

Featured Article

The Relationship of Human Wound Vascular Endothelial Growth Factor (VEGF) After Breast Cancer Surgery to Circulating VEGF and Angiogenesis

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Abstract

Purpose: The assessment of locally produced proangiogenic cytokines may be an indicator of the stromal response of an individual to wounding or cancer. This study describes the profile of VEGF production in human surgical wounds in both breast cancer patients and reduction mammoplasty controls, and assesses the changes in systemic VEGF levels and platelet profiles perioperatively.

Experimental Design: Perioperative surgical wound fluid samples and blood were collected daily up to 13 days from 52 patients undergoing breast cancer surgery (local tumor burden), delayed breast reconstruction (previous tumor burden but none present at the time of surgery), or breast reduction surgery (noncancer control). Samples were analyzed for VEGF by ELISA.

Results: VEGF levels in surgical wound fluid were lowest on day 1 followed by an early peak on day 2 of >900% the corresponding serum value. There was a trend in the VEGF response at the day-2 time point: reduction > reconstruction > cancer subgroups, with a significant difference between the reduction and cancer subgroups ($P < 0.05$). There was a 20–30-fold variation in the response between days 1 and 2, and within subgroups.

Conclusions: Much higher local concentrations of angiogenic factors may need to be antagonized for effective antiangiogenic therapy, and there is great heterogeneity between patients. The small peripheral blood changes compared with large tumor fluid changes show that there is a tissue barrier. This has relevance for design of antiangiogenic

therapy trials, highlighting the need for individually tailored treatment with biologically targeted interventions.

Introduction

Surgery is still the main curative therapeutic modality for breast cancer. However, local and distant recurrence of breast cancer may be because of the perioperative stimulation of residual cancer cells (1).

Angiogenesis plays a key role in both wound healing and the ability of a cancer to survive and grow, so investigation into the angiogenic response may help guide surgical approaches and timing of antiangiogenic therapy.

Normal wound repair generates an angiogenic response to deliver nutrients and inflammatory cells to injured tissue. The angiogenic response enables the removal of debris and is central to the development of a granulation tissue framework for wound closure (2). The mediators of wound angiogenesis include soluble factors such as VEGF,² tumor necrosis factor α , transforming growth factor β , fibroblastic growth factor, and platelet-derived growth factor identified in several wound models (3). Angiogenic agonists (e.g. VEGF) and antagonists (e.g. thrombospondin-1) have been described at various times during repair (4–6), suggesting that the net angiogenic stimulus may be a balance of factors changing to favor either vessel growth or regression (7).

Previous studies have shown that surgical wound fluid collected within a few hours of operation is potently angiogenic. fibroblastic growth factor levels have been shown to peak immediately after surgery and then fall by the second postoperative day (8). This immediate release has been suggested to function to initiate wound angiogenesis. In later wounds VEGF is the predominant angiogenic mediator (4). An up-regulation of VEGF production in wound repair has been demonstrated in keratinocytes in skin wounds in rat, guinea pig, and mouse models (9).

Work on the production of proangiogenic cytokines in early human wound fluid has been done using drain fluid from patients undergoing cancer surgery (4, 8). These studies are based on the premise that wound fluid would be generally representative of the growth environment of the wound. However, none have studied breasts that have had previous cancer surgery *versus* breasts immediately after cancer surgery.

Our study compares the profile of VEGF production after surgical intervention in the presence of tumor burden (breast cancer surgery) in patients with a prior history of cancer but no current tumor burden (delayed breast reconstruction) and in the normal breast (breast reduction surgery).

Despite a large body of literature describing the production

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² The abbreviations used are: VEGF, vascular endothelial growth factor; PD, platelet count drop.

of angiogenic growth factors in wounds and the release of VEGF from platelets, none have studied the relationship between levels of wound fluid VEGF and platelet counts. Substantial quantities of VEGF are released from platelets during formation of a hemostatic plug *in vivo* (10), and platelet release of VEGF has been a source of much controversy in terms of sample collection when analyzing the prognostic implications of VEGF in cancer patients (11–16).

