

Key words: *Bevacizumab; platelet function; haemorrhage; thrombosis; PFA-100; soluble P-selectin; von Willebrand antigen*

Introduction

Angiogenesis is a key element in growth and metastasis of cancer. Vascular endothelial growth factor (VEGF), its receptors and signalling pathways are the target of several novel anti-cancer drugs. Bevacizumab is a recombinant humanised monoclonal neutralizing antibody against VEGF, which has shown clinical benefits and efficacy in several types of malignancies including metastatic colorectal cancer, advanced breast and lung cancer [1–3]. The inhibition of VEGF has been noted to cause serious adverse events such as wound dehiscence, bowel perforation and, in addition, an increased rate of congestive heart failure in breast cancer patients has recently been demonstrated [4]. The use of bevacizumab has also been associated with an increased risk of developing venous and arterial thromboembolic events and haemorrhage. In a meta-analysis, patients treated with bevacizumab had an increased risk of venous thromboembolism with a relative risk (RR) of 1.33 and an overall incidence rate of 11.9% [5]. The risk of arterial thromboembolic events such as a myocardial infarction or stroke was found to be increased 2-fold with an event rate of 5.5 per 100 person-years [6]. Another recently published meta-analysis of 20 randomized trials confirmed that bevacizumab was associated with an increased risk of arterial ischaemia with an overall RR of 1.46 [7]. In a large observational treatment study in patients with metastatic colorectal cancer the incidence rates of clinically significant bleeding associated with bevacizumab was 2.4% [8]. In a randomized phase III trial of patients with advanced non-small-cell lung cancer significant haemorrhage was reported in 4.4% of patients treated with bevacizumab [2]. Increased treatment related mortality of bevacizumab in combination with chemotherapy or biological therapy (RR of 1.43) as compared to chemotherapy alone has been demonstrated in a recently published meta-analysis of 16 randomized controlled trials with incidence rates of fatal adverse events (FAE) of 2.5% versus 1.7%. Haemorrhage was the most common FAE linked to the use of bevacizumab with 23.5% of all FAEs [9].

The pathophysiological mechanisms leading to these side effects are poorly understood. Data from in vitro experiments and animal models point to a possible influence of bevacizumab in primary haemostasis and platelet function. Recently VEGF and VEGF receptors (VEGF-R) have been found to be relevant mediators of platelet aggregation [10, 11]. Both of these targets represent potential sites at which bevacizumab could potentially interact with primary haemostasis. The presence of VEGF-R messenger-RNA and the membranous localisation of VEGF on platelets have been known for some years [12, 13]. In addition, the presence and functions of VEGF-R1 (fms-like tyrosine kinase receptor, Flt-1) and VEGF-R2 (kinase-insert domain region, KDR) on human platelets has been demonstrated. In vitro tests have shown a stimulatory effect of VEGF on thrombin-induced platelet activation. This suggests that

the endogenously secreted platelet VEGF may function as a positive feedback regulator during platelet activation [10]. Theoretically, the interaction of bevacizumab with the platelet VEGF during platelet activation could result in impaired primary haemostasis, increasing the risk of haemorrhage.

Recently, the activation of the platelet FcgammaRIIa receptor and induction of platelet aggregation, degranulation and thrombosis through immune-complex formation of bevacizumab with VEGF and unfractionated heparin has been demonstrated in a transgenic mouse model [11]. However, platelet activation was not observed with bevacizumab alone, with VEGF alone, with bevacizumab and VEGF alone, or when using a VEGF variant lacking the heparin binding domain [11]. So far, this preclinical observation serves as a possible explanation of the increased rate of arterial and venous thrombus formation in mice. However, its clinical relevance in humans remains unclear.

On the basis of these preclinical data we hypothesized that primary haemostasis is a possible site of interaction of bevacizumab and VEGF resulting in and explaining some of its adverse effects. We decided to test whether the effects observed in experimental settings could be confirmed by observations in clinical practice. To test for disorders in platelet function the platelet function analyser PFA-100® was employed to obtain quantitative data on primary haemostasis in vitro and in a clinical observational study. In addition, soluble P-selectin (sP-selectin) serum levels and von Willebrand Factor antigen (vWF:Ag) were measured as a serological marker of platelet activation and endothelial activation in the clinical part of the study.

