Experimental

Platelet Quantification and Growth Factor Analysis from Platelet-Rich Plasma: Implications for Wound Healing

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Growth factors released from activated platelets initiate and modulate wound healing in both soft and hard tissues. A recent strategy to promote the wound-healing cascade is to prepare an autologous platelet concentrate suspended in plasma, also known as platelet-rich plasma, that contains growth factors and administer it to wound sites. The purpose of this study was to quantitate platelet number and growth factors released from a prepared platelet concentrate. Whole blood was drawn from 10 healthy patients undergoing cosmetic surgery and concentrated into platelet-rich plasma. Platelet counts on whole blood and platelet-rich plasma were determined using a Cell-Dyn 3200. Platelet-derived growth factor-BB, transforming growth factor-β1, vascular endothelial growth factor, endothelial growth factor, and insulin-like growth factor-1 were measured in the platelet-rich plasma using the enzyme-linked immunosorbent assay method. In addition, platelet activation during the concentration procedure was analyzed by measuring P selectin values in blood serum. An 8-fold increase in platelet concentration was found in the platelet-rich plasma compared with that of whole blood (baseline whole blood, $197 \pm 42 \times 10^3$ platelets/μl; platelet concentrate, $1600 \pm 330 \times 10^3$ platelets/μl). The concentration of growth factors also increased with increasing platelet number. However, growth factor concentration varied from patient to patient. On average for the whole blood as compared with platelet-rich plasma, the platelet-derived growth factor-BB concentration increased from 3.3 ± 0.9 ng/ml to 17 ± 8 ng/ml, transforming growth factor-β1 concentration increased from 35 ± 8 ng/ml to 120 ± 42 ng/ml, vascular endothelial growth factor concentration increased from 155 ± 110 pg/ml to 955 ± 1030 pg/ml, and endothelial growth factor concentration increased from 129 ± 61 pg/ml to 470 ± 320 pg/ml. No increase was found for insulin-like growth factor-1. In addition, no increase in platelet activation occurred during the concentration procedure as determined by the platelet surface receptor P selectin (45 ± 16 pg/ml to 52 ± 11 pg/ml, $p = 0.65$). In conclusion, a variety of potentially therapeutic growth factors were detected and released from the platelets in significant levels in platelet-rich plasma preparations. Sufficient concentrates and release of these growth factors through autologous platelet gels may be capable of expediting wound healing in a variety of as yet undetermined specific wound applications. (*Plast. Reconstr. Surg.* 114: 1502, 2004.)

Autologous platelet-rich plasma has gained popularity as a clinical treatment in a variety soft- and hard-tissue applications in almost all fields of surgery, most notably in acute surgical conditions and in the management of chronic nonhealing wounds.[1–7] Surgeons are using various platelet-rich plasma concoctions to take advantage of an autologous fibrin clot that will aid in hemostasis along with providing growth factors in the form of platelet releasate to potentially promote healing. Depending on the application, the surgeon may be preferentially seeking one of these biological applications or both.

Despite their expanding clinical availability and the euphoria for their use, their clinical effectiveness is scientifically unproven, and most patient data are empiric. Before one even considers applying it to a patient, it would seem logical to characterize the content of platelet-rich plasma gel, including its variability from patient to patient. The purpose of this study, therefore, was to accurately count platelet numbers, determine whether platelet activation occurs during platelet-rich plasma preparation, and quantitate growth factors released from the platelets from one commercially available platelet concentration preparation system.

Materials and Methods

Preparation of Platelet-Rich Plasma

Ten study participants were selected from plastic surgery patients ranging in age from 29 to 58 years (eight women and two men). Five cubic centimeters (1 cc = 1 cm^3 = 1 ml) of Anticoagulant Citrate Dextrose Solution (Solu-

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tion A Citra Anticoagulant, Inc., Braintree, Mass.) was drawn into a 60-cc syringe, followed by 55 cc of whole blood. Samples were gently agitated to thoroughly mix the anticoagulant with the blood. Blood samples for baseline measurements were drawn into 12-cc syringes with 1 cc of Anticoagulant Citrate Dextrose Solution and 11 cc of whole blood.