To better understand the mechanism of wound angiogenesis and its significance in tumor biology and surgical intervention, we describe the temporal profile of VEGF in fluid collected from human surgical wound drains placed at the time of breast operations, the corresponding plasma, and serum VEGF and circulating platelet counts. We show an early VEGF response, the relationship to VEGF levels in the circulation, platelet counts, and the differences between patients with malignancy *versus* controls.

Materials and Methods

Patients. This study was approved by the Aylesbury Vale Research Ethics Committee at Stoke Mandeville Hospital. Informed consent was obtained from all of the patients. Female patients were recruited consecutively from those undergoing breast surgery procedures for cancer, reduction, or reconstruction, presenting to the Breast Surgery and Plastics and Reconstructive Surgery Departments at Stoke Mandeville Hospital. Exclusion criteria were age <16 years or if the procedure was not expected to result in the placement of a drain.

Blood Samples. Venous blood samples were obtained using a 21-gauge needle, with blood aliquoted into vacutainer bottles. No additive was used for serum VEGF collection, and sodium citrate 3.2% (Greiner Labortechnik) was used for plasma collection resulting in a 10% dilution of the final sample. Plasma samples were processed within 1 h, whereas serum was processed at 1 h. All of the VEGF samples were collected preoperatively at room temperature and centrifuged at $3000 \times g$ for 5 min at 22°C. Aliquots of 250 μ l were immediately frozen and stored at -70°C before analysis, storage time ranged between 1 week and 15 months. An EDTA sample was taken for full blood count measurement using a Cell-Dyn coulter counter (Abbott Diagnostics) recording all of the parameters including the mean platelet volume. The VEGF content per platelet ($\text{VEGF}^{\text{PLAT}}$) was calculated as:

Preop $\text{VEGF}^{\text{PLAT}}$

$$= \frac{\text{average (serum VEGF pg/ml) - plasma VEGF pg/ml}}{\text{average platelet count } 10^9/\text{l}} \times 1000$$

Wound Fluid Samples. If a patient had >1 drain in place then each was coded and sampled individually. Drain fluid was collected at the same time each day, from postoperative day 1 onwards. Using standard aseptic techniques the drain bag was detached from the tubing connection and replaced with a new bottle. After inverting the bottles several times to facilitate mixing, samples were aliquoted into plain serum tubes for transfer to the laboratory. The validation of wound fluid collection was the subject of a pilot study (see below). In the final

analysis, all of the wound fluid results for that day were averaged.

Protein Analysis. Serum and wound fluid samples were analyzed for albumin and total protein content. Samples were collected in serum tubes with clot activator (Becton Dickinson Vacutainer Systems, Oxford, United Kingdom) and analyzed by a Johnson & Johnson Vitros 750 analyzer.

VEGF ELISA. VEGF concentrations were determined using a quantitative sandwich enzyme immunoassay technique (Quantikine Human VEGF Immunoassay; R&D Systems, Abingdon, United Kingdom) that detects the soluble isoforms of human VEGF (VEGF 165 and 121). The system uses a solid-phase monoclonal and an enzyme-linked polyclonal antibody raised against recombinant human VEGF. For each analysis, 100 μ l of sample were used. All of the analyses and calibrations were carried out in duplicate. The calibration on each microplate used recombinant human VEGF standards. Optical densities were determined using a microplate reader (Titertek Multiskan PLUS – MK II) at 450 nm with a correction at 570 nm. The blank was subtracted from the duplicate readings for each standard and sample. A standard curve was created using Deltasoft 3 by plotting the logarithm of the mean absorbance of each standard *versus* the logarithm of the VEGF concentrations. VEGF concentrations are reported as pg/ml. The sensitivity of the assay is 9 pg/ml with the reliable standard curve range from 31.25 pg/ml to 2000 pg/ml. Raw ELISA values were adjusted for 10% dilution as a liquid anticoagulant was used (any value below the standard range was then allocated the 31.25 minimum value).

Statistical Analysis

Analysis of wound fluid data was done using untransformed data, *i.e.*, as concentration of VEGF per ml of wound fluid.

A professional statistician performed statistical analysis. A general estimating equation was adopted for panel data, with paired *t* test and Wilcoxon matched-pairs signed-ranks test applied to paired identical samples. All of the *P*s are two-tailed, and a *P* < 0.05 was taken as being statistically significant. All of the tests were performed using Stata 5.0 (Stata Corporation).

Results

Pilot Study: Validation of Collection Methodology

Collection methodology for blood sampling with respect to subsequent VEGF measurement has already been a subject of analysis and review by our laboratory (17). Seven patients were recruited for the pilot study; three underwent breast reductions, two mastectomies, and two breast reconstructions.

VEGF Recovery from Drain Fluid. Drain fluid from day 1 and day 2 was spiked with a known amount of VEGF made up from reconstituted standards. The samples were then subjected to serial doubling dilutions with calibrator diluent RD6U (validated for serum and plasma analysis) in the same manner as the standards and analyzed for VEGF content.

VEGF recovery was 97%, and samples were diluted in parallel to the standard curve (data not shown).

Fresh versus Overnight Wound Fluid Collection.

Drain fluid was collected at the same time each day, from postoperative day 1 onwards. Using standard aseptic techniques the drain bag was detached from the tubing connection and replaced with a new bottle. After 10 min of collection the drain bottle was again exchanged. Both the 24-h overnight and the fresh 10-min samples were aliquoted into plain serum tubes and placed in ice for transfer to the laboratory. Drain samples were centrifuged at $3000 \times g$ for 5 min at 4°C . Samples were aliquoted in 250 μl aliquots for subsequent analysis and stored at -70°C . This was repeated daily until the drain was removed.

From the pilot study there was no significant difference ($P = 0.6$) between the fresh (mean $1114.6 \pm \text{SD } 755.1$ pg/ml) and 24-h collection (mean $940.7 \pm \text{SD } 898.2$ pg/ml).

To assess potential sample degradation, stored 10-min samples from study A ($n = 4$) were defrosted and allowed to stand at room temperature for 24 h, and the sample was then reanalyzed for VEGF content. A single random overnight drain was also sampled ($t = 0$) and then left at room temperature and sampled at $t = 24$ and $t = 48$ h; samples were analyzed fresh and after a single freeze-thaw cycle. There was no significant difference ($P = 0.6$) between the paired samples.

The pilot study indicated that 24-h drain collection is a valid method to assess VEGF production.

Main Cohort

An additional cohort of 52 patients was recruited, which consisted of 20 patients undergoing breast cancer surgery (10 mastectomy and 10 wide local excision), 18 patients undergoing breast reduction surgery, and 14 patients undergoing delayed breast reconstruction for breast cancer surgery. The group ranged from 17–86 (median 49) years of age, median time between primary breast cancer surgery and delayed breast reconstruction was 22 months, 319 blood samples were collected perioperatively, and a total of 300 wound fluid samples were collected between postoperative days 0 and 13. Most of the wound fluid samples collected beyond 4 days were from the cancer or reconstructive groups, because of the tendency to remove drains earlier in the reduction group. No wounds were complicated by hematoma formation, infection, or flap necrosis. All of the blood samples were collected at room temperature as above and centrifuged at 22°C .

Results

VEGF Levels in Surgical Wound Fluid. The temporal profile of VEGF levels in wound fluid samples in comparison to corresponding serum values ($n = 300$) is shown in Fig. 1. On the first postoperative day wound VEGF was 314% the corresponding serum value (mean 1052 ± 750 SD versus mean 330 ± 249 SD pg/ml; $P = 7 \times 10^{-9}$) and rose to a peak by day 2 of 927% (mean 2590 ± 1742 SD versus mean 299 ± 253 SD pg/ml; max, 7700 pg/ml; $P = 3 \times 10^{-12}$). A plateau followed of a 547% relative increase (mean 2134 ± 1326 SD versus mean 399 ± 332 SD pg/ml; $P < 0.001$), and then there was a smaller peak of 671% on day 8 (mean 2904 ± 1310 SD versus mean 433 ± 179 SD pg/ml; $P < 0.005$). The trend did not change when serum and wound fluids were corrected for total protein content, and a significant rise on day 2 persisted (data not shown).

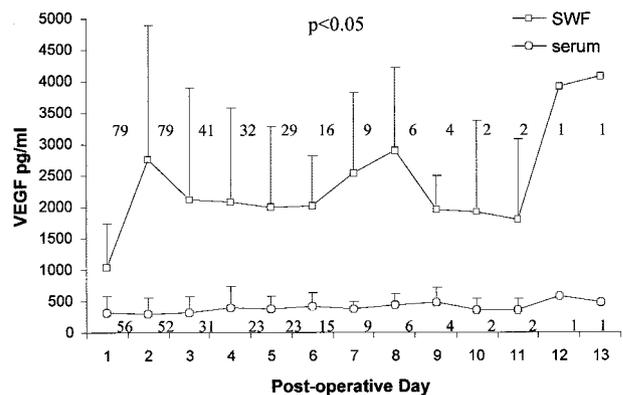


Fig. 1 Graph to show mean VEGF levels from all patients in surgical drain fluid and serum; bars, \pm SD; number of samples at each time point annotated (all samples were analyzed in duplicate and the average given).

Wound Fluid VEGF Response versus Patient Category.

An ordered pattern of response was evident between subgroups: cancer < reconstruction < reduction subgroups. This reached statistical significance when the reduction subgroup was compared with either the cancer subgroup ($P = 0.03$ day 2 and day 3) or the pooled cancer and reconstructive subgroups ($P = 0.04$ day 2 and day 3) but not the reconstructive group alone ($P > 0.05$; Fig. 2).

The day-2 peak in VEGF release is present in both of the subgroups without a local tumor burden (Fig. 2) but is different in the cancer group. The cancer group demonstrates the initial day-2 increase and then a pattern of steady expression of VEGF levels over time with a much lower response at the day-2 time point than in the other groups ($P < 0.05$; Fig. 2). The extent of the wound differs in the primary cancer patients between those with mastectomy and those with wide local excision, but there was no significant difference between their days 1, 2, or 3 VEGF wound fluid concentrations, both categories showing an increase on day 2. However, the levels were higher on days 2 and 3 in the former [2187 pg/ml (± 1288 SD; $n = 10$) day 2; 1944 pg/ml (± 1267 SD; $n = 10$) day 3 versus 1692 pg/ml (± 1308 SD; $n = 8$) day 2; 1410 pg/ml (± 1325 SD; $n = 7$) day 3], and these were still lower than the reduction or reconstruction cases.

VEGF Response and Age. Despite a statistically significant age difference between each group ($P = 0.01$), with cancer group older (mean 60.95 years ± 11.7 SD years) than reconstruction group (mean 50.46 years ± 9.3 SD years) older than reduction group (mean 35.2 years ± 11.7 SD years), there was no overall relationship between age and day-2 wound fluid VEGF production ($r = -0.20$; $P = 0.16$). However, when comparisons were based on a similar age range (40–56 years) the difference between the higher VEGF response in the normal controls and the muted response in the cancer subgroup was statistically significant ($P = 0.03$).

VEGF Response and Platelet Count. The platelet count showed an initial drop maximal on day 1 (preop mean 288 versus day 1 mean 235×10^9 /liter; $P < 0.001$) and then a gradual increase in numbers to a peak between day 10–13 (Fig.

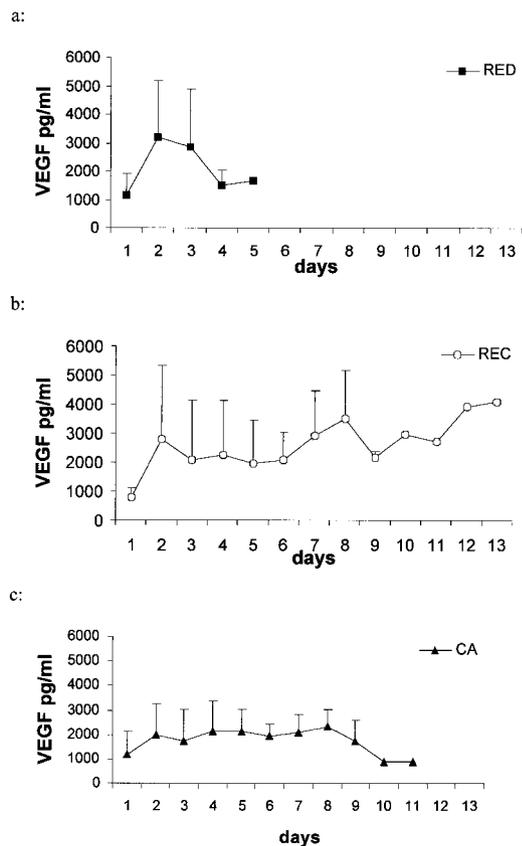


Fig. 2 Graph to show subgroup variation in mean surgical drain fluid VEGF; bars, \pm SD.

3a) returning to preoperative values over time. This trend was reproduced if the total circulating platelet volume was calculated (platelet count \times mean platelet volume: mean preop 2.524 versus day 1 1.761 ml; $P < 0.001$). Mean platelet volume dropped over time, and $\text{VEGF}^{\text{PLAT}}$ varied inversely with platelet count (Fig. 3, b and c). There was no direct correlation between platelet counts and wound fluid VEGF.

Previous kinetic studies have shown significant platelet accumulation in wounds soon after injury (18). VEGFs available from the platelets lost to the wound may be approximated with knowledge of the total circulating PD [PD \times blood volume (4.2l): estimated as 7% of normal body weight taking an average of 60 kg for a female] and the VEGF content per platelet ($\text{VEGF}^{\text{PLAT}}$) as shown below:

$$\text{PC drop} = \text{PC (preop-day 1)} = 52.64 \times 10^9/\text{l platelets}$$

$$\text{Total circulating PD} = 52.64 \times 4.2 = 221.07 \times 10^9 \text{ platelets}$$

$$\text{Preop } \text{VEGF}^{\text{PLAT}}$$

$$= \frac{\text{average (serum VEGF} - \text{plasma VEGF}^*) 1000}{\text{average platelet count}}$$

$$= \frac{(365.3 - 60.88) 1000}{288} = 1059 \text{ per } 10^9 \text{ platelets/l}$$

Platelet VEGF available to the wound = $221.07 \times 1059 = 234,000 \text{ pg}$ (234 μg) in total (*plasma value adjusted for hematocrit and dilution effect of the anticoagulant).

This could account for part of the early peak in VEGF up to 7800 pg/ml, if it was contained in a volume of 30 ml.

Plasma VEGF. Overall there is a significant change in plasma VEGF levels compared with preoperative values, with time with an initial increase over the first 2 days [preop < day 1: $P < 0.001$, preop < day 2: $P = 0.01$; Fig. 4a].

Significantly lower plasma values occur in cancer patients compared with reduction controls at day 1 (cancer versus reduction group: $P < 0.001$; and pooled cancer and reconstruction group results versus reduction normal controls: $P < 0.03$; Fig. 4b). There was no correlation of plasma VEGF with wound fluid VEGF.

Serum VEGF. Overall there was a significant change in serum VEGF levels compared with preoperative values with time with an initial drop over the first 3 days (preop versus day 1: $P = 0.03$; preop versus day 2: $P < 0.001$; preop versus day 3: $P = 0.006$; Fig. 5); thereafter levels recovered. There was no direct correlation between serum VEGF and wound fluid VEGF.

Protein Levels. Whereas serum protein remained relatively constant (mean 61.7 g/dl \pm 4.7 SD) wound protein levels dropped from a serum equivalent to a new significantly lower baseline (mean 34.9 g/dl \pm 4.5 SD; $P < 0.0001$).

Discussion

Analysis of the local wound response in our study has shown that VEGF levels in the wound environment are much higher than the serum equivalent from as early as the first postoperative day. They then peak at day 2 and remain at a higher level thereafter for several days.

This fits well with previous studies of wound vascular permeability, which in an animal study of healing split thickness wounds was maximal at 2–3 days and persisted as late as day 7 postinjury (9, 19). Furthermore, a rat aorta *in vitro* model of angiogenesis suggests that the vasoformative capacity of the rat aorta is restricted to the first days after dissection (19). The quantitative data are not available over the immediate postoperative period in their study (day 1 then day 5); however, the immunoblot data available at days 1, 3, and 5 suggest high VEGF levels over this period reducing thereafter, in keeping with our results. A porcine model (20) and human study (4) suggest a graduated increase in VEGF production with time without the day 2 peak demonstrated in our results. These studies may not be representative, as the animal model involved the creation of a wound compartment with silicone sheeting and may mimic a chronic as opposed to an acute wound response because of the presence of foreign material, and the latter had smaller numbers in the study group with a predominance of cancer patients, which may not be representative of the “normal” healing process.

There are several potential sources for the wound fluid VEGF that may be from sequestered platelets or locally released and produced. Platelet counts dropped immediately after injury thereafter more than recovering their preoperative values as would be expected in a wound response. Similarly, serum values show a significant reduction in the first 3 days compared with

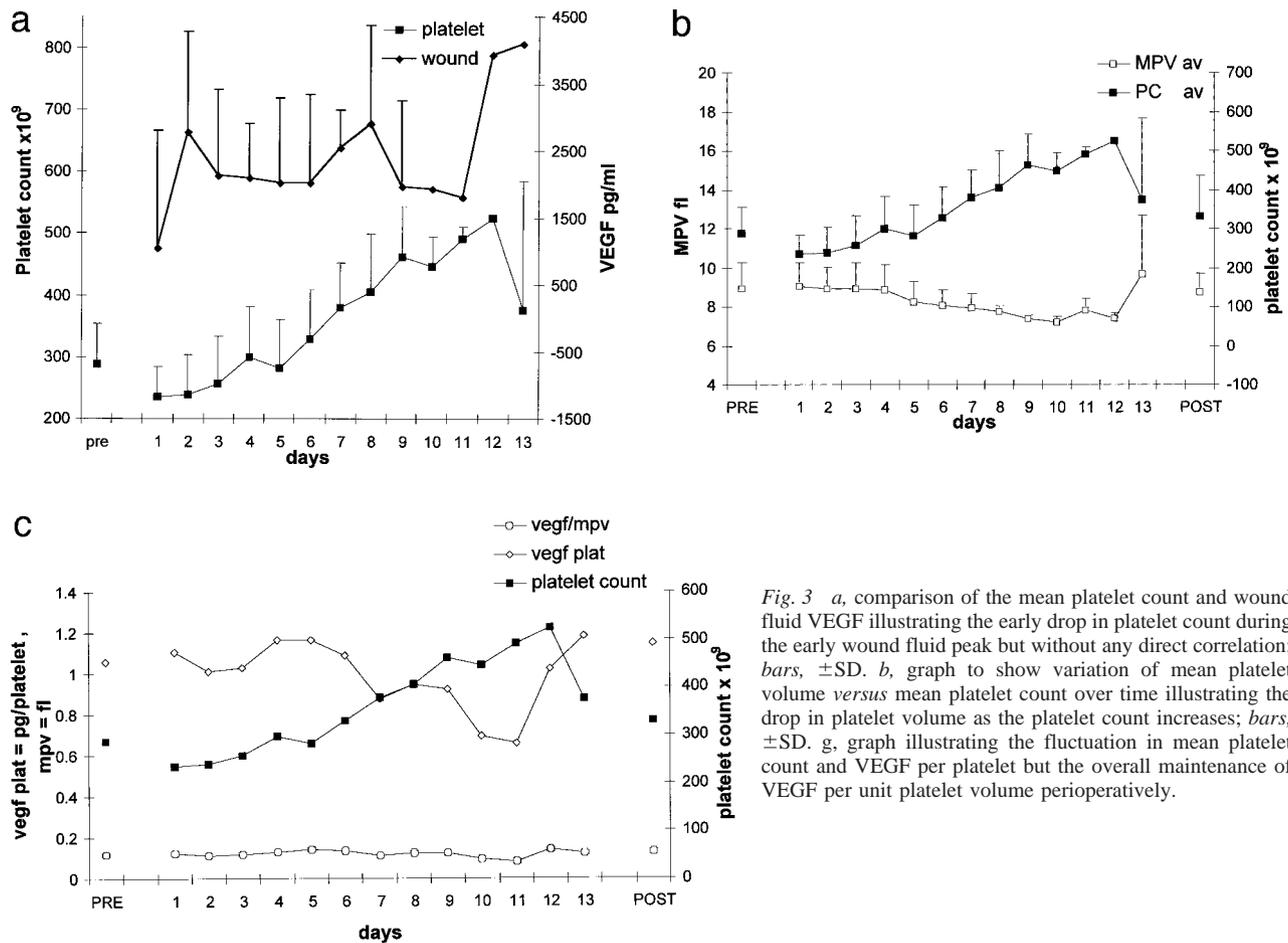


Fig. 3 a, comparison of the mean platelet count and wound fluid VEGF illustrating the early drop in platelet count during the early wound fluid peak but without any direct correlation; bars, \pm SD. b, graph to show variation of mean platelet volume versus mean platelet count over time illustrating the drop in platelet volume as the platelet count increases; bars, \pm SD. c, graph illustrating the fluctuation in mean platelet count and VEGF per platelet but the overall maintenance of VEGF per unit platelet volume perioperatively.

preoperative values. The calculations for the serum VEGF drop and the drop in platelet count and, hence, potential VEGF delivery to the wound show some similarities. Platelets are the main source of serum VEGF, and there is reasonable concordance between the two calculations in terms of the amount of VEGF sequestered in the wound. Slichter *et al.* (18) estimated an increased platelet accumulation of up to a maximum of 161% of normal circulating counts by day 2 postoperatively and a reduction in platelet survival from 5 days to 24–48 h. Potential platelet VEGF delivery as a consequence of these measured responses could account at least in part for the wound VEGF levels.

There was no direct or inverse correlation between serum VEGF and wound fluid VEGF. This may reflect local VEGF production or difficulties in assessing platelet redistribution and turnover.

Analysis of the systemic impact of surgery on plasma VEGF showed an initial small but significant increase in plasma VEGF levels. However, this was not maintained despite the extremely high local concentration in the wound. The brief increase in plasma VEGF at a time of massive local wound VEGF production suggests local mechanisms for preventing egress. This may be binding of VEGF to receptors and extracellular matrix, as well as internalization of VEGF. Evidence for

platelet scavenging has already been suggested in the literature (12, 14, 21). In our study despite an increasing platelet count with a lower mean platelet volume, the VEGF concentration per platelet was maintained over the first 5 days after injury. Although platelet scavenging may counteract any overspill of VEGF into the circulation there was no increase in platelet VEGF concentration, arguing that the wound VEGF does not overspill significantly after the first 2 days.

The surgical wound itself is a unique extravascular compartment with increased vascular permeability and a high surface area:volume ratio. That reabsorption of wound fluid constituents occurs is evident from antibiotic studies (22–24). If reabsorption occurs freely from the surgical wound site changes in local VEGF concentrations should be reflected in the circulation, *i.e.*, the plasma levels. Our study shows that there is little protein egress across a new wound barrier after the first few days, which provides an environment with extremely high angiogenic factors.

Subgroup analysis in our study establishes that there is a hitherto unrecognized difference in the VEGF response of patients with and without local tumor burden at the time of surgery. VEGF production in the cancer subgroup follows a different temporal pattern with an increase by day 2, with a continuing steady increase thereafter. Age differences between

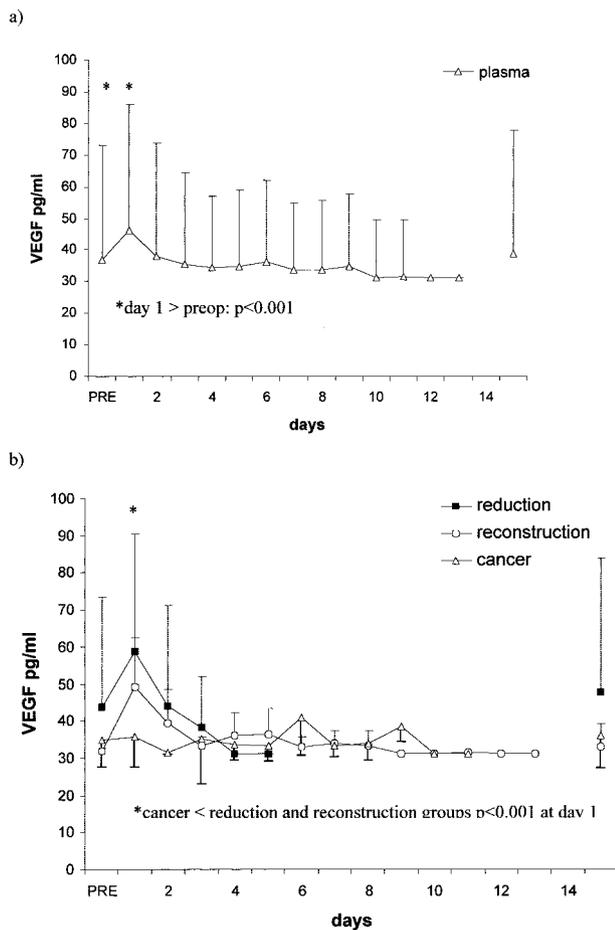


Fig. 4 Graph to show change in mean plasma VEGF (a) overall and (b) between subgroups over time; bars, \pm SD.

