Abstract

Purpose: Platelet concentrates are important for support of patients with malignancies requiring myelotoxic chemotherapy. During storage, 10% to 15% of platelets may become activated resulting in the release of α-granules, which contain growth factors. We hypothesize that, during storage, growth factors accumulate in the plasma, specifically platelet-derived growth factor, vascular endothelial growth factor (VEGF), transforming growth factor-β, and fibroblast growth factor-2, which may adversely affect cancer patients.

Experimental Design: The concentrations of growth factors were measured by ELISA from the plasma of apheresis platelets serially throughout storage (days 1, 3, 5, and 7) and compared with concentrations in fresh plasma from healthy blood donors. Washing was evaluated as a method of growth factor removal, and an in vitro model of platelet transfusion in a patient receiving Bevacizumab (Avastin) using immunoprecipitation was employed to determine if Bevacizumab would be bound by the VEGF in apheresis platelets.

Results: VEGF, platelet-derived growth factor, and transforming growth factor-β were increased on day 1 versus fresh plasma and throughout storage reaching a relative maximum at outdate (P < 0.01, day 5 or 7). Fibroblast growth factor-2 concentrations were significantly increased on day 7 alone versus day 1 or to fresh plasma (P < 0.01). Washing removed 41 ± 11% to 56 ± 2% of the growth factors. Bevacizumab effectively bound the VEGF from apheresis platelets, with significant amounts of VEGF remaining in the supernatant.

Conclusions: Significant amounts of growth factors are present in apheresis platelets due to the isolation procedures, and these concentrations increase over storage, which may be partially removed by washing. In addition, apheresis platelet transfusion could affect cancer treatment by binding monoclonal antibodies directed against growth factors of tumor origin.

Thrombocytopenia is a common complication of many chemotherapeutic regimens to treat malignancies; thus, platelet transfusions are a mainstay in supportive care for cancer patients. Most blood centers provide platelet concentrates collected by apheresis techniques, and these units are functionally pre-storage leukoreduced (containing <10⁶ leukocytes per unit; ref. 1). Platelet concentrates are stored at 20°C to 24°C with constant gentle agitation for as long as 5 days, although newer data suggest that storage may be extended to 7 days based on in vivo platelet viability with minimal increased risk of bacterial contamination (1–4). During storage, 10% to 15% of platelets become activated, and substances within the α-granules, including several growth factors, are released extracellularly (3, 6). Several of these proteins, especially vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF), have been associated with tumor growth (7–9).

Allogeneic transfusions cause immunomodulation in the host documented by the association of transfusions with (a) acceptance of renal allografts, (b) ability of women who suffer multiple spontaneous abortions to successfully give birth to normal progeny, and (c) a decline in immune function in HIV-infected patients (10–14). Moreover, transfusion-related immunomodulation is associated with tumor growth, and many animal models have shown such a causal relationship, although causality has been more difficult to prove clinically (15–20). Recently, transfusion of adults (<50 years old) with acute myelogenous leukemia with washed platelets (type-specific) and packed RBC showed a significant survival advantage compared with patients who received unwashed units (21). Because human platelets contain angiogenic and oncogenic growth factors and may become activated during storage, we hypothesize that, during routine storage, growth factors accumulate in the plasma fraction of platelet concentrates, which have the capacity to adversely affect patients with malignancies.

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Materials and Methods

Materials. Unless otherwise specified, all reagents were purchased from Sigma. Sterile couplers, sterile saline for injection (USP), and sterile water for injection (USP) were purchased from Baxter Healthcare. ELISA for VEGF, transforming growth factor-β (TGF-β), PDGF-AB, and fibroblast growth factor-2 (FGF-2) and an antibody against VEGF were procured from R&D Systems. All buffers were made from injection grade (USP) solutions in sterile water obtained from the following manufacturers: 10% CaCl2, Fujisawa, 23.4% NaCl, 20 μg/mL of KCl, and 50% MgSO4 (American Regent Laboratories) and sodium phosphates and 50% dextrose (Abbott Laboratories). Test tubes, Nunc microplates, and solvents for extraction and separation of liquids were purchased from Fischer Scientific. Nitrocellulose membranes were obtained from Life Sciences, and additional materials for SDS-PAGE were purchased from Bio-Rad. Bevacizumab (Avastin, Genentech BioOncology) was purchased directly from the commercial supplier, and an additional grade (USP) solutions in sterile water obtained from the following suppliers: Pfizer, and 50% MgSO4 (American Regent Laboratories) and sodium phosphates and 50% dextrose (Abbott Laboratories). Test tubes, Nunc microplates, and solvents for extraction and separation of liquids were purchased from Fischer Scientific. Nitrocellulose membranes were obtained from Life Sciences, and additional materials for SDS-PAGE were purchased from Bio-Rad. Bevacizumab (Avastin, Genentech BioOncology) was purchased directly from the commercial supplier, and an additional commercial antibody to VEGF was purchased from Abcam. Enhanced chemiluminescence reagents were bought from Amersham.

Sample collection. Ten regular, volunteer blood donors were recruited anonymously without knowledge of the subject of this project and plasma or apheresis platelet concentrates were drawn only after informed consent was obtained that the units would be used for research under a protocol approved by the Combined Institutional Review Board at the University of Colorado School of Medicine. Platelet concentrates were collected from these 10 healthy donors using a Cobe Trima apparatus, and all 10 units were significantly leukoreduced with cell counts ≤ 5 x 10⁸ units. Samples were taken serially on days 1, 3, 5, and 7 from five of these apheresis platelets via sterile couplers and centrifuged at 5,000 x g for 7 min to obtain acellular plasma followed by a second spin at 5 min at 12,500 x g to remove any acellular debris (1, 22). The plasma was aliquoted and stored at -70 °C. Importantly, no plasma samples were employed twice for any assays. The remaining apheresis platelets were used to determine if washing eliminated growth factors on day 7 of storage. In these experiments, a sample was obtained before washing, and the plasma was isolated. The apheresis platelets were washed using a Cobe 2991 Cell Processor per published methodology and the plasma fraction (supernatant) was isolated, aliquoted, and frozen using centrifugation at g-forces recommended for plasma isolation per American Association of Blood Banks criteria with a final spin at 12,500 x g to remove acellular debris (1, 23). Twelve fresh plasma controls were obtained from the platelet donors (3) and from 9 healthy subjects via venipuncture with 18-gauge needles, after obtaining informed consent, and the plasma was isolated via centrifugation at g-forces per American Association of Blood Banks criteria for the isolation of plasma, aliquoted, and stored at -70 °C (1). These fresh plasma samples were not obtained via apheresis equipment in order to better assess the effect of the apheresis isolation procedure.

Measurement of growth factors. The concentrations of VEGF, TGF-β, FGF-2, and PDGF were measured in duplicate via ELISA throughout platelet storage on days 1, 3, 5, and 7 and compared with fresh plasma controls, including the plasma fraction from washed and unmodified day 7 apheresis platelets. Importantly, all growth factors were measured in triplicate, which was repeated employing two different ELISA kits to ensure accuracy.

Western blotting and immunoprecipitation of VEGF. Plasma proteins from washed and unmodified apheresis platelets were separated by SDS-PAGE, transferred to nitrocellulose, and probed with Bevacizumab and immunoreactivity was visualized with a goat anti-human horse-radish peroxidase–conjugated secondary antibody and enhanced chemiluminescence (24). To ensure that the immunoreactivity visualized was VEGF, the membranes were stripped and probed with a commercial polyclonal antibody to VEGF followed by visualization with a mouse anti-rabbit horseradish peroxidase–conjugated secondary antibody and enhanced chemiluminescence.

To mimic platelet transfusion in a 70 kg patient with a plasma volume of ~3 L receiving Bevacizumab at a dosage of 5 mg/kg (1.17 mg) Bevacizumab was conjugated to agarose beads to ~1 mL plasma from such a patient. Ten percent apheresis platelet plasma was used because a 70 kg adult would have ~3 L plasma volume and these apheresis platelet units had an average volume of 300 mL. To determine the percentage of Bevacizumab bound to the agarose beads, the amount of protein was measured via a modified Lowry protein assay (Pierce ref. 24). Immunoprecipitations of VEGF were done in a total volume of 1 mL of 5% defatted and globulin-free human albumin control or human albumin control with 10% plasma from day 5 or 7 washed or unmodified apheresis platelet plasma (24). Immunoprecipitation of the plasma with Bevacizumab was then completed by standard techniques (24). Following dissolution of the VEGF from the beads, the immunoreactivity, recognized by the commercial antibody, would approximate the amount of VEGF bound by Bevacizumab with the amount of VEGF in the supernatant would represent the amount of VEGF remaining in the plasma. Additional controls included an equal

Table 1. Measurement of VEGF, PDGF, TGF-β1 and FGF-2 in platelet concentrates

(A) Concentration of growth factors during platelet storage

<table>
<thead>
<tr>
<th>Day</th>
<th>VEGF (pg/mL)</th>
<th>PDGF (ng/mL)</th>
<th>TGF-β1 (ng/mL)</th>
<th>FGF-2 (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh</td>
<td>8.9 ± 1.4</td>
<td>0.10 ± 0.01</td>
<td>0.2 ± 0.01</td>
<td>4.2 ± 0.4</td>
</tr>
<tr>
<td>Day 1</td>
<td>173.5 ± 2.6</td>
<td>7.0 ± 1.2</td>
<td>8.0 ± 0.9</td>
<td>7.2 ± 1.0</td>
</tr>
<tr>
<td>Day 3</td>
<td>204.1 ± 25.6</td>
<td>15.0 ± 1.9</td>
<td>15.4 ± 0.9</td>
<td>5.9 ± 1.3</td>
</tr>
<tr>
<td>Day 5</td>
<td>304.3 ± 67.1</td>
<td>24.8 ± 2.7</td>
<td>23.1 ± 1.2</td>
<td>7.9 ± 1.6</td>
</tr>
<tr>
<td>Day 7</td>
<td>405.6 ± 47.9</td>
<td>36.6 ± 4.0</td>
<td>30.2 ± 2.0</td>
<td>14.0 ± 0.9</td>
</tr>
</tbody>
</table>

(B) Washing decreases growth factor concentration in plasma from day 7 apheresis platelets

<table>
<thead>
<tr>
<th>Day 7 apheresis platelets</th>
<th>VEGF (pg/mL)</th>
<th>PDGF (ng/mL)</th>
<th>TGF-β1 (ng/mL)</th>
<th>FGF-2 (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unmodified</td>
<td>419.2 ± 65.8</td>
<td>25.7 ± 4.3</td>
<td>35.5 ± 7.0</td>
<td>14.7 ± 1.1</td>
</tr>
<tr>
<td>Washed</td>
<td>184.2 ± 58.8</td>
<td>14.7 ± 2.6</td>
<td>18.5 ± 4.7</td>
<td>7.2 ± 1.7</td>
</tr>
</tbody>
</table>

NOTE: (A) Data are expressed as the mean ± SE (pg/mL or ng/mL) of the measured growth factors from 5 units of apheresis platelets measured serially over the storage interval (7 days). (B) Data are expressed as the mean ± SE (pg/mL or ng/mL) of the measured growth factors from 5 units of apheresis platelets that were unmodified or washed on day 7 of storage.

1* P < 0.01, compared with control plasma drawn from 12 healthy donors, which includes 2 of the platelet donors before apheresis.

2* P < 0.01, compared with the prior day to storage (e.g., day 3 versus 1 or day 5 versus 3, etc.).
amount of unbound Bevacizumab to the nitrocellulose and 10% fresh plasma in the place of apheresis platelet plasma.

**Statistical analyses.** Logistic regressions were done on the growth factor standards and used for measurements of growth factors from the plasma samples. Statistical differences were determined using paired analyses of variance followed by either the Bonferroni or Newman-Keuls post hoc test for multiple comparisons depending on the equality of variance. Statistical significance was defined as \( P < 0.05 \) among the controls and different storage and treatment groups, and all data are expressed as the mean ± SE.

**Results**

**Measurement of TGF-β, PDGF-AB, VEGF, and FGF-2.** Compared with concentrations from plasma obtained via venipuncture from both healthy controls and the platelet donors, there were statistically significant increases in TGF-β, PDGF-AB, and VEGF in the day 1 samples, and the levels of these growth factors significantly increased throughout the storage interval (Table 1A, \( P < 0.01 \)). Concentrations of each of these growth factors in apheresis platelet plasma from days 3, 5, and 7 were different from both day 1 as well as the fresh plasma samples and the concentrations from each subsequent day of storage: day 7 versus 5, day 5 versus 3, and day 3 versus day 1. (Table 1A, \( P < 0.01 \)). Conversely, FGF-2 concentrations did not significantly increase until day 7 of storage compared with fresh plasma and to all other storage times (Table 1A, \( P < 0.01 \)). Importantly, all apheresis platelets had WBC counts \( < 5 \times 10^6 \) leukocytes per unit as documented by flow cytometry (results not shown).

Washing of day 7 apheresis platelet concentrates caused significant diminution for each growth factor measured by \( 41 ± 11\% \) to \( 56 ± 2\% \) (Table 1B, \( P < 0.01 \)) with a loss of 10% to 14% of platelets as determined by pre- and post-washing counts and estimation of the total volume. Washing did not eliminate these growth factors from the plasma fraction but did reduce the concentrations to the day 3 levels for each growth factor.

**In vitro modeling of the effects of apheresis platelet transfusion on a patient with recent Bevacizumab treatment.** Immunoblotting with Bevacizumab as the primary antibody showed significant immunoreactivity to the VEGF present in the plasma fraction of day 7 apheresis platelets (Fig. 1A). In our in vitro model of platelet transfusion on a 70 kg patient, Bevacizumab was able to immunoprecipitate VEGF from the unmodified day 7 apheresis platelet plasma and this immunoreactivity was remarkably decreased when washed components were used (Fig. 1B). Moreover, the supernatants contained a large band of VEGF immunoreactivity, identified by a commercial antibody to VEGF, which was not precipitated by Bevacizumab and was not present in the fresh plasma controls or in the washed day 7 apheresis platelets.

**Discussion**

Apheresis platelets contain significant amounts of free PDGF, TGF-β, and VEGF in the plasma on day 1 of storage, presumably as a function of the apheresis techniques employed for isolation of a concentrated product, compared with fresh plasma isolated from whole blood drawn directly into a heparinized tube from healthy controls, which included the platelet donors. These data should not be compared with serum levels of these growth factors, which would be released during the cellular (platelet) phase of clotting resulting in much higher levels of these factors compared with plasma (25–30). Importantly, healthy donors do not have significantly increased amounts of VEGF, TGF-β, PDGF, or FGF-2 in their plasma, and the presented data support this previous work (25, 30). These concentrations increased progressively during the storage interval, showing significant accumulation in the plasma fraction compared with the previous day, and reached a relative maximum at component outdate (day 5 or 7). In contrast, FGF-2 only showed significantly increased levels on day 7 of storage compared with fresh plasma or the plasma from day 1 apheresis platelets. These results are curious because FGF-2 is contained within the α-granules; however, FGF-2 is a heparin-binding protein known to bind to activated platelets in vitro (31). Given the fact that a percentage of the platelets become activated during storage, such binding properties could readily decrease the amount of free FGF-2 in the plasma fraction of stored platelet concentrates, when the platelets are separated from the plasma by centrifugation (31). Little data are available with regard to accumulation of growth factors in apheresis platelets; however, platelets may be activated in vivo via the binding of a platelet agonist (e.g., thrombin, ADP, or collagen) to receptors on the platelet surface and this activation causes exocytosis of the α-granule contents, including VEGF, PDGF, FGF-2, and TGF-β, as well as several others, including...
insulin-like growth factor and epidermal growth factor (32–35). Platelets may also be activated via contact with the artificial membranes of the tubing of hemodialysis machines, tubing that is identical to that of the apheresis equipment (36).

Washing of apheresis platelet units removed significant amounts of the accumulated growth factors but did not totally remove them. Such procedures are labor intensive, may cause platelet activation, and incur losses significant platelet losses, up to 30% (37–42). However, these data were consistent with the observation that these growth factors were released into the plasma fraction and hence their removal by washing, and such procedures could decrease the amount of growth factors in the platelet concentrate.

Growth factors and their receptors are at the center of many new targeted, therapeutic agents for various malignancies. For example, PDGF is contained within the α-granules of platelets and may exist as the A form, the B form, or the AB “dimer” (7, 36, 43). PDGF-AB contributes to the tumorigenic potential of various hematologic and solid malignancies including Philadelphia chromosome-positive (Ph1+) leukemias (7, 43–45). Treatment of patients with imatinib mesylate (Gleevec), a low-molecular-weight inhibitor of the PDGF receptor, is also postulated to inhibit the abl tyrosine kinases as well as other receptor tyrosine kinases, has been used in combination with traditional chemotherapy and has resulted in a better survival of patients with Ph1+ acute lymphoblastic leukemia (46, 47). This increased PDGF and PDGF receptor expression is seen in a variety of cancers, including Ewing’s sarcoma, gastric carcinoma, and desmoplastic small round blue cell tumor (45, 48, 49). Imatinib can inhibit growth of these cell lines in vitro as well as using xenograft models, showing the effect of PDGF receptor blockade (48–50). In addition, VEGF, which may exist in six forms (A-E and placental growth factor), exists predominantly in the A form in human blood, is contained within the platelet α-granule, and is expressed in 50% of all colorectal tumors as well as in liver, lung, breast, ovary, and several other malignancies (8, 9, 44, 51–54). Increased serum levels correlate with tumor growth rate in Ewing’s sarcoma and colorectal cancer as well as metastatic potential and poor clinical prognosis, and similar data have been shown in patients with Hodgkin’s disease and women with breast cancer (8, 9, 30, 49, 51, 53, 55, 56).

TGF-β contributes to metastasis by regulating cell adhesion and motility allowing for solid tumor invasion of surrounding tissues and has been implicated in several malignancies because the gene appears to be mutated in colon and gastric cancers as well as in gliomas (57–61). TGF-β has also been implicated in the genesis of graft versus host disease following bone marrow transplantation (62). Lastly, FGF-2 levels in healthy humans are negligible and its appearance in the circulation often marks the presence of a malignancy, especially in the gastrointestinal tract. The utility of following FGF-2 levels is under investigation (52, 63–66). Because of its role in angiogenesis, FGF-2 may play a role in metastatic disease, much like VEGF, but further work is needed to understand its possible role in the growth and spread of solid tumors (52, 63–67).

Bevacizumab, a humanized, monoclonal antibody to VEGF, is part of standard treatment protocols at doses varying 5 to 15 mg/kg for several malignancies, including primary malignancies of the colon (68–72). It is also being used in multiple experimental protocols for cancer treatment, including Ewing's sarcoma (44, 49). The patients on these studies are receiving platelet transfusions, if necessary, and the number of platelet transfusions received is not being accounted for in determining treatment effect. Therefore, an in vitro approximation of platelet transfusion was designed, assuming (a) the patient weighed 70 kg, (b) the plasma volume was ~ 3 L, and (c) this individual had recently received 5 mg/kg Bevacizumab (lowest dose being used clinically). Other assumptions include that all of the Bevacizumab infused would reside in the plasma and the apheresis platelet concentrate would have a volume of 300 mL. The data from this experiment showed that all the available Bevacizumab would be bound to the transfused VEGF present in the stored apheresis platelet concentrates if a platelet transfusion occurred temporally related to Bevacizumab infusion. Moreover, there was still significant VEGF immunoreactivity present in the plasma. Therefore, a platelet transfusion temporally related to Bevacizumab may obviate the clinical utility of this antibody at this dose and could affect the treatment efficacy at higher doses. However, these in vitro experiments do have their limitations: (a) they assume that all of the Bevacizumab would reside in the plasma compartment and (b) that conjugation to agarose beads does not mask the ability of this humanized monoclonal antibody to bind VEGF. Larger patients would have a larger plasma volume, but the amount of VEGF infused would still bind most of the Bevacizumab if one assumes that there is a 1:1 stoichiometry of Bevacizumab IgG to VEGF molecule. Importantly, recent data have shown that platelets have the ability to take up Bevacizumab (73). These data are not surprising for platelets actively take up IgG and IgA at concentrations similar to the circulating amounts of immunoglobulins as well as other proteins, package these immunoglobulins in α-granules, and release them on thrombin stimulation (74–78). Such data are important in determining the effective volume of distribution of antibody therapy for treatment in humans as well as in animal models of disease, because all mammalian platelets may actively transport several proteins across their plasma membrane and store them in the α-granules (74–78).

In conclusion, further studies should be done to determine the clinical effect on tumor growth and treatment outcome in patients who receive significant amounts of platelet transfusions. In addition, the clinician should consider the timing of transfusion of patients with platelets who are being treated with monoclonal antibodies to VEGF because this intervention may decrease the effectiveness of this therapy. Further experimental protocols using these types of treatment should also consider the number of platelet transfusions patients receive while on these studies in determining their clinical efficacy. In addition, these data also provide in vitro evidence to support the data from a series of patients with acute myelogenous leukemia whose survival was beneficially affected when washed, type-specific products were used, versus standard transfusion, which provided a survival advantage for acute myelogenous leukemia patients less than 50 years old (21). Further work is required to determine the effects of transfused TGF-β and FGF and to determine if the exposure to increased amounts of PDGF could detrimentally affect the efficacy of imatinib mesylate in Ph1+ leukemia patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.
References


Oncogenic and Angiogenic Growth Factors Accumulate during Routine Storage of Apheresis Platelet Concentrates

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