The platelet-rich plasma was produced using the Gravitational Platelet Separation System (Cell Factor Technologies, Biomet, Warsaw, Ind.). Sixty cubic centimeters of blood was injected into the Gravitational Platelet Separation disposable unit and then centrifuged for 12 minutes at 3200 rpm (Centra CL2, IEC International Equipment Company, Needham Heights, Mass.). After centrifugation, the buffy coat layer, consisting of platelets and white blood cells, was sequestered in a volume of 6 cc of plasma.

Hematology Analysis of Platelet-Rich Plasma

Samples were retained in counting tubes and left on a shaker for 15 minutes to ensure complete mixing. Care was taken to continually agitate the samples, as platelets would begin to settle to the bottom of the tube immediately. Complete blood counts were obtained for each sample with an Abbott Cell-Dyn 3200 (Abbott Laboratories, Abbott Park, Ill.) at Follas Laboratories, Inc. (Indianapolis, Ind.). A preliminary study (data not shown) was performed to validate platelet counts on this Cell-Dyn 3200, with samples counted manually with a hemacytometer.

The entire volume of the whole blood was added to a single sample tube without dividing the sample to reduce error. The samples were counted and then the remaining blood was retained for growth factor measurements. Each sample was counted in triplicate and then averaged (mean ± 1 SD). This step was repeated for the platelet-rich plasma samples. The linear limit of the Cell-Dyn 3200 is 1.9×10^6 platelets/μl. Any sample that was initially 1.8×10^6 platelets/μl or higher was diluted with phosphate-buffered saline and recounted.

Quantification of Growth Factors in Platelet-Rich Plasma

The same undivided samples used for platelet counts were then used to measure growth factor and P selectin content. One milliliter from each baseline and platelet-rich plasma sample was separated into a centrifuge tube and was retained for P selectin measurement. To collect samples for the growth factors platelet-derived growth factor (PDGF)-BB, transforming growth factor (TGF)-β1, vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), and insulin-like growth factor (IGF)-1 measurements, the remaining whole blood and platelet-rich plasma samples were activated with 1000 units of bovine thrombin (Sigma Co., St. Louis, Mo.) per milliliter of 10% $CaCl_2$ (Sigma). After a firm blood clot formed, the samples were centrifuged for 5 minutes at 3200 rpm. The supernatant was frozen at −70°C.

In addition, P selectin, a platelet surface receptor that is expressed once platelets become activated, was measured in sample plasma to determine the activation of the platelets during gel preparation. For the P selectin measurements, the 1-ml samples that were separated as described above were centrifuged without thrombin activation. The plasma was collected and frozen as previously described. Frozen samples for all enzyme-linked immunosorbent assay analyses were shipped to Burleson Research Technologies (Raleigh, N.C.) for biochemical analysis.

The growth factor and P selectin levels were determined using the enzyme-linked immunosorbent assay method. All kits were purchased from R&D Systems (Minneapolis, Minn.) and were tested according to the manufacturer's instructions. In general, the test procedure included adding standards and samples to a microplate precoated with an antibody against each growth factor. Any growth factor present was bound by the immobilized receptor. After any unbound substances were rinsed away, an enzyme-linked polyclonal antibody specific for each growth factor was added to the wells. After a second wash, a substrate solution was added, and color developed in proportion to the amount of bound growth factor in the first step. The color development was stopped, and the intensity of the color was measured using the SpectraMax 340 microplate reader (Molecular Devices Corp., Sunnyvale, Calif.).

Statistical Analysis

Averages are presented as mean ± 1 SD. Statistical significance between concentrated and baseline values were determined with a paired *t* test ($\alpha = 0.05$). Linear regression techniques were used to evaluate correlation be-

tween platelet number and growth factor concentrations.

RESULTS

Hematology Analysis of Platelet-Rich Plasma

Complete blood count analysis was performed on whole blood and platelet-rich plasma samples from each study participant. The platelet concentration procedure increased the platelet numbers on average from 197,000 ± 42,000 platelets/μl to 1,603,000 ± 330,000 platelets/μl. The whole blood baseline measurements ranged from 142,000 to 263,000 platelets/μl and were all within the normal human range. The concentration procedure resulted in an averaged 8-fold increase in platelet concentration. There was an 85 ± 19.0 percent recovery of platelets in the platelet-rich plasma from the whole blood. The platelet-rich plasma group was significantly higher in platelet number than the baseline whole blood group, with a value of $p < 0.001$.

