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Neoadjuvant bevacizumab persistently inactivates VEGF at the time of surgery despite preoperative cessation

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BACKGROUND: When anti-VEGF (vascular endothelial growth factor) antibody bevacizumab is applied in neoadjuvant treatment of colorectal cancer patients with liver metastasis, 5–6 weeks between last bevacizumab dose and liver resection are currently recommended to avoid complications in wound and liver regeneration. In this context, we aimed to determine whether VEGF is inactivated by bevacizumab at the time of surgery.

METHODS: Fifty colorectal cancer patients with liver metastases received neoadjuvant chemotherapy ± bevacizumab supplementation. The last dose of bevacizumab was administered 6 weeks before surgery. Plasma, subcutaneous and intraabdominal wound fluid were analysed for VEGF content before and after liver resection (day 1–3). Immunoprecipitation was applied to determine the amount of bevacizumab-bound VEGF.

RESULTS: Bevacizumab-treated individuals showed no increase in perioperative complications. During the entire monitoring period, plasma VEGF was inactivated by bevacizumab. In wound fluid, VEGF was also completely bound by bevacizumab and was remarkably low compared with the control chemotherapy group.

CONCLUSION: These data document that following a cessation time of 6 weeks, bevacizumab is fully active and blocks circulating and local VEGF at the time of liver resection. However, despite effective VEGF inactivation no increase in perioperative morbidity is recorded suggesting that VEGF activity is not essential in the immediate postoperative recovery period.

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Colorectal cancer (CRC) represents one of the most common causes of cancer related deaths in the western world (Jemal *et al*, 2011). Approximately 50% of patients with CRC will develop liver metastases during the course of their disease and 25% present with liver metastasis at diagnosis (Van Cutsem and Oliveira, 2009). The median survival of patients with metastatic CRC increased substantially within the past decade (Kopetz *et al*, 2009). This is largely attributed to improved surgical techniques, more effective chemotherapy (CTx) and the introduction of biologicals (Simmonds *et al*, 2006; Kemeny, 2007; Gallagher and Kemeny, 2010).

Surgical removal of liver metastases is considered the only potentially curative treatment option for patients with resectable liver metastases and no extrahepatic disease (Van Cutsem and Oliveira, 2009). As the majority of patients have unresectable disease at presentation, neoadjuvant CTx regimens are now frequently applied to improve secondary resectability (Adam, 2003; Gallagher and Kemeny, 2010). Several clinical trials have documented an increased response rate in metastasised CRC patients receiving Avastin (Genentech – Roche, San Francisco, CA, USA), the monoclonal antibody bevacizumab (BV) against vascular endothelial growth factor (VEGF) in combination with chemotherapy (Hurwitz *et al*, 2004; Kabbinavar *et al*, 2005; Giantonio *et al*, 2007; Hochster *et al*, 2008). In accordance, the addition of VEGF inhibitors to neoadjuvant chemotherapy results in high resectability rates (Bertolini *et al*, 2011; Wong *et al*, 2011).

Vascular endothelial growth factor is a central regulator of blood vessel development (Ferrara, 2009). While angiogenesis is indispensable for neoplastic growth and metastasis, it is also involved in physiological processes, such as wound healing or hepatic regeneration (Folkman and Shing, 1992; Nissen *et al*, 1998; Reynaert *et al*, 2001). Accordingly, the neoadjuvant use of BV has been suspected to increase perioperative complications, and discontinuation of BV treatment for at least 7–8 weeks before surgery was suggested to avoid adverse events (Ellis *et al*, 2005). Applying this treatment schedule, several clinical studies have documented that neoadjuvant therapy with BV is a safe and feasible treatment, without substantial increase in perioperative morbidity after resection of colorectal liver metastases (D'Angelica *et al*, 2007; Reddy *et al*, 2008; Okines *et al*, 2009). Furthermore, when the BV-free period before surgery was reduced to 6 weeks or less, no significant rise in complications was observed (Gruenberger *et al*, 2006, 2008; Tamandl *et al*, 2010). Of note, there was no correlation between weeks of BV cessation and postoperative complication rates (Scappaticci *et al*, 2005; Kesmodel *et al*, 2008; Kozloff *et al*, 2009).

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Thus, 5–6 weeks of BV 'wash-out' before surgery are recommended at present (Hompes and Ruers, 2011). Based on the clinical experience and the half life of BV, 6 weeks between last BV dose and elective surgery are commonly assumed to restore VEGF activity and allow for postoperative wound healing and hepatic regeneration. In the present study, we submit evidence that following a cessation time of 6 weeks, BV is fully active in patient blood and VEGF is largely complexed by the antibody at the time of surgery. Furthermore, as circulating VEGF may not necessarily reflect the site of wound healing and hepatic regeneration, the results were confirmed for VEGF in subcutaneous and intraabdominal wound fluid. Despite complete VEGF inactivation, perioperative complication rates and postoperative liver function failure were not increased, as previously reported for a cessation time of 5–6 weeks after neoadjuvant BV treatment of metastatic CRC patients (Gruenberger *et al*, 2006, 2008; Kozloff *et al*, 2009).

MATERIALS AND METHODS

Patient collective and treatment

From March 2007 to February 2011, 50 patients with CRC metastases restricted to the liver (with or without primary *in situ*) were enroled in this prospective, translational study. The disease was identified as colorectal carcinoma stage IVA (the cancer may or may not have grown through the wall of the colon or rectum (any T), and it may or may not have spread to nearby lymph nodes (any N). It has spread to one distant organ – the liver (M1a)). The patients selected were at high risk for early recurrence of metastatic disease (Fong *et al*, 1999) and were therefore assessed for response to neoadjuvant therapy before being eligible for potentially curative surgical treatment. Characteristics of patients who underwent surgery are listed in Table 1.

Patients received neoadjuvant chemotherapy with or without the addition of BV. Assignment to the two treatment arms was not based on disease characteristics, but caused by recruitment modalities. CTx patients who were primarily referred to our clinic by other hospitals were continued on their chemotherapy treatment schedule without BV, whereas all patients starting treatment at our facility received combination CTx with BV at $5\,mg\,kg^{-1}$ biweekly for five cycles. Bevacizumab treatment was ceased 6 weeks before liver resection. Reflecting the clinical application, several different CTx regimens were included (compare Table 1). CTx regimens were conducted as follows. XELOX/XELIRI: Oxaliplatin at $85\,mg\,m^{-2}$ or irinotecan at $200\,mg\,m^{-2}$ was given i.v. on day 1 of each cycle. The dose of capecitabine was $1500\,mg\,m^{-2}$ administered twice daily for the first week, followed by 1 week of rest period. Patients were treated for six 2-week cycles where the sixth cycle was given without BV, resulting in a BV-free period of 6 weeks. The FOLFOX6 regimen consisted of oxaliplatin at $100\,mg\,m^{-2}$, leucovorin at $400\,mg\,m^{-2}$, a bolus of $400\,mg\,m^{-2}$ 5′-fluorouracil (5-FU) on day 1 as well as $2800\,mg\,m^{-2}$ 5-FU infusion. The treatment was repeated every 14 days for six courses. Regarding FOLFIRI/FOLFOXIRI treatment, patients received irinotecan at $165\,mg\,m^{-2}$ and $200\,mg\,m^{-2}$ leucovorin followed by a $400\,mg\,m^{-2}$ bolus of 5-FU. Furthermore, 5-FU at $3200\,mg\,m^{-2}$ was administered via 46 h continuous infusion. In the FOLFOXIRI group, oxaliplatin at $85\,mg\,m^{-2}$ was given additionally. All substances were administered on day 1 of a 2-week cycle for six courses.

Sample collection

Plasma samples were obtained during the perioperative period (1 day before and on the first, second and third day after surgery). Accordingly, wound fluid was collected on all three postoperative time points. Drains were placed subcutaneously and intraabdominally (directly subhepatic) to reflect the actual site of cutaneous wound healing and hepatic regeneration, respectively.

Table 1 Demographic and clinical characteristics of study participants

	CTx + BV ($N = 32$)		CTx ($N = 10$)		
Parameter	***N***	**%**	***N***	**%**	***P***
Sex					*0.715*
Male	19	59	7	70	
Female	13	41	3	30	
Site of primary					*0.999*
Colon	19	59	6	60	
Rectum	13	41	4	40	
Patients with concomitant primary resection	*7*	*22*	*0*	*0*	*0.164*
Hepatic resection					
Major	20	63	8	80	0.451
Minor	11	34	2	20	0.466
Exploration	1	3	0	0	0.999
Chemotherapy regimen					
XELOX	22	69	3	30	0.062
XELIRI	1	3	0	0	0.999
FOLFOXIRI	5	16	0	0	0.315
FOLFOX	4	12	3	30	0.328
FOLFIRI	0	0	2	20	0.052
None	0	0	2	20	0.052
	Median	**Range**	**Median**	**Range**	
CTx cycles	6	3–10	6	4–12	0.649
BV cycles	5	3–9			
Weeks CTx to surgery	3.8	2.1–7.3	5.0	3.3–10.6	0.038
Weeks BV to surgery	6.1	3.9–8.3			
Age (years)	64	42–78	68	55–80	0.215

Abbreviations: CTx = neoadjuvant chemotherapy; CTx + BV = neoadjuvant chemotherapy plus bevacizumab; XELOX = Xeloda + oxaliplatin containing CTx; XELIRI = Xeloda + irinotecan containing CTx; FOLFOXIRI = 5-FU + oxaliplatin + irinotecan containing CTx; FOLFOX = 5-FU + oxaliplatin containing CTx; FOLFIRI = 5-FU + irinotecan containing CTx; None = no CTx at least 6 months before study inclusion.

Preparation of plasma and wound fluid

Platelet-poor plasma was prepared as previously described (Brostjan *et al*, 2008; Starlinger *et al*, 2010b, 2011). Briefly, blood (10 ml) was drawn into prechilled CTAD tubes containing sodium citrate, theophylline, adenosine, and dipyridamole, was kept on ice and further processed within 30 min. After an initial centrifugation step at 1000 **g** and 4 °C for 10 min, the plasma supernatant was subjected to further centrifugation at 10 000 **g** and 4 °C for 10 min and stored in aliquots at − 70 °C. To achieve comparability with plasma samples, wound fluid was transferred to CTAD tubes and processed by the same procedure.

The analysis of blood and wound fluid samples was approved by the Institutional Ethics Committee (#300/2006, #437/2006, #791/2010); all patients gave written informed consent.

Vascular endothelial growth factor measurements

Plasma and wound fluid samples were analysed for VEGF content by enzyme-linked immunosorbent assay (ELISA) following the manufacturer's instructions (Invitrogen Corp., Carlsbad, CA, USA). The ELISA was confirmed to detect VEGF despite BV complexation. However, binding of BV to VEGF reduced the detection sensitivity.

Immunoprecipitation

Removal of human IgG (including BV) from plasma samples and wound fluid was carried out as recently described (Brostjan *et al*,

2008). A total of 200 μl of plasma were combined with 100 μl of protein A/G PLUS-agarose (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). After 4 h of sample rotation at 4 °C and centrifugation for 5 min at 1000 **g**, 200 μl of supernatant were again mixed with 100 μl of protein A/G PLUS-agarose and subjected to rotation over night. After two consecutive centrifugation steps, plasma and wound fluid (supernatant) was analysed by ELISA for VEGF content. The established concentrations were multiplied by a factor of 1.6 to adjust for the dilution of samples in the immunoprecipitation (IP) procedure.

Statistical analysis

Statistical analyses were carried out with SPSS 17 Software (SPSS, Inc., Chicago, IL, USA) and were based on nonparametric tests (Mann–Whitney *U* test, Wilcoxon test). Fisher's exact test was applied to compare patient characteristics and therapy modalities between treatment arms. Boxplot illustrations are given without outliers and extreme values to improve the resolution of interquartile ranges.

RESULTS

Of the initial 50 patients, 42 were eligible for liver resection of hepatic metastases after neoadjuvant therapy. These patients are listed in Table 1. Age ($P=0.215$) and sex distribution ($P=0.715$) were comparable between treatment arms with and without BV administration. Major hepatic resection was performed in 80% of patients in the CTx group and in 63% of patients who received neoadjuvant BV combination therapy ($P=0.451$). The remaining patients underwent minor hepatic resection ($P=0.466$). In addition, in the CTx plus BV arm, 22% of patients received concomitant primary tumour and liver resection, which was not performed in the CTx treatment arm ($P=0.164$). Surgery was suspended in one BV patient due to extrahepatic disease discovered during laparotomy.

Neoadjuvant treatment and BV schedule

In representation of the current diversity of clinical applications different CTx backbones were included (Table 1). The median application of chemotherapy were six cycles (range 3–10) in the CTx plus BV group and six cycles (range 4–12) in the CTx control arm ($P=0.649$). The median BV administration lasted for five cycles (range 3–9) and BV therapy was ceased for a median of 6 weeks (range 4–8 weeks) before surgery. The treatment arms differed in the median time between last chemotherapy to liver resection ($P=0.038$), with BV patients having shorter (3.8 weeks) break periods than CTx patients (5 weeks).

Postoperative morbidity and mortality

No patient died during the perioperative period. A total of 13 patients (41%) experienced postoperative complications after neoadjuvant BV combination therapy as opposed to 7 patients (70%) in the control group who did not receive BV (Table 2). The incidence of complications was comparable between treatment arms ($P=0.109$). In particular, CTx + BV patients showed no increased rates of wound complication ($P=0.954$), anastomotic leak ($P=0.576$), thrombosis ($P=0.074$), bile leak ($P=0.423$), or hyperbilirubinemia ($P=0.206$) and there were no cases of liver function failure.

Table 2 Perioperative complications

	CTx + BV (*N* = 32)		CTx (*N* = 10)		
Complication[a]	***N***	**%**	***N***	**%**	***P***
No complication	19	59	3	30	0.109
Wound complication[b]	3	9	1	10	0.954
Anastomotic leak	1	3	0	0	0.576
Ascites	1	3	1	10	0.379
Thrombosis	1	3	2	20	0.074
Fever	5	16	2	20	0.749
Bile leak	2	6	0	0	0.423
Pleural effusion	2	6	1	10	0.691
Hyperbilirubinemia[c]	9	28	5	50	0.206
Liver function failure[d]	0	0	0	0	

Abbreviations: CTx = neoadjuvant chemotherapy; CTx + BV = neoadjuvant chemotherapy plus bevacizumab. [a]Some patients had multiple complications. [b]Wound complications were generally wound infections, and one case of wound dehiscence in the CTx + BV group. [c]Hyperbilirubinemia was defined as bilirubin levels $>2\,mg\,dl^{-1}$ within the first postoperative week. [d]Liver function failure was classified by bilirubin levels $>5\,mg\,dl^{-1}$ and prothrombin times <50% within 3 months after surgery.

Perioperative VEGF concentration in plasma and wound fluid of patients

Plasma samples of CRC patients were obtained after neoadjuvant chemotherapy with or without concomitant BV administration. Blood was drawn immediately before surgery and on the first 3 postoperative days. VEGF plasma levels of patients who had received BV during neoadjuvant therapy were about five-fold ($P<0.001$) increased compared with CTx controls before surgery (Figure 1A). Furthermore, VEGF levels stayed significantly elevated on all 3 days after liver resection (day 1: $P<0.001$, day 2: $P<0.001$, day 3: $P=0.002$). Of note, as plasma levels do not necessarily reflect VEGF concentrations at the site of wound healing and liver regeneration, wound fluid was additionally analysed from subcutaneous and intraabdominal drainages. In striking contrast to plasma VEGF levels, we found a highly significant reduction of VEGF levels in subcutaneous as well as intraabdominal wound fluid of patients who had received BV during neoadjuvant CTx (subcutaneous day 1: $P=0.003$, intraabdominal day 1: $P=0.001$). The difference between treatment arms (Figure 1B and C) was highly significant during the entire postoperative monitoring period (subcutaneous day 2: $P=0.007$, intraabdominal day 2: $P<0.001$, subcutaneous day 3: $P=0.005$, intraabdominal day 3: $P<0.001$).

Perioperative VEGF complexation by BV

Despite the total increase in plasma VEGF in BV patients, it is the degree of biologically active, unbound VEGF, which is of physiological importance. To evaluate the proportion of free *vs* antibody-bound VEGF, plasma and wound fluid samples were comparatively analysed after IP, a procedure removing all plasma IgG including the BV antibody (and the VEGF molecules in complex with the antibody). Samples were then reanalysed concomitantly with the corresponding untreated controls for VEGF content (Figure 2A). For patients who had been treated with BV, the majority of VEGF found in blood or at wound sites was bound (i.e. inactivated) by the antibody, as reflected in a significant difference between VEGF concentrations measured before and after the IP procedure (plasma preoperatively: $P=0.002$; plasma postoperatively: $P=0.001$; intraabdominal wound fluid: $P=0.018$; subcutaneous wound fluid: $P=0.005$). There was no significant difference between postoperative days 1 to 3 (data not shown). Of note, the levels of unbound, biologically active VEGF were consistently low (close to the ELISA detection limit) and were comparable in plasma and wound fluid of BV patients.

When subjecting samples of CTx patients to IP, the VEGF concentration was only moderately reduced, indicating that the IP procedure did not result in unspecific loss of VEGF molecules. We recorded a significant difference between BV patients and CTx controls in preoperative plasma (Figure 2B; before IP: $P=0.001$; after IP: $P=0.160$), in postoperative plasma (Figure 2C; before IP: $P=0.003$; after IP: $P=0.006$), in intraabdominal wound fluid

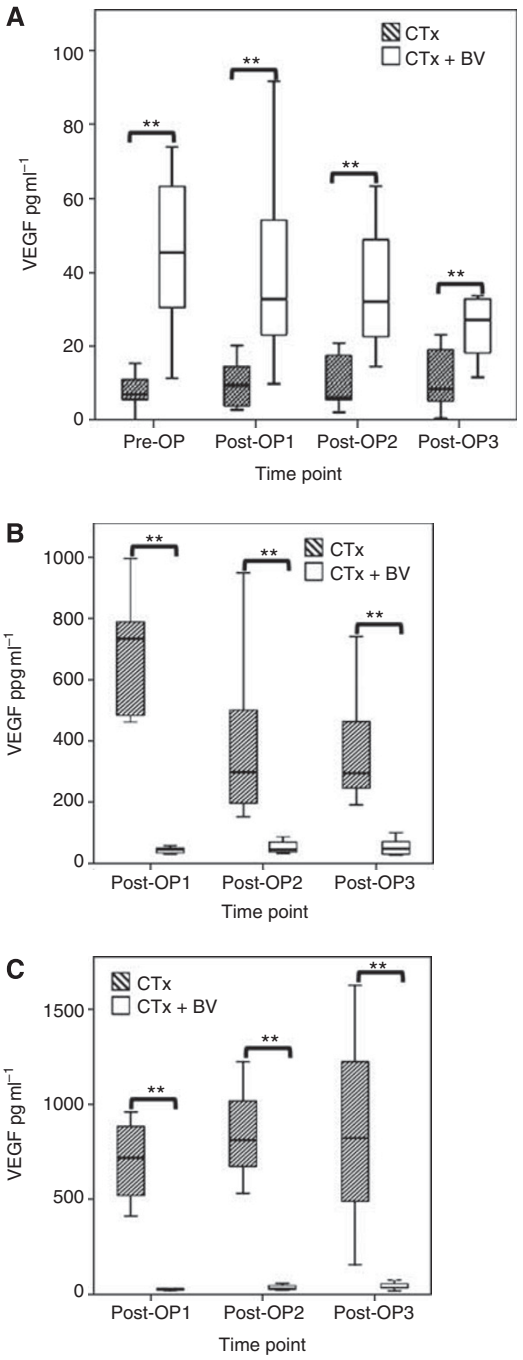

Figure 1 Circulating levels of VEGF in plasma and wound fluid during the perioperative period. VEGF concentrations were determined before surgery (pre-OP) and on the following three postoperative days (post-OP1, 2, 3) in plasma (**A**), intraabdominal (**B**) or subcutaneous (**C**) wound fluid, and are illustrated by boxplot for the neoadjuvant treatment groups with (CTx + BV) or without (CTx) bevacizumab supplementation (*$P \leqslant 0.05$; **$P \leqslant 0.01$).

(Figure 2D; before IP: $P = 0.001$; after IP: $P = 0.001$), and in subcutaneous wound samples (Figure 2E; before IP: $P = 0.002$; after IP: $P = 0.002$).

Thus, total VEGF was significantly increased in the blood of CTx + BV patients, but unbound (biologically active) VEGF was comparable to or even lower than recorded for CTx controls. With respect to wound fluid, BV-treated individuals showed remarkably decreased concentrations of total VEGF and further reduced levels of unbound VEGF in comparison with CTx patients.

DISCUSSION

The effect of neoadjuvant BV treatment on the perioperative availability of VEGF was investigated in metastatic CRC patients. In this prospective, translational study, we were able to demonstrate that following a median BV cessation time of 6 weeks, circulating VEGF was mostly bound by the antibody and therefore biologically inactive. Besides pre- and postoperative plasma, also wound fluid of subcutaneous and intraabdominal drainages showed a comparable extent of VEGF complexation by BV. Despite effective VEGF inactivation at the time of surgery, no increase in postoperative mortality or morbidity after hepatic resection of liver metastases was observed, which is essentially in line with earlier studies based on larger patient collectives (D'Angelica *et al*, 2007; Kesmodel *et al*, 2008; Reddy *et al*, 2008; Mahfud *et al*, 2010). We would like to emphasise that this study was not designed to compare complication rates between treatment groups with and without BV, as this has been subject to investigation in numerous reports demonstrating comparable complication rates. The primary objective of this study was to evaluate the availability of VEGF in the perioperative period following neoadjuvant BV therapy. The unbalanced distribution of patients in the two treatment arms and the variety of chemotherapeutic agents applied were determined by the current clinical practice at the hospital facility. Despite this disparity, the BV-related effects on VEGF availability were highly specific and clearly distinct from CTx controls, thus generally resulting in significance levels markedly below 0.01.

As we have previously demonstrated (Brostjan *et al*, 2008; Starlinger *et al*, 2011), total VEGF increases significantly in patient blood after neoadjuvant BV treatment, which is considered a feedback response and pharmacodynamic marker of VEGF inactivation by BV. However, despite the elevated levels of plasma VEGF, we found that BV patients experience a general VEGF blockade by the antibody during the entire perioperative period. Of interest, at the actual site of wound healing and liver regeneration total VEGF was significantly lower in BV-treated individuals as compared with CTx controls: While control patients showed VEGF concentrations in subcutaneous and intraabdominal wound fluid, which were 70 times higher than the corresponding plasma values, VEGF detected in the wound fluid of BV-treated individuals ranged at a level comparable to plasma VEGF. Importantly, the remaining VEGF molecules were mostly antibody-bound, confirming a complete VEGF inactivation in the wound fluid of BV patients.

This study is the first to demonstrate the remarkable discrepancy between increased blood concentrations but low wound levels of VEGF in BV-treated individuals. A possible explanation for this observation may relate to a differential stability of VEGF–antibody complexes in circulation as compared with wound sites. While antibodies in blood are protected from elimination by the endothelial neonatal Fc receptor, they are selectively degraded in distinct organs, such as the liver and the skin (Keizer *et al*, 2010). Thus, VEGF in complex with the BV antibody may be stabilised in blood but subject to rapid degradation at wound sites of liver and skin when compared with unbound VEGF. Alternatively, platelets may account for the effects observed. They are a major source of VEGF, which is released upon platelet degranulation during injury and wound healing. Verheul *et al* (2007) reported that platelets are able to scavenge BV and VEGF from circulation. The uptake of BV may reduce their VEGF storage capacity and therefore lead to a lower level of total VEGF released by platelets at wound sites as compared with circulating VEGF detected in plasma. However, these hypotheses remain tentative and await further investigation.

Several preclinical studies have documented the importance of VEGF for functional wound healing and hepatic recovery

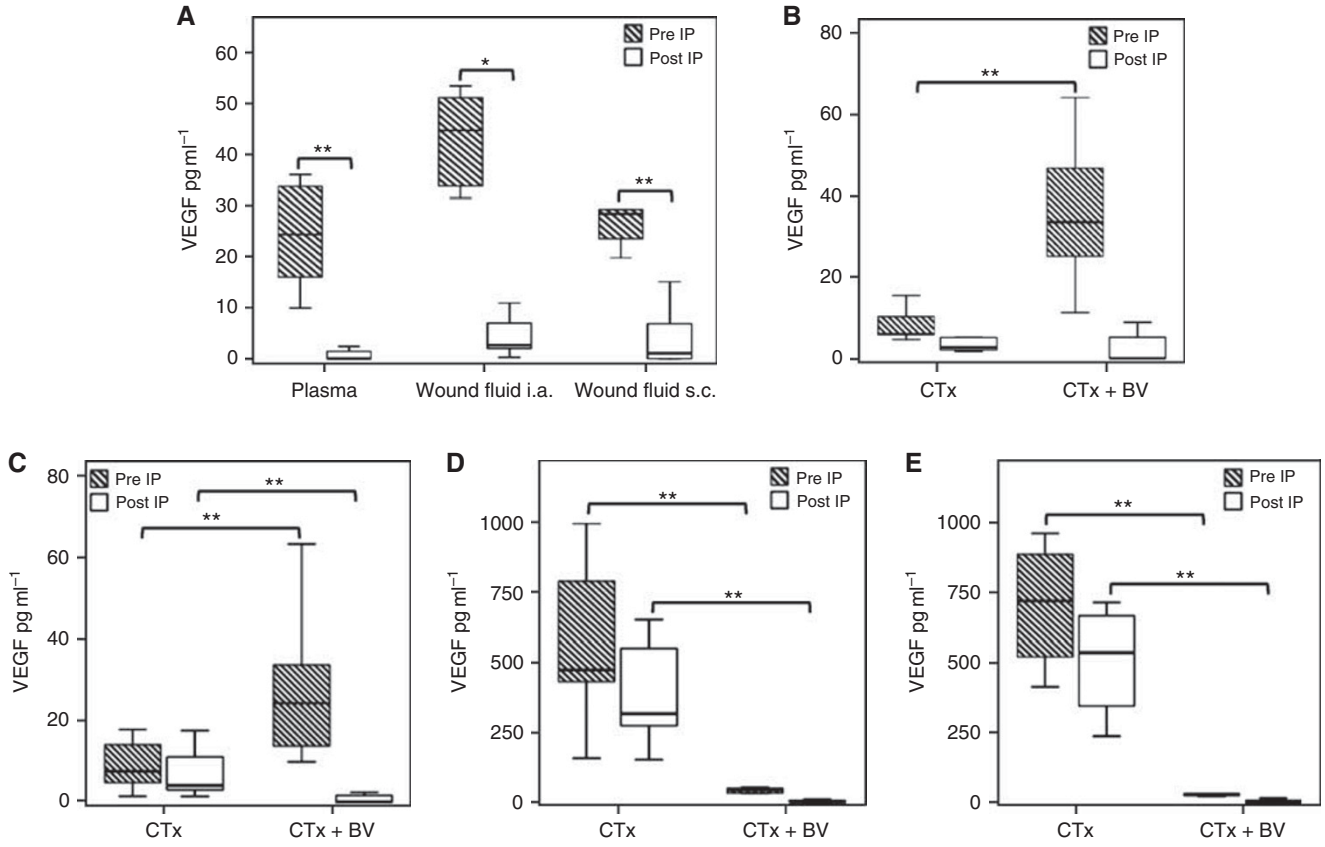

Figure 2 Free *vs* antibody-bound VEGF in plasma and wound fluid during the perioperative period. An IP procedure was applied to determine the proportion of biologically inactivated, bevacizumab-bound VEGF in patient samples. The VEGF concentrations determined before IP represent totally detectable VEGF, whereas VEGF levels after IP reflect free, unbound VEGF molecules. (**A**) CTx + BV patients were investigated for total (pre IP) *vs* unbound (post IP) VEGF in plasma and wound fluid of postoperative days 1 to 3. Samples of CTx + BV patients were compared with CTx controls (before and after IP) for preoperative plasma (**B**), postoperative plasma (**C**), intraabdominal (**D**) and subcutaneous (**E**) wound fluid (*$P \leqslant 0.05$; **$P \leqslant 0.01$).