groups could not explain this; not only was there was no overall correlation between age and VEGF response, but when age was eliminated as a factor the significance of the response in the cancer subgroup persisted. Mastectomy creates a wound with a larger surface area than wide local excision, but there was no significant difference overall between the values but the same temporal pattern.

This effect may mark an interaction between residual tumor-derived local inhibitors resulting in an initially depressed normal stromal angiogenic response that recovers over time. This would be in keeping with the evidence that tumor cells secrete factors that provide negative "feedback" regulation and serve to suppress vascular growth, restraining the growth of secondary tumors or metastases (25–27). It seems reasonable to suggest that tumors may have a similar regional effect as well. Surgical clearance of cancer involves regional extirpation, and residual tissues may still be under the influence of tumor-derived inhibitors delaying the normal angiogenic wound response. This effect is seen not just locally in the wound bed but is reflected in the plasma response, a measure of the "in vivo" circulating levels, with a significantly lower response in the cancer subgroup. These results also suggest measuring plasma VEGF, as a marker for tumor VEGF is likely to be misleading,

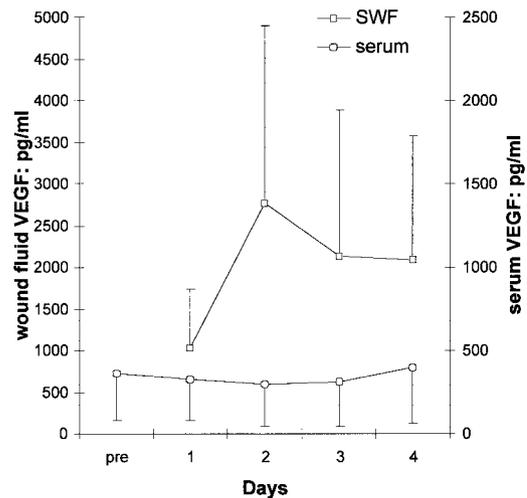


Fig. 5 Mean VEGF levels in surgical wound fluid and serum over the first 3 days; bars, \pm SD.

because tissue concentrations can be so much higher and do not correlate with plasma VEGF.

The mechanism underlying these observations requires additional investigation and may relate to the half-life of angiogenic stimulators compared with inhibitors, to a local effect on the stroma when the angiogenic drive from the tumor is removed or because of impaired influx of blood and platelet release at the time of injury. This muted response in cancer patients may represent an opportunity to complete surgical treatment while minimizing stimulation of metastatic disease, a biological argument in favor of immediate reconstruction after, for example, breast cancer surgery.

Experimental evidence suggests that a growth factor-rich environment enables the survival of cancer cells left in an area of cancer extirpation or in the circulation (28–34). However, as wounds age the surgical site becomes less favorable to tumor implantation, and when healing is complete injected tumor cells do not localize to the surgical site (35). Thus, local recurrence found in conjunction with widespread metastatic disease is likely to have been established by perioperative seeding rather than as a late phenomenon. Furthermore, a growth factor-stimulated microenvironment affects growth of established residual tumor foci *in vivo* and cell lines *in vitro* (27, 36–45).

Antiangiogenic therapy is currently undergoing clinical trials, and in the future perioperative systemic therapy or local therapy may include use of such therapy. However, our findings indicate that very high local concentrations may need to be antagonized and also show how poor plasma levels of VEGF may be in reflecting tissue levels.

Quantification of this *in vivo* biological response should facilitate the design of wound healing experiments to more closely represent the "human" response (44–49), and drainage systems may offer an opportunity to manipulate the early wound environment and reduce local cancer recurrence rates in the future.

Acknowledgments

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