Additional blood parameters measured by the complete blood count illustrated that the centrifugation procedure increased the white blood cell concentration by 5.4-fold (5.8 ± 2.2 × 10^3/μl to 31.1 ± 10.7 × 10^3/μl) and decreased the red blood cell concentration by 40 percent (3.8 ± 0.6 × 10^6/μl to 1.4 ± 0.9 × 10^6/μl). The increase in white blood cell count was significant ($p < 0.001$), as were the decreases in red blood cell and hematocrit percentages ($p < 0.001$ each).

Quantification of Growth Factors in Platelet-Rich Plasma

Enzyme-linked immunosorbent assay quantification of growth factor content from whole blood and platelet-rich plasma samples was determined for each patient. The PDGF, TGF-β1, VEGF, and EGF cytokines were all significantly greater in the platelet-rich plasma samples than in the whole blood baseline samples. On average, the PDGF-BB increased from 3.3 ± 0.9 ng/ml to 17 ± 8 ng/ml, TGF-β1 increased from 35 ± 8 ng/ml to 120 ± 42 ng/ml, VEGF increased from 155 ± 110 pg/ml to 955 ± 1030 pg/ml, and EGF increased from 129 ± 61 pg/ml to 470 ± 317 pg/ml. No statistically significant increase was found in IGF-1 in this study, which was 67 ± 22 pg/ml in the baseline samples and 72 ± 25 pg/ml in the platelet-rich plasma samples ($p = 0.22$). In addition, no platelet activation occurred during the concentration procedure as determined by the lack of increase in platelet surface receptor P selectin (45 ± 16 pg/ml to 52 ± 11 pg/ml) ($p = 0.65$).

The increase was calculated per patient for both platelet number and growth factor levels. The greatest increase is seen with VEGF (6.2-fold increase), followed by PDGF-BB (5.1-fold increase) and EGF and TGF-β1 (3.9- and 3.6-fold, respectively). Figure 1 gives the averaged increase for each of the measured parameters.

A linear regression analysis was used to determine whether a correlation existed between

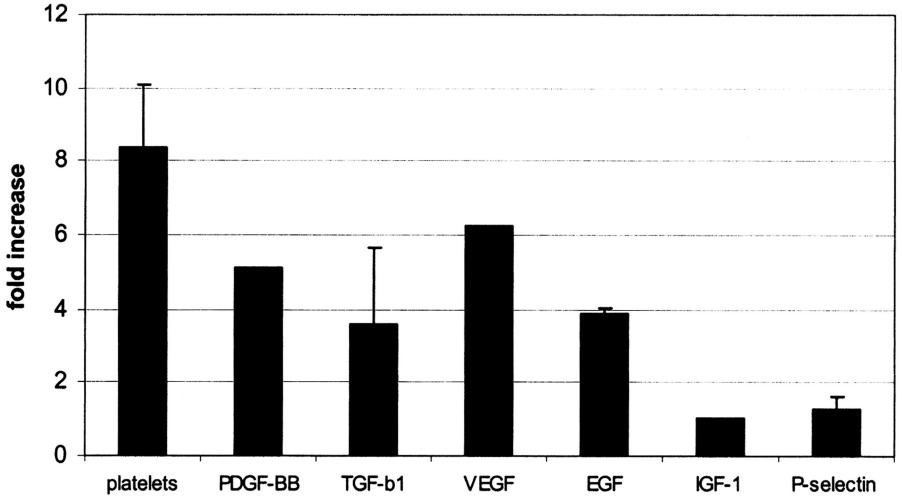

FIG. 1. Increase of platelet concentration and growth factor measurement was calculated from averaged means for the study patients. Platelet counts were acquired on a Cell-Dyn 3200 and counted before and after concentration per patient. The growth factor measurements were achieved by the enzyme-linked immunosorbent assