Neoadjuvant Treatment of Colorectal Cancer with Bevacizumab: The Perioperative Angiogenic Balance Is Sensitive to Systemic Thrombospondin-1 Levels

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Abstract
Purpose: Colorectal cancer patients receiving neoadjuvant treatment with bevacizumab, a monoclonal antibody neutralizing vascular endothelial growth factor (VEGF), may suffer from wound healing complications after surgery as the antibody persists in patient blood. We characterized the systemic angiogenic balance in the perioperative period to evaluate its effect on physiologic angiogenesis.

Experimental Design: Nineteen patients receiving combination chemotherapy and bevacizumab for six neoadjuvant cycles were compared with 14 patients receiving chemotherapy without bevacizumab. Plasma from perioperative days -1, +1, +7, and +21 was analyzed for VEGF, thrombospondin-1 (TSP-1), and PD-ECGF concentrations. The angiogenic capacity was further tested in an \textit{in vitro} assay of endothelial cell proliferation and migration.

Results: On day +1, the onset of wound healing was reflected in a change of balance, i.e., an increase of proangiogenic factors VEGF and platelet-derived endothelial cell growth factor compared with low TSP-1 inhibitor levels in both treatment groups. Patients with bevacizumab therapy showed significantly higher blood levels of total VEGF throughout the evaluation period. However, most VEGF molecules were inactive, i.e., complexed with antibody. Nevertheless, the capacity to stimulate endothelial growth was higher for these plasma samples and was reflected in low TSP-1 levels and an altered TSP-1 sensitivity. When purified TSP-1 protein was added, plasma samples of the bevacizumab but not the chemotherapy group showed reduced endothelial growth.

Conclusions: Feedback mechanisms of bevacizumab therapy are not restricted to VEGF expression but seem to involve additional factors, such as TSP-1, which influences the systemic angiogenic balance and permits endothelial growth.

At the time of diagnosis, ~20% of colorectal cancer (CRC) patients with metastases confined to the liver are eligible for surgery (1). In the remaining cases, palliative chemotherapy primarily based on 5'-fluorouracil combinations is applied to achieve a resectable state and eradicate potential micrometastases before surgery (2). In the attempt to further improve the number of patients who may become resectable after first-line therapy, regimens have recently been extended by the antiangiogenic compound bevacizumab, i.e., Avastin (3). Bevacizumab is a humanized monoclonal antibody that neutralizes all isoforms of human vascular endothelial growth factor A (VEGF) and has proven efficacy in the management of metastatic CRC when combined with established chemotherapies (4). A significantly increased response rate and improved overall and progression-free survival have been reported in phase II and III trials in metastatic CRC (5, 6). The effects of bevacizumab on tumor physiology include a reduction of microvessel density, vascular volume, and tumor perfusion (7). With respect to combination chemotherapy, the bevacizumab-mediated "vessel normalization", and related decrease in interstitial fluid pressure seem to improve the delivery of therapeutic agents (8). VEGF is a central player in pathologic as well as physiologic neovascularization, i.e., controls proliferation, migration, and survival of endothelial cells in the angiogenic process. Thus, safety concerns have been raised with regard to the effect of VEGF blockade by bevacizumab therapy on wound healing and liver regeneration after surgery (9). Particularly, with respect to the neoadjuvant setting, the long antibody half-life of 21 days (10) raises the issue of the appropriate timing between last bevacizumab administration and tumor resection. At the commonly applied, repetitive antibody dosage in CRC of...
5 mg/kg biweekly and the currently accepted interval of 8 weeks to surgery, the remaining level of bevacizumab around the time of surgical intervention is presumably sufficient to clear all free VEGF from circulation (9, 10). Therefore, it has been of major interest to compare wound healing problems in patients after bevacizumab treatment and elective surgery with patients after chemotherapy without VEGF blockade (11, 12). Interestingly, a moderate increase in wound healing complications was observed in the preoperative combination therapy with bevacizumab (13%) as opposed to chemotherapy alone (3.4%) if emergency surgery was required during treatment (13).

The Department of General Surgery at the Medical University of Vienna comparably conducts neoadjuvant therapy of CRC patients with both initially resectable and unresectable liver metastases. Patients receive oxaliplatin- and capecitabine-based chemotherapy with concomitant bevacizumab treatment. If response is achieved, surgery is done 5 weeks after the last administration of the antibody. To date, we have not encountered any substantial, bevacizumab-related increase in perioperative or postoperative bleeding and wound healing complications (12). We were thus interested in investigating circulating angiogenic factors during the perioperative period of wound healing, hypothesizing that bevacizumab-treated patients were capable of initiating a physiologic angiogenic response that would be reflected in systemic changes of the angiogenic balance comparable with the wound healing process in CRC patients exposed to chemotherapy without the addition of bevacizumab.

A massive production of VEGF is detectable in wound fluid within 2 days after surgery and generally remains elevated or further increases over the course of the following 2 weeks (14, 15). A significant “spillover” to circulation is shown in an increase of plasma VEGF concentrations on postoperative days 1 to 2, which provides a limited time window for systemic investigations on the wound healing process (14). Serum measurements primarily reflect VEGF stored in and released by platelets upon serum preparation and have thus been found to mimic the initial decrease of platelet counts in hemostatic plug formation rather than the local VEGF surge in wound tissue (14, 16, 17). Detectable increases in serum VEGF levels have only been reported for major surgery (16, 18, 19), which argues for plasma analyses to investigate changes in the systemic angiogenic balance during wound healing. A change in balance involving the increase of proangiogenic factors and a concomitant decrease of antiangiogenic molecules is required to promote vessel growth (15, 20). Thrombospondin-1 (TSP-1) is a well-characterized endogenous inhibitor of neovascularization that plays a role in wound repair (20, 21). It is strongly reduced and shows inverse regulation to VEGF expression by hypoxia or oncogenic mutations promoting angiogenesis (22–24). TSP-1 inhibits endothelial cell migration and proliferation (25) and can induce apoptosis (26), thus, limiting vessel formation (27).

In contrast, platelet-derived endothelial cell growth factor (PD-ECGF) sustains neovascularization via its activity as thymidine phosphorylase, i.e., by indirectly promoting endothelial cell proliferation and migration (28, 29). Apart from its angiogenic properties, PD-ECGF has an effect on cancer chemotherapy because it is involved in the conversion of 5′-fluorouracil prodrugs into the active compound (30).

In this study, we have thus chosen to investigate the perioperative plasma levels of VEGF, TSP-1, and PD-ECGF of CRC patients after neoadjuvant treatment with oxaliplatin, capecitabine, and bevacizumab. For comparison, a collective of patients receiving chemotherapy alone was analyzed. Systemic changes in the angiogenic balance during the postoperative wound healing period were evaluated.

### Materials and Methods

#### Neoadjuvant CRC treatment protocol.

The patients selected were at high risk for early recurrence of metastatic disease and were therefore assessed for response to neoadjuvant therapy before being eligible for potentially curative surgical treatment. Bevacizumab patients received combination chemotherapy consisting of bevacizumab at 5 mg/kg biweekly and XELOX (capecitabine, 3,500 mg/m²/d on days 1 to 7 plus oxaliplatin, 85 mg/m² on day 1 of a 2-wk cycle) for six cycles resulting in 3 mo of therapy. The sixth cycle did not include bevacizumab, providing a gap of 5 wk between the last antibody dose and liver resection compared with 2 and 3 wk of interval between last capecitabine and oxaliplatin to surgery, respectively. Chemotherapy patients received XELOX without the addition of bevacizumab.

#### Preparation of platelet-poor plasma.

Blood (10 mL) was drawn into prechilled CTAD-tubes containing sodium citrate, theophyllin, adenosine, and dipryridamole, was kept on ice and further processed within 30 min. After an initial centrifugation step at 1,000 × 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 (to remove “contaminating” platelets). The supernatant was stored in aliquots at -70°C to avoid repeated cycles of freezing-thawing before analysis.

The analysis of blood samples was approved by the Institutional Ethics Committee; all patients and healthy volunteers gave written informed consent.

#### Determining plasma concentrations of angiogenic factors.

Plasma samples were analyzed by ELISA to determine the concentration of circulating angiogenic factors. Commercially available ELISA tests were applied for VEGF (Quantikine; R&D Systems) and TSP-1 (Accucyte; Cytimmune Sciences, Inc.). A comparable “sandwich” ELISA system for PD-ECGF (detection range, 1-100 ng/mL) has previously been reported by us (31) and is based on the following antibodies: gelatin-free goat polyclonal α-human PD-ECGF antiserum (sc-9523; Santa Cruz Biotechnology, Inc.) diluted to 5 μg/mL for coating, and murine monoclonal IgG1 clone P-GF.44C diluted to 500 ng/mL for detection (Neomarkers Lab Vision Corp.). The detection antibody was biotinylated using the FluorReporter Mini-Biotin-X Protein Labeling kit (Molecular Probes, Inc.) according to manufacturer’s instructions and was further complexed with a conjugate of streptavidin–horseradish peroxidase (Pierce Biotechnology). Plasma samples were measured undiluted at 50 μL per well and compared with a standard dilution series of human recombinant PD-ECGF (R&D Systems).

#### Immunoglobulin precipitation of plasma samples.

Removal of human IgG (including bevacizumab) from plasma samples was carried out as recently described (32). Two hundred microliters of plasma were combined with 100 μL of protein A/G PLUIS-agarose (Santa Cruz Biotechnology, Inc.). After 4 h of sample rotation at 4°C and centrifugation for 5 min at 1,000 × g, 200 μL of supernatant were again mixed with 100 μL of protein A/G PLUIS-agarose and subjected to rotation over night. After two consecutive centrifugation steps, the plasma (supernatant) was analyzed by ELISA for VEGF content. The established concentrations were multiplied by a factor of 1.6 to adjust for the dilution of plasma samples in the immunoprecipitation procedure.

#### In vitro wound healing (“scratch”) assay.

Endothelial cells were isolated from human foreskin samples by dispase digest, purified to >98% purity via α-CD31 antibody coupled Dynabeads (Invitrogen Corp.) and cultured in fibronectin-containing EGM2-MV (Cambrex Corp.) without VEGF supplementation. Cells were seeded at 1.4 × 10⁵ in 24-well plates to yield a confluent monolayer within 24 h. A bare
area for regrowth of endothelial cells was generated with a cross-like scratch. Medium was then exchanged for EBM2 (Cambrex Corp.) basal medium supplemented with 10% of patient plasma and heparin (18 IU/mL). Where indicated, purified human TSP-1 (Calbiochem-Merck KGaA) was added to a final concentration of 1,500 ng/mL. Digital images of the regrowth area were taken at baseline and after 6 and 20 h with an Olympus IMT-2 microscope supplied with a Nikon Coolpix 4500 camera. The area covered by cells was evaluated with the ImageQuant 5.2 software (Molecular Dynamics) and translated to a growth rate (slope) k of percentage area covered between 0 and 6 h of incubation. Twenty-hour readings were not included in the calculation because several samples had reached confluence by then, which affects their growth rate.

Statistical analysis. Statistical analyses were carried out with SPSS 10.0.07 Software (SPSS, Inc.) and were based on nonparametric tests (Mann-Whitney U test, Wilcoxon test, and Spearman test). The Bonferroni-Holm procedure was applied to correct for errors of multiplicity; changes in the significance of P values are indicated where applicable. Boxplot illustrations are given without outliers and extreme values to improve the resolution of interquartile ranges.

Results

Angiogenic profile of a healthy control collective. To determine the physiologic range of plasma concentrations for VEGF, TSP-1, and PD-ECGF, a healthy control collective of 36 individuals (17 males and 19 females; median age, 34 years; distribution, 17-77 years) was first evaluated. Median values ranged at 2.0 pg/mL VEGF (interquartile range, 0.6-6.3 pg/mL; 95th percentile, 15.6 pg/mL), 1,698 ng/mL TSP-1 (interquartile range, 1,437-2,705 ng/mL; 5th percentile, 1,088 ng/mL), and 2.1 ng/mL PD-ECGF (interquartile range, 1.5-3.4 ng/mL; 5th percentile, 0.0 ng/mL).

Characterization of the patient collective. The CRC patients undergoing neoadjuvant therapy were divided into the chemotherapy group of 14 subjects (8 males and 6 females) receiving XELOX chemotherapy and into the bevacizumab group of 19 individuals (10 males and 9 females) exposed to combination therapy with bevacizumab. The age distribution of these groups was similar, i.e., ranging from ages 44 to 82 years (median, 63 years) for the bevacizumab group compared with ages 47 to 81 years (median, 60 years) for the chemotherapy group.

The extent of metastasis ranged from 1 to 8 foci (median, 2 foci) in either treatment arm. No wound healing complications were encountered in the bevacizumab group, compared with one case of wound infection and secondary closure as well as one incidence of operative intraabdominal abscess drainage in the control group. No increased intraoperative or postoperative bleeding was recognized for the bevacizumab versus chemotherapy treatment arm. There were two patients in each group with postoperative septic signs (fever and increased infectious blood results) where no septic source was identified and patients recovered under antibiotics. One patient with urinary tract infection was recorded for the bevacizumab collective.

Perioperative fluctuations of VEGF, TSP-1, and PD-ECGF plasma levels. Plasma samples were obtained from CRC patients after neoadjuvant therapy. Blood was drawn immediately before surgery (on the day of surgery or up to 3 days before), on the 1st day after surgery, as well as once between postoperative days 5 and 8, and markedly thereafter, between postoperative days 17 and 43. According to the median days, these time points of analysis were categorized as -1 d, +1 d, +7 d, and +21 d.

The median preoperative concentrations of VEGF, TSP-1, and PD-ECGF were 48 pg/mL, 328, and 1.1 ng/mL, respectively. As compared with the healthy control group (by Mann-Whitney U test), a significant elevation of circulating VEGF (P < 0.001) and a significant decrease of TSP-1 (P < 0.001) as well as PD-ECGF (P < 0.001) was observed for the patient collective. When healthy cutoff levels were defined by 95% of control values, 85% of patient samples were above the VEGF cutoff (15.6 pg/mL) and 82% were below the TSP-1 cutoff level (1,088 ng/mL). Notably, most preoperative CRC plasma samples were close to the PD-ECGF detection limit.

One day after resection, the angiogenic balance was altered considerably, indicating the onset of wound healing (Fig. 1A-C). Although proangiogenic molecules increased significantly (VEGF, P < 0.001 for +1 d versus -1 d, +1 d versus +7 d, and +1 d versus +21 d; PD-ECGF, P < 0.001 for +1 d versus -1 d, +1 d versus +7 d, and P = 0.024 for +1 d versus +21 d), the angiogenesis inhibitor TSP-1 was low (P = 0.017 for +1 d versus +7 d, Wilcoxon rank test). Within a week postsurgery, all factors returned to preoperative levels.

Comparison of bevacizumab and chemotherapy treatment groups with respect to perioperative plasma fluctuations of angiogenic molecules. When VEGF, TSP-1, and PD-ECGF concentrations were evaluated separately for the bevacizumab and chemotherapy treatment groups (Fig. 1D-F), no significant difference between the two types of therapy was found for plasma PD-ECGF and TSP-1 at the time points investigated (Mann-Whitney U test). However, the median thrombospandin levels of bevacizumab patients remained low throughout the observation period, which was in contrast to the fluctuations detected for the chemotherapy group. TSP-1 values dropped on the day after surgical intervention and were restored to preoperative levels 1 week postsurgery (P = 0.019 for +1 d versus +7 d, Wilcoxon rank test)—an increase that was not seen for the bevacizumab group.

With respect to VEGF blood levels, the two therapy groups showed a significant difference at all time points of evaluation (P ≤ 0.001, Mann-Whitney U test). Before surgery, VEGF plasma concentrations were already elevated in the treatment arm that had been subjected to bevacizumab (P < 0.001), displaying a 3-fold median value (59 versus 19 pg/mL). The VEGF increase on the day after resection was more pronounced for the chemotherapy (P = 0.003 for +1 d versus -1 d) than for the bevacizumab group (P = 0.036 for +1 d versus -1 d, Wilcoxon rank test; P = 0.072, when adjusted according to Bonferroni-Holm). Nevertheless, total VEGF concentrations remained higher in the plasma of bevacizumab-treated patients compared with chemotherapy patients 1 day postsurgery (P < 0.001, Mann-Whitney U test). Although VEGF levels decreased thereafter to preoperative values in the chemotherapy group, plasma VEGF concentrations of bevacizumab patients fell well below starting levels at 1 and 3 weeks after surgical intervention (median, 52 and 30 pg/mL, respectively).

Evaluation of VEGF, TSP-1, and PD-ECGF plasma levels before and after neoadjuvant therapy. Because this study was designed for perioperative monitoring of angiogenesis factors, blood samples of patients were not routinely obtained before neoadjuvant treatment. However, to confirm that the elevation of VEGF plasma levels observed in bevacizumab patients was due to the neoadjuvant therapy, six corresponding pretreatment samples of individuals in the bevacizumab group were collected.
and analyzed (Fig. 2). VEGF concentrations markedly increased ($P = 0.028$, Wilcoxon test; $P = 0.084$, when adjusted according to Bonferroni-Holm) in response to neoadjuvant bevacizumab combination therapy. Although based on a limited number of available samples, our results were in line with the previously reported capacity of bevacizumab to stimulate an increase of total circulating VEGF in patient blood (7, 33, 34).

Analysis of free versus antibody-bound VEGF in CRC plasma samples. Despite the total increase in circulating VEGF in bevacizumab patients, the majority of VEGF molecules are antibody bound and, thus, inactive (10, 32). To evaluate the proportion of free versus antibody-bound VEGF, eight bevacizumab and two chemotherapy plasma samples with comparable VEGF content were chosen and subjected to an immunoprecipitation procedure removing all plasma IgG (including the bevacizumab antibody). Samples were then reanalyzed concomitantly with the corresponding untreated controls for VEGF content (Fig. 3). Although the VEGF concentration of chemotherapy samples was not altered by the procedure, the VEGF values of bevacizumab samples dropped significantly ($P = 0.012$, Wilcoxon test), indicative of a small proportion of free VEGF. The median VEGF content decreased from 52 to 12 pg/mL, i.e., below the healthy cutoff level. When bevacizumab samples were separately analyzed for plasma obtained 1 day before and 1 day after surgery, a comparable result was obtained, i.e., very low amounts of free VEGF were detectable.

Effect of the systemic angiogenic balance on reendothelialization. After having established the plasma concentrations of PD-ECGF, TSP-1, as well as free versus inactive VEGF in patient
plasma, we were interested in the effect of the systemic angiogenic balance on endothelial cells. In a common \textit{in vitro} wound healing (scratch) assay, we monitored the proliferation and lateral migration of an endothelial cell monolayer supplied with patient plasma. Samples of the bevacizumab and chemotherapy group obtained on postoperative day 1, i.e., at the onset of wound healing, were evaluated. Figure 4 compares the +1 d plasma values for total VEGF, TSP-1, and PD-ECGF to the established growth rate of endothelial cells. Surprisingly, the reendothelialization in the presence of bevacizumab plasma was not reduced but proceeded faster than for chemotherapy plasma (median $k$ values of 4.2 versus 3.2; $P = 0.040$, Mann-Whitney $U$ test; $P = 0.120$, when adjusted according to Bonferroni-Holm). Interestingly, endothelial cell growth rates did not correlate with any of the proangiogenic factors but showed an inverse correlation with plasma TSP-1 content (Fig. 5; $P = 0.017$; $k = -0.412$, Spearman test; $P = 0.051$, when adjusted according to Bonferroni-Holm).

\textbf{Effect of TSP-1 addition in the \textit{in vitro} wound healing assay.} To test whether changes in the TSP-1 concentration could alter the angiogenic balance in patient plasma and affect endothelial cell growth, we chose seven samples of the bevacizumab as well as the chemotherapy group with comparably
low thrombospondin content. The TSP-1 concentration was adjusted by addition of purified thrombospondin protein to 1,500 ng/mL (the average concentration of chemotherapy plasma samples with high TSP-1 content). Original and TSP-1–supplemented samples were then compared in the in vitro wound healing assay (Fig. 6). The endothelial growth rate in untreated plasma was faster for the bevacizumab than the chemotherapy group (median k values of 5.4 versus 3.6; $P = 0.006$, Mann-Whitney U test). However, only bevacizumab samples were sensitive to the addition of TSP-1 as shown by a significant decrease in reendothelialization to a level close to chemotherapy samples (median k value of 4.5; $P = 0.018$, Wilcoxon test).

Discussion

The angiogenic variables investigated in this study were initially determined for a healthy control collective to define basal blood concentrations of VEGF, TSP-1, and PD-ECGF for the method of plasma preparation and the quantitative assays.
applied in this analysis. When preoperative plasma samples of CRC patients were compared with the healthy control collective, a significant difference was observed for all angiogenic variables: circulating VEGF was highly elevated, whereas TSP-1 and PD-ECGF were reduced. The data are in accordance with a number of detailed reports on the correlation of VEGF tissue or blood levels with diagnosis and prognosis of colorectal carcinoma (17, 35, 36). Moreover, the lack of TSP-1 and concomitant tissue expression of VEGF was found to reflect a poor prognosis for CRC patients (37). The low plasma concentration of PD-ECGF, however, was unexpected (38) and might relate to the fact that all patients had been exposed to capcitabine-based chemotherapy before analysis. Conversion of this 5'-fluorouracil prodrug requires PD-ECGF activity, i.e., specifically targets PD-ECGF-expressing cancer cells. The decreased PD-ECGF plasma levels (median, 1.1 ng/mL) compared with the healthy control collective (median, 2.1 ng/mL) might thus reflect the effects of neoadjuvant chemotherapy. The limited number of patient samples obtained before neoadjuvant treatment showed a median PD-ECGF concentration comparable with the healthy control collective (median, 1.9 ng/mL).

When angiogenic factors were monitored in the perioperative period, the induction of an angiogenic response at the early onset of wound healing was reflected in plasma samples of the postoperative day 1. Higher concentrations of the proangiogenic molecules VEGF and PD-ECGF were accompanied by a decrease in the angiogenesis inhibitor TSP-1, thus indicating that the systemic levels determined within 24 hours after surgery reflected the local change of balance between proangiogenic and antiangiogenic factors required for tissue neovascularization (39). One week thereafter, the changes in the investigated variables were no longer detectable in patient samples. The time course observed is in accordance with the perioperative measurements of plasma VEGF conducted by Hormbrey et al. (14), showing a significant increase within 24 to 48 hours after cancer surgery and a rapid decline to preoperative concentrations by day 4. Other groups have reported an additional peak or plateau of plasma VEGF around postoperative days 8 to 10, which we have not observed (19, 40). The difference may relate to the extent and type of surgery in the respective studies (16) or —as suggested by one of the authors— may be due to variations in plasma preparation (19). The latter argument seems highly feasible because different methods of plasma preparation were shown to be prone to platelet contamination and degranulation (41, 42), and the extended postoperative increase in plasma VEGF correlated with an increase in platelet counts and serum VEGF levels in this particular study (19).

The changes in blood concentrations of the proangiogenic factors during the perioperative monitoring period were more prominent than for TSP-1, as reflected in the statistically significant increase of VEGF and PD-ECGF plasma levels on day +1. Although median TSP-1 values dropped on the 1st postoperative day and were restored to baseline 1 week thereafter, a statistically significant difference in TSP-1 plasma levels was only recorded for d+1 to d+7 but not for d-1 to d+1. The latter may be due to a smaller regulatory range of TSP-1 expression (43) and a wide distribution of physiologic TSP-1 blood levels, possibly necessitating a higher number of study participants to reliably reflect a wound healing-related decrease of circulating TSP-1 on postoperative day 1.

With respect to the two treatment groups receiving neoadjuvant chemotherapy with or without addition of bevacizumab, a significant difference was determined for VEGF plasma concentrations. Patients of the bevacizumab group showed substantially elevated circulating VEGF—an effect that has previously been described for clinical applications of the antibody (7, 33, 34). Neutralization of VEGF seems to trigger a feedback mechanism, which results in the enhanced production of VEGF presumably initiated to compensate for the loss of VEGF activity. In the perioperative monitoring period, total VEGF moderately increased in bevacizumab samples on postoperative day +1, thus reflecting the onset of physiologic angiogenesis in the wound healing process. Both, chemotherapy and bevacizumab samples showed a comparable pattern of postoperative fluctuation. Although the relative increase on day +1 was even more pronounced for the chemotherapy treatment group, total VEGF concentrations were consistently higher for the bevacizumab-treated patients. Considering the short half-life of VEGF, this result would indicate that the remaining presence of neutralizing antibody in bevacizumab patients leads to a continuous enhancement of VEGF production (based on the above mentioned feedback mechanisms). The effect declines but is still detectable at 3 weeks postsurgery, i.e., 8 weeks after the last antibody administration.

Despite the total increase in circulating VEGF molecules of bevacizumab-treated patients, the availability of “free”, i.e., functional, VEGF is dramatically reduced due to the continued presence of the antibody (10, 32). Similarly, we have observed a substantial difference between total and free VEGF in plasma samples of the bevacizumab group drawn before as well as immediately after surgery. Thus, 5 weeks after the last bevacizumab treatment, blood concentrations of the antibody were sufficient to inactivate nearly all systemically detectable VEGF and to further neutralize the wound-related increase in...
circulating VEGF on postoperative day 1. Nevertheless, bevacizumab patients exhibited normal wound healing comparable with the chemotherapy treatment group. It has previously been suggested that tissue concentrations of VEGF in healing wounds may greatly exceed the detectable plasma levels and may require higher local concentrations of inhibitors than suggested by plasma measurements (14). Although this hypothesis would certainly explain the discrepancy between systemic VEGF blockade and local angiogenesis, the results of our in vitro wound healing assay led us to an additional line of reasoning. Plasma of bevacizumab versus chemotherapy patients was applied to promote endothelial cell proliferation and migration. Despite the substantially reduced content of free, i.e., functional VEGF, bevacizumab samples were even more potent than plasma derived from chemotherapy patients in sustaining endothelial cell growth and spread. In a comparable endothelial cell proliferation assay, postoperative serum samples of CRC patients were recently shown to be of prognostic value (44). In our setting, in vitro growth rates significantly correlated with TSP-1 content of plasma samples rather than VEGF or PD-ECGF levels, and endothelial cells supplied with bevacizumab plasma were more sensitive to the addition of thrombospondin than endothelial cells exposed to chemotherapy samples.

Fig. 6. Angiogenic profile of bevacizumab versus chemotherapy plasma samples and the capacity for endothelial cell growth after TSP-1 supplementation. VEGF (A), TSP-1 (B), and PD-ECGF (C) concentrations as determined for seven bevacizumab and seven chemotherapy plasma samples of postoperative day 1 with comparably low TSP-1 content. The endothelial growth rate k (D) was determined for untreated plasma and after addition of TSP-1 to a final concentration of 1,500 ng/mL. Median values and interquartile ranges are illustrated by boxplot. An experimental example is given for a bevacizumab plasma sample (E and F) to illustrate the growth and morphology of endothelial cells without (E) or with (F) TSP-1 supplementation at 0, 6, and 20 h of incubation.
The comparison of a limited number of plasma samples before and after bevacizumab therapy revealed a lower median TSP-1 level after neoadjuvant treatment. Similarly, when monitoring the perioperative TSP-1 fluctuations, we found that bevacizumab patients showed consistently low median values of TSP-1, thus, following the inverse pattern of VEGF plasma levels. Although median TSP-1 concentrations were minimal on postoperative day 1 for both treatment groups, thrombospondin levels rapidly recovered in the chemotheraphy treatment arm, whereas they stayed low in the bevacizumab group of samples. Despite the fact that these “trends” did not reach statistical significance, they point to a possible inverse effect of bevacizumab administration on TSP-1 as opposed to VEGF expression in CRC patients. The proposed feedback mechanism of VEGF neutralization by the antibody thus might not only induce VEGF production but also trigger the inhibition of TSP-1 expression. Considering that the concomitant but opposing regulation of VEGF and TSP-1 expression has been reported for various other stimuli (22–24), a comparable regulatory mechanism seems feasible. With VEGF being complexed and inactivated by excess antibody, this would consequently explain the increased sensitivity of bevacizumab compared with chemotherapy plasma samples to thrombospondin levels in the in vitro wound healing assay.

We thus believe that our observations offer a possible and exciting explanation for the effective angiogenic response in surgical wounds despite the systemic inactivation of VEGF by bevacizumab. The hypothesis that bevacizumab therapy might result in regulation of angiogenesis factors apart from VEGF, which can affect the systemic as well as local angiogenic balance, merits further investigation. In particular, plasma levels of TSP-1 before and after bevacizumab treatment should be analyzed for a larger number of patients (compared with total and free VEGF). Although the change in proangiogenic versus antiangiogenic balance observed in plasma samples of postoperative day 1 strongly argues for the relevance of systemic measurements, the concomitant evaluation of these angiogenic mediators in wound fluid might shed further light on the consistency or discrepancy of the local and systemic angiogenic response.

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