(Reynaert *et al*, 2001; Rossiter *et al*, 2004). But subsequent investigations indicated that VEGF blockade may indeed be of limited impact on wound and liver regeneration. In this context, Jacobi *et al* (2004) demonstrated that VEGF promotes wound angiogenesis but is not required for wound closure in mice. Comparably, VEGF inhibition did not prevent cutaneous wound healing (Ko *et al*, 2005) and showed a marginal effect on liver regeneration (Van Buren *et al*, 2008) in rodent models. These preclinical studies support our observation of normal wound and liver recovery in BV patients despite the lack of biologically active VEGF. The results indicate that VEGF is not essential in the immediate postoperative period of wound healing and liver regeneration. Alternatively, resistance mechanisms may develop during BV therapy, which compensate for the functional loss of VEGF, as has been extensively investigated over the past years (Bergers and Hanahan, 2008; Brostjan *et al*, 2008; Starlinger *et al*, 2010a). For example, an increase in basic fibroblast growth factor was shown to compensate for VEGF inactivation and reduce the efficacy of anti-angiogenic therapy (Casanovas *et al*, 2005). A comparable mechanism may also promote wound and liver regeneration and account for the unaffected perioperative morbidity after neoadjuvant BV therapy.

To avoid the potential of surgical complications, it is currently recommended that BV is suspended for 5–6 weeks before elective surgery (Hompes and Ruers, 2011). However, based on our own experience (Gruenberger *et al*, 2006, 2008; Tamandl *et al*, 2010) and other reports (Scappaticci *et al*, 2005; Kesmodel *et al*, 2008; Kozloff *et al*, 2009), the postoperative complication rate does not increase with shorter cessation times and does not correlate with the timing of BV discontinuation. Here we demonstrate that BV is fully active and efficiently blocks VEGF at the recommended 6 weeks of BV 'wash-out' without significant increase in postoperative morbidity. We thus hypothesise that shorter waiting periods may be feasible for clinical practice when warranted by emergency surgery or other clinical indications. However, larger prospective clinical trials would be required to conclusively establish the safety of shorter cessation times between last BV dose and liver resection.

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