Patients and methods

In vitro study

Blood was drawn atraumatically from an antecubital vein from ten healthy, non-smoking Caucasian volunteers (four females, six males, age 20–59 years), using a standard tourniquet with a cuff pressure of 40 mm Hg, an 18 gauge butterfly needle and a standard vacutainer system with 1 part 0.126 M sodium citrate for 9 parts of blood.

Up to at least one week prior to the blood being taken, the volunteers had not taken any medication known to influence platelet function (e.g. acetylsalicylic acid, non-steroidal anti-inflammatory drugs). After measuring the haematocrit level, the exact volume of citrated whole blood per aliquot was adjusted in order to obtain exactly 3 ml plasma per aliquot. The aliquots were incubated with different amounts of bevacizumab (Genentech Inc, San Francisco, CA, USA) diluted in phosphate buffered saline, resulting in the following plasma concentrations of bevacizumab: 0 µg/ml (control), 125 µg/ml, 250 µg/ml, 500 µg/ml, and 1000 µg/ml. After an incubation period of 30 minutes the platelet function was analysed.

In a second series of experiments aliquots of citrated whole blood prepared as described above were incubated with VEGF (Santa Cruz, Biotechnology) diluted in phosphate buffered saline resulting in VEGF plasma concentrations of 0 µg/l (control), 100 µg/l, 500 µg/l and a combination of 500 µg/l VEGF followed by 500 µg/ml bevacizumab. Platelet function was analysed after an incubation period of 30 minutes.

Analysis of platelet function

Platelet adhesive and aggregatory function was tested with a platelet function analyser (PFA-100®, Dade Behring, Düdingen, Switzerland). In this instrument blood is aspirated at a constant negative pressure through a capillary with a diameter of 200 µm at a high shear rate of 5000–6000 s^{-1} into a 150 µm membrane pore coated with collagen type I and either epinephrine bitartrate (EPI) or adenosine diphosphate (ADP) as an additional platelet activator. Platelets adhere to the membrane pore, become activated, then aggregate and form an occluding plug in the pore thus stopping blood flow. The time measured from the start of the test until the formation of an occluding platelet plug is called closure time (CT). Consequently, the duration of the CT is inversely related to the platelet adhesiveness and aggregation. All measurements were done in duplicate and the mean value was calculated [14].

Routine analysis of haemoglobin levels, haematocrit and platelet count were performed with an electronic particle counter (Sysmex K-1000, Digitana AG, Horgen, Switzerland).

Clinical observation study

In the clinical part of the study all patients with advanced metastatic disease, who received a first infusion of bevacizumab and chemotherapy at the Kantonsspital Graubünden, Switzerland, were considered eligible. Patients taking acetylsalicylic acid were excluded. Patients receiving anticoagulation in a therapeutic dose were not included, because safety data for this group of patients were lacking at the time of the trial. Informed consent was obtained from all patients. The study was approved by the local ethical committee, was conducted according to good clinical practice guidelines (GCP) and the Declaration of Helsinki (National Cancer Institute Registration: NCT00898794).

Six of the 20 patients included had a previously implanted central venous access device with a small reservoir (CVAD; "Port-a-cath"), which was used for blood drawing and administration of drugs. In the remaining patients blood was drawn in a similar way as described above before and 15 minutes after the first i.v. application of bevacizumab and again after 2-3 infusions of bevacizumab, depending on the schedule (6 weeks of treatment). The duration of the first infusion was 90 minutes. No other medication was given between the two blood samples. A portion of the blood samples was processed on the same day according to current operational standards for the PFA-100® [14]. Another portion of the blood samples was immediately centrifuged and the serum was stored at –80° Celsius until being further processed for the sP-selectin measurements. SP-selectin and vWF:Ag measurements were performed at the Laboratory for Coagulation Studies at the University Hospital of Zürich, Switzerland, using commercially available immunoassay-kits (R&D Systems, Inc., Minneapolis, MN, USA and Asserachrom vWF:Ag, Diagnostica Stago Inc., France). The researchers performing

the sP-selectin and vWF:Ag analysis were blinded for the PFA-100® results.

Statistics

For the in vitro study sample sizes were calculated to detect a difference of 20 seconds (s) in collagen/ADP CTs and of 35 sec in collagen/EPI CTs which can be equated with an excess of the normal limits of the PFA-100® CTs (68–121 s for collagen/ADP and 84–160 s for collagen/EPI) with a power of 90% and a level of significance of 5%. For the clinical observation study sample size calculations were based on the assumption of a larger standard deviation in the patient population and take a drop out rate of 20% into account.

Statistical analysis were performed with the help of Statistica® 8.0 and Instat® 3.1a computer software (SoftStat, Inc., OK, USA and GraphPad Software, Inc., CA, USA) using repeated measures analysis of variance (ANOVA), Bonferroni Multiple Comparisons Test and Friedman-ANOVA. A p-value <0.05 was considered as statistically significant.

Results

In vitro study

The results of the in vitro experiments are shown in table 1. Note that the normal range of the PFA-100® closure times (CTs) for collagen/EPI is 84–160 s and for collagen/ADP 68–121s. Increasing bevacizumab concentrations up to 1000 µg/ml did not affect CTs with either epinephrine or ADP as a platelet activator, i.e. there was neither a CT prolongation indicating a platelet inhibition, nor CT shortening indicating platelet activation. Moreover the increase of the physiological VEGF plasma concentration by 100 or 500 µg/l had no effect on platelet function. The combination of VEGF and bevacizumab was also without an effect (table 1).

Clinical observation study

Twenty patients, who were treated with bevacizumab for the first time, were recruited. The clinical characteristics of these patients are shown in table 2.

Of the 20 patients, the results of 18 (90%) were considered evaluable for statistical analysis. One technical sampling error and one error in processing led to the exclusion of two patients for the evaluation of PFA-100® CTs. One patient was excluded from the evaluation of sP-selectin levels and vWF:Ag due to a technical sampling error. After six weeks the results of 15 patients were evaluable (2 technical errors, 2 continued treatment at another institution, 1 drop out due to disease progression).

The median CTs before and after the first bevacizumab application and after six weeks of treatment are shown in table 3. No statistically significant changes of CTs were observed ($p > 0.05$).

Six of 20 patients, who had a previously implanted CVAD were exposed to small amounts of unfractioned heparin, because the small reservoir of this device was filled with 3–4 ml of heparin containing solution after its use. In a post hoc analysis comparing those patients exposed to unfractioned heparin via the CVAD, with the other patients, who had no exposure to unfractioned heparin, no statistically significant difference was observed. CT-EPI difference from baseline were –1.3 s (range –26.9 – 24.3) and – 1.8 s (–16.4 – 12.7), CT-ADP difference from baseline were + 5 s (range –11.9 – 21.9) and + 3.1 s (–3.6 – 9.9) for heparin exposed (n = 6) and heparin-non-exposed (n = 12) patients, respectively ($p > 0.05$).

The median sP-selectin and vWF:Ag serum levels before and after the first bevacizumab application and after six weeks of treatment are also shown in table 3. Mean sP-selectin serum levels were significantly reduced by 18% ($p = 0.045$) after the first bevacizumab application. The changes in mean vWF:Ag after the first bevacizumab application and after six weeks of treatment were not statistically significant. Note that the normal reference range for vWF:Ag is 40–200%.

Discussion

Our hypothesis, based on preclinical data [10, 11], was that bevacizumab could influence human platelet function by either inhibiting or stimulating primary haemostasis. Our data indicate however, that this is not the case, i.e. bevacizumab did not affect platelet aggregation as assessed by the platelet function analyser PFA-100® in either direction.

