Vascular endothelial growth factor is mainly synthesized in endothelial and various tumor cells in humans and dogs but also in platelets and leukocytes. This growth factor is one of the most potent for blood vessel formation. Thus, it is essential for embryonic development and wound healing. On the other hand, VEGF also provides circulatory access to tumor cells and facilitates metastatic spread. Human tumor patients have higher concentrations of circulating VEGF than do healthy individuals, and a similar phenomenon has also been reported for dogs.

As various cells (mostly leukocytes and platelets) disintegrate during storage of human blood and blood components, bioactive substances are released such as VEGF, but also histamine, myeloperoxidase, plasminogen activator inhibitor-1, and eosinophilic cationic protein. In addition, leukocytes produce cytokines, which accumulate extracellularly during storage. Therefore, filtration of human blood products with leukocyte filters prior to storage has many advantages (eg, decrease in the incidence of transfusion reactions, transmission of infections, inflammatory responses in recipients, and rates of tumor relapse).

The accumulation of VEGF in stored human blood products can be reduced through use of leukocyte filters. Leukocyte-reduced RBC products are preferred in human patients and particularly in those with high plasma VEGF concentrations (ie, primarily tumor patients) because additional VEGF might improve the environment for micrometastases and thus may contribute to a shorter life expectancy.

Several studies of leukoreduction in dogs have been reported. One study involved the evaluation of various methods for leukoreduction of donor plate-
lets to prevent alloimmune platelet refractoriness in a model of transfusion in dogs. Another study was conducted to determine the effect of leukoreduction filtration on canine RBC units. Recently, the in vivo effects of filtration were evaluated after administration of leukoreduced versus nonleukoreduced packed RBCs. In that study, significant increases in WBC counts, plasma fibrinogen concentration, and serum C-reactive protein concentration were detected in dogs receiving nonleukoreduced packed RBCs but not in dogs receiving leukoreduced packed RBCs. Therefore, in dogs with profound inflammatory responses due to underlying illness (eg, sepsis or immune-mediated hemolytic anemia), use of leukoreduced blood could improve the safety of transfusion treatment.

In canine medicine, no data are available on the accumulation of VEGF in stored canine blood products and the prevention of this accumulation with leukoreduction filters. Thus, the purpose of the study reported here was to evaluate the accumulation of VEGF in nonfiltered and filtered canine blood products during storage.

**Materials and Methods**

**Animals**—Since 1996, the Clinic of Small Animals of the Freie Universität Berlin has had a voluntary blood donor program. Ten client-owned healthy dogs (2 male and 8 female; median age, 6 years [range, 2 to 11 years old]; median body weight, 29.5 kg [range, 25 to 55 kg]) were included in the study. For the purpose of the study, no blood samples other than for a pretransfusion health check were collected. The donated blood was not discarded, and the blood components were administered to recipients.

All CBC and plasma biochemical results for all dogs were within reference limits, with the exception of 1 dog that had mild leukocytosis (13.9 × 10^6 leukocytes/µL). The 10 dogs were randomly assigned to 2 groups of 5 dogs each. In group 1, the whole blood donated was not filtered, and in group 2, the whole blood donated was filtered before separation into packed RBCs and FFP units.

**Procedure for blood donation**—During blood donation, approximately 10 mL of whole blood was collected per kilogram of body weight from each dog. Maximum volume was 450 mL (range, 8.2 to 10.7 mL/kg; median, 9.7 mL/kg). In 5 dogs, withdrawal of blood was performed with a collection system involving 3 bags (group 1). In the other 5 dogs, blood was withdrawn with a collection system involving an integrated leukoreduction filter between the primary and secondary bag (group 2). The first bag contained 63 mL of CPD as anticoagulant and RBC stabilizer. In situations in which < 450 mL blood was withdrawn, a corresponding volume of CPD was removed from the bag prior to blood donation via the needle belonging to the collection system.

**Blood filtration**—Filtration of whole blood was performed at room temperature (approx 22°C) immediately after blood withdrawal prior to separation of whole blood into packed RBCs and FFP units. The leukoreduction filter used was a third-generation polyester filter. This process took 3 to 5 minutes.

The whole blood that remained between the filter and the secondary bag was used for measuring the blood cell counts. The detection limits reported by the manufacturer of the hematology analyzer were 0 to 2.5 × 10^3 leukocytes/µL and 0 to 2 × 10^6 platelets/µL.

**Separation and storage**—Separation of the whole blood was performed immediately after withdrawal. The blood was centrifuged at 3,600 × g for 15 minutes at 4°C. Subsequently, the plasma was pressed via a plasma separator through the satellite line into the plasma bag. The plasma bag and the packed RBC units were sealed with an aluminum clip after adding SAGM. After labeling of the bags, packed RBC units were stored at 4°C until used. Bags of FFP were stored at −33°C.

**Obtaining and processing of samples**—To establish prestorage values, VEGF concentration was measured in plasma samples from the 10 dogs prior to blood donation and in plasma samples and in the supernatant of packed RBC samples immediately after separation of the whole blood to establish prestorage values. For this purpose, 3 mL of donor whole blood was withdrawn into tubes containing sodium citrate-theophylline-adenosine-dipyridamole. Samples from blood products containing anticoagulant were transferred into plastic tubes without additive.

To measure changes in VEGF concentrations during storage, aliquots of the 10 packed RBC units were obtained on day 0 (day of original blood collection) and days 7, 14, and 21 afterward from a small bag (sample pouch) belonging to the blood collection system. Twenty milliliters of packed RBCs (with SAGM) remaining in the blood collection bag was removed in as sterile a manner as possible via a syringe and then transferred into the sample pouch that was sealed with an aluminum clip. Thus, it was possible to obtain samples without opening the blood bag. At each of the specified measurement points, 3 mL was removed by opening the locking cap and allowing the blood to drip off into an empty tube. The supernatant was obtained from the packed RBC aliquot by centrifugation at 3,600 × g for 5 minutes. Subsequently, the supernatant was stored at −78°C until examination.

Extraction of plasma by centrifugation from blood and packed RBC samples was completed within 20 minutes after obtaining the samples. To avoid thawing and thus discarding the FFP, VEGF measurement was only performed twice (ie, after preparation but before freezing and after thawing just before administration). Two filtered FFP products were thawed and then assayed for VEGF concentration on days 26 and 42 after blood collection, and 2 nonfiltered FFP products were assayed on days 29 and 42.

**Determination of VEGF concentrations**—A commercial ELISA for measurement of human VEGF concentration in packed RBC and FFP units was used in accordance with the manufacturer’s instructions. The minimum detectable VEGF concentration was 9 pg/mL. The intra- and interassay coefficients of variation were 6.7% and 8.8% at VEGF concentrations of 53.7 and 64.5 pg/mL, respectively.
Statistical analysis—Statistical analysis was accomplished with a statistical computer application. The Shapiro-Wilk test was used to assess the distribution of the data. Study results are reported as median and range for nonnormally distributed data (VEGF concentration and leukocyte count) or as mean ± SD for normally distributed data (RBC and platelet counts). The Mann-Whitney U rank test was performed to evaluate differences in VEGF concentrations between the 2 treatment groups. Differences between the pre- and postfiltration cell counts were analyzed with the nonparametric Wilcoxon signed rank test (leukocyte count) or the paired Student t test (RBC and platelet counts). Values of P < 0.05 were considered significant for all analyses.

Results

Plasma VEGF concentrations of blood donors—In 4 of the 5 blood donors from group 1 (unfiltered whole blood), VEGF concentrations were lower than the detection limit. One dog had a VEGF concentration of 12.4 pg/mL; its WBC count was 12.9 × 10^6 WBCs/µL. Plasma VEGF concentrations of all 5 donors from group 2 (whole blood filtered before separation in packed RBCs and FFP) were lower than the detection limit of the VEGF assay (Figure 1).

VEGF concentrations in nonfiltered blood products—After separation into packed RBC and FFP units, VEGF concentrations in the plasma samples from all units were lower than the detection limit. Median VEGF concentrations were as follows: day 7 after blood collection, 19.0 pg/mL (range, 0 to 139 pg/mL); day 14, 163 pg/mL (range, 0 to 333 pg/mL); and day 21, 110 pg/mL (range, 36.9 to 357 pg/mL). Four of the 5 samples had an increase in concentration to higher than the assay detection limit after 1 week of storage. In the fifth sample, an increase was only detected after 3 weeks of storage. In 2 samples, VEGF concentrations increased continuously throughout storage, whereas samples from 2 other donors had an increase after 1 and 2 weeks of storage and a slight decrease after 3 weeks.

After storage for 1 week, all samples had distinct signs of hemolysis. The degree of hemolysis did not differ macroscopically between the samples. For samples from 2 donors, FFP was available for examination after 29 and 42 days of storage. Concentrations of VEGF in both samples were lower than the detection limit.

Cell depletion in whole blood after filtration—To establish the degree of leukocyte depletion and loss of platelets over time, filtered whole blood samples were re-examined hematologically, and the results were compared with the blood values of the respective donor (Figure 2). There was only a minor difference in the RBC counts of the donor sample (6.61 ± 0.52 × 10^6 RBCs/µL), compared with the RBC counts after filtration (7.45 ± 0.81 × 10^6 RBCs/µL). However, leukocyte counts after the filtration process (0 to 0.20 × 10^3 cells/µL [median, 0 × 10^3 cells/µL]) were significantly lower than before filtration (9.08 to 15.9 × 10^3 cells/µL [median, 11.2 × 10^3 cells/µL]).
The same was true for platelet counts (3.6 ± 4.0 × 10^3 cells/µL after filtration vs 361 ± 84 × 10^3 cells/µL before filtration).

VEGF concentration in filtered blood products—After separation into packed RBC and FFP units, VEGF concentrations in the supernatant of packed RBC samples and in plasma were lower than the VEGF assay detection limit in products from all 5 donors of group 2. In contrast to the nonfiltered samples, VEGF concentrations in the supernatants of filtered packed RBC samples did not increase during the 3 weeks of storage and remained lower than the detection limit. For 2 donors, VEGF concentration did not increase after 26- and 42-days of storage. Both samples yielded VEGF concentrations that were lower than the assay detection limit.

Discussion

Although human circulating VEGF has 1 amino acid more than canine VEGF; the receptor binding region on the 2 molecules is identical. Canine VEGF activates human endothelial cells to the same extent as human VEGF. The human VEGF assay used in the present study has been already used in other studies for measurement of VEGF concentration in canine plasma. The use of the assay to detect VEGF specifically in CPD-adenine–anticoagulated blood samples has not been reported yet and was not evaluated in our study. However, this assay has been used in several studies to analyze samples treated with various anticoagulants, such as sodium citrate and citrate-theophylline-adenosine-dipyridamole, and additives such as SAGM. Moreover, differences in the recovery rates reported by the assay manufacturer among serum and EDTA-treated, heparinized, and citrated plasma were 10%, which is regarded as acceptable.

Platelets are activated during the coagulation process and release VEGF into the serum. Therefore, plasma is the preferred medium for VEGF measurement. Addition of theophylline, adenosine, and dipyridamole into sodium citrate tubes, which were used for blood withdrawal, was used to provide maximum platelet stabilization.

Plasma VEGF concentrations were lower than the assay detection limit in 9 of 10 dogs in the present study. This finding is in agreement with results of other studies in which most dogs had concentrations lower than the detection limit of the assays used. In 1 study, only 1 of 17 healthy dogs had a detectable plasma VEGF concentration of 17.7 pg/mL, and no correlation between VEGF concentration and Hct or WBC or platelet count was found. In human medicine, a possible correlation between platelet number and serum VEGF concentration has been discussed. Moreover, the VEGF concentration within platelets appears to vary as well. A study in humans revealed a concentration of 2.51 ± 2.30 pg/10^10 platelets. Another study revealed concentrations of 0.16 to 4.96 pg/10^10 platelets (median, 1.39 pg/10^10 platelets).

Results of the present study corroborated those of earlier studies, which showed that healthy humans have higher mean plasma concentrations of VEGF than do healthy dogs. Mean concentrations of physiologically circulating VEGF in dogs are reportedly lower than assay detection limits. In humans, these concentrations are considerably higher (reported median values are 9.0 pg/mL, 13 to 37 pg/mL, and 17 to 38 pg/mL).

Immediately following separation of the whole blood units, all VEGF concentrations for nonfiltered and filtered packed RBC and FFP units were lower than the assay detection limit in the present study. Samples of nonfiltered packed RBCs were obtained on the day of blood donation and at days 7, 14, and 21 of subsequent storage. In the supernatants of packed RBCs, the VEGF concentrations increased, as has been described in human medicine. In 1 study, VEGF concentrations were investigated in various human medical blood products such as nonfiltered whole blood, revealing that VEGF concentration increased from a median of 8.6 ng/blood unit (range, 4.6 to 28.7 ng/blood unit) before storage to 65.2 ng/blood unit (range, 35.8 to 290.4 ng/blood unit) after 35 days of storage at 4°C.

We chose not to directly obtain samples from bags of packed RBCs because this would have required an opening of the bag, rendering it unusable for further storage and transfusion. Withdrawal of packed RBCs from the sample pouches allowed for sterile extraction from part of the packed RBCs, which were stored separately from the unit. Bags of packed RBCs and the sample pouches were stored at 4°C in the same refrigerator under the same conditions. Therefore, the sample handling was unlikely to have influenced the study findings. Supernatants of the packed RBC samples were used for the VEGF measurements.

The main reason that VEGF concentrations typically increase during storage of cell-containing blood is likely the disintegration of platelets. Platelets undergo a loss of membrane integrity and activation, causing a release of stored substances in blood components, comparable to the apoptosis of nucleus-containing cells. However, various WBCs, such as neutrophilic granulocytes, also contain an intracellular pool of VEGF. In the present study, prestorage filtration of canine whole blood with a leukoreduction filter prevented the accumulation of VEGF in stored packed RBC units, as has been shown in human medicine.

Use of filters designed for leukocyte reduction in human blood products for reduction in canine blood products has been described. In 1 study, 450 mL of whole blood was obtained from 5 dogs twice, at 8-week intervals. One bag of blood from each dog was filtered directly after withdrawal and after 4 hours of storage at 4°C to examine the effect of cooling on the efficiency of filtration. The filtering system was a third-generation filter, which was the type used in our study. Cell removal is accomplished in third-generation filters not by pores but by adsorption. Consequently, it is not the dimensions of the cells but their electrical charge that is important. Because platelets interact with retained leukocytes, they are retained indirectly by adsorption.

Filtering took 7.0 and 15.6 minutes for the noncooled and cooled blood, respectively, in the other study. In the present study, only 250 to 300 mL was donated, so the filtration process took only 3 to 5 minutes for the noncooled blood. The temperature did not
have any effect on the mean blood loss in the cooling study,\(^4\) which was approximately 52 mL. After the whole blood in that study was cooled, the degree of leukocyte reduction was more effective. This finding may be explained by an increase in viscosity of the cooled blood, which would not affect adhesion but would enhance the mechanical filtering process because of the resulting decrease in flow velocity.\(^3\) The RBC, platelet, and WBC counts prior to and after filtration as measured in the study\(^5\) of cooled and noncooled blood corresponded to the results obtained in the present study.

Although the manufacturer of the hematology analyzer provided a leukocyte count of 0 leukocytes as the lower detection limit, this value should be scrutinized because hematology analyzers are not sensitive enough for the precise detection of residual leukocytes in a leukoreduced product. Use of Nageotte counting chambers or flow cytometry would have been the superior approach. A possible release of VEGF by activation of platelets did not occur during filtration. This corresponds to findings of a human study\(^6\) in which activation of platelets due to filtration appeared unlikely.

Bioactive substances in FFP have been investigated in humans. In that study,\(^1\) concentrations of histamine, eosinophilic cationic protein, eosinophilic protein X, and myeloperoxidase were significantly higher in thawed FFP samples than in plasma samples from the corresponding blood donors and plasma samples before freezing. Concentrations in filtered FFP units were comparable with those of the blood donors. It follows that bioactive substances are present in FFP and to some extent accumulate because of freezing and thawing. We presumed that bioactive substances were released from the remaining leukocytes in nonfiltered units in our study. In humans,\(^2\) FFP is not acellular and can have relevant leukocyte counts justifying filtration. In blood from the healthy study dogs, an increase in VEGF concentration could be detected neither in nonfiltered nor in filtered FFP units; however, the sample size was small, which may have obscured any difference that might have been present. On the whole, use of leukoreduction filters was effective in preventing the release of VEGF during the storage of canine RBC products. Therefore, in dogs with hemangiosarcoma or other tumors that might result in an increase in VEGF concentration, use of leukoreduced blood could improve the safety of transfusion treatment.

References


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