Steady state bevacizumab concentrations after one infusion are in the order of 200 µg/ml (e.g., 500 mg diluted in 2.5 l plasma). Hence with in vitro concentrations of 0–1000 µg/ml we covered a sufficiently broad range of clinically relevant bevacizumab concentrations. In the in vitro study no dose effect was observed, which corresponds to certain clinical observations. In a randomized phase III trial with two different bevacizumab doses in metastatic breast can-

Table 1: PFA-100® closure times using either collagen/epinephrine (EPI) or collagen/adenosine diphosphate (ADP) as a platelet activator in *in vitro* experiments with bevacizumab, VEGF, or a combination thereof (mean ± SD, n = 10; n.s. = not significant).

		Closure times (s)	
		EPI	ADP
Bevacizumab (µg/ml)	0	120.8 ± 25.7	92.8 ± 7.7
	125	106.2 ± 21.7	88.5 ± 9.9
	250	123.2 ± 31.9	88.4 ± 18.2
	500	121.3 ± 23.5	93.5 ± 17.5
	1000	111.9 ± 24.2	100.2 ± 17.2
VEGF (µg/l)	0	112.2 ± 35.8	93.7 ± 11.8
	100	117.6 ± 24.5	92.6 ± 26.7
	500	113.7 ± 27.3	98.3 ± 13.8
VEGF (500 µg/l) + Bevacizumab (500 µg/ml)		108 ± 23.4	91.8 ± 10.0
		n.s.	n.s.

cer the rates of side effects amongst the two different treatment groups were not statistically different [15]. In a meta-analysis, the rate of venous thrombosis was also not dependent on the bevacizumab dose [5].

The target of bevacizumab, VEGF, given alone had no effect on platelet aggregation despite the fact that platelets have VEGF receptors on their surface [10]. Apparently, a binding of VEGF to its platelet receptor is not a signalling event for epinephrine – or ADP-induced platelet aggregation tested in this study. This does not exclude some other form of platelet activation as described for other pathways such as thrombin [10] or immunocomplex-induced activation [11]. However, considering that sP-selectin serum levels were not increased after the application of bevacizumab, such other forms of platelet activation seem unlikely.

Our results are in agreement with the work of Meyer et al. [11], who did not observe platelet activation with bevacizumab alone, VEGF alone or bevacizumab and VEGF alone. In the presence of unfractionated heparin, however, immune complexes consisting of bevacizumab, VEGF and heparin were formed, which activated platelets via Fc gamma receptors very similarly to platelet activation in heparin-induced thrombocytopenia caused by heparin-platelet factor 4 complexes. Our post-hoc analysis of six patients who had their heparin-containing Port-a-cath system rinsed shortly before bevacizumab application suggests that this amount of heparin was not sufficient to trigger this elegantly shown mechanism nor that it is clinically relevant.

The PFA-100® has a good sensitivity and specificity for the detection of primary haemostatic disorders [16]. It has replaced the classic bleeding time test in clinical practice in view of improved reproducibility and better test performance [14, 17, 18]. The test is highly sensitive for the detection of platelet disorders [19] and von Willebrand disease [14]. The range of additional clinical and research applications is also expanding [20–23]. Whilst the PFA-100® has been designed to detect an impaired haemostatic function of platelets, it has become apparent that it is also capable of detecting an increased adhesive and aggregatory platelet function [24–27].

Given the complexity of platelet function testing we considered it to be appropriate to additionally employ sP-selectin as a serological marker of platelet activation. P-selectin is a member of the selectin family localised in the membranes of α-granules of platelets and the Weibel-Palade bodies of endothelial cells [28]. A soluble form of P-selectin can be found in the serum as a circulating protein [29]. Elevated levels of sP-selectin may reflect platelet activation [30], because P-selectin is proteolytically shed from the plasma membrane in vivo shortly after activation [31, 32]. Therefore, serum levels of sP-selectin have been considered a useful tool to predict thrombotic consumptive platelet disorders [33–36]. Physiological functions of sP-selectin have not been elucidated to a large extent, but preclinical data support the view that sP-selectin may also be a direct inducer of pro-coagulant activity associated with vascular and thrombotic disease [37].

We have found an 18% decrease of sP-selectin serum levels after bevacizumab infusion. This decrease is unlikely to be due to an expansion of the plasma volume and a dilution effect by the concomitant saline infusion, because no significant effect on the haematocrit was observed. Since the half-life of sP-selectin is approximately two hours [38], a true decrease of sP-selectin is possible. This would suggest a decreased rather than an increased platelet activation. However, it seems unlikely that this has a lasting effect on primary haemostasis in view of the unchanged platelet aggregation and thrombus formation in our study.

SP-selectin may also be shed from endothelial cells upon their activation [39, 40], an observation which has, however, not been confirmed by others [41]. Therefore, measurement of vWF:Ag was additionally employed as a marker of endothelial activation. In our patient cohort vWF:Ag was not significantly changed after the first bevacizumab administration and after six weeks of treatment. This points to different origins of vWF (endothelium) and sP-selectin (platelets). However the study was not designed to address the interaction of bevacizumab with endothelial function and therefore the results of the vWF:Ag alone should be interpreted with caution.

Table 2: Patient characteristics in the clinical observation study.

Age	Median: 64.4 years (range 37–82)
Gender	Females: 7/20 (35%)
	Males: 13/20 (65%)
Bevacizumab dose	Median: 8.25 mg/kg bodyweight (range 5–15)
Disease	Metastatic colorectal cancer: 14/20 (70%)
	Metastatic breast cancer: 4/20 (20%)
	Other: 2/20 (10%)
Adverse events	1 pulmonary embolism after 2 months of treatment
	No bleeding events

Table 3: Results from the clinical observation study, PFA-100® closure times, soluble P-selectin serum levels and haematological parameters (mean value with 95% confidence interval); n.s. = not significant.

		Before first bevacizumab	After first bevacizumab	After 6 weeks of treatment	
PFA-100® closure times (s)	EPI	112.6 (99.8 – 125.4)	110.9 (98.5 – 123.3)	110.8 (94 – 127.7)	n.s.
	ADP	79.9 (71.9 – 86.7)	82.1 (72.7 – 90.8)	81 (72.8 – 89.2)	n.s.
Soluble P-selectin plasma level (ng/ml)		49.6 (39.5 – 59.6)	40.8 (31.7 – 49.8)*	40.5 (29.5 – 51.6)	*p = 0.045
vWF:Ag (%)		189.7 (146.2 – 233.2)	196.9 (130.4 – 263.4)	221.9 (168.4 – 275.4)	n.s.
Thrombocyte count (G/l)		190 (149 – 232)	188 (150 – 225)	159 (121 – 195)	n.s.
Haematocrit (%)		38.2 (35.2 – 41.1)	36.8 (34.2 – 39.4)	36.0 (32.7 – 39.4)	n.s.

This study has some further limitations. It was designed as a clinical observation study without a control group because the design of any control group (e.g. patients not receiving bevacizumab or healthy volunteers receiving an anti-cancer drug) was considered unethical and impossible. The sample size appears small but was adequately calculated for changes of PFA-100® CTs exceeding the normal limits with sufficient power. However, we refrained from increasing the number of patients to observe smaller effects on PFA-100® CTs in order to minimise the burden on patients and resources. The study was not designed to compare differences in platelet aggregation after bevacizumab treatment in patients exposed to unfractioned heparin with patients who were not exposed to heparin. However, we consequently aimed at addressing this question by the means of a small post hoc analysis after the work of Meyer et al. was published when our patient accrual was almost completed [11].

To conclude, our in vitro and clinical observation data taken together suggest that the interaction of bevacizumab with platelet aggregation can not be regarded as a major contributing factor to either thrombotic events or bleeding. Consequently other mechanisms must be involved, such as the interaction of bevacizumab with endothelial cell function and the plasma coagulation cascade. It is therefore worthwhile considering these other potential mechanisms of interactions in further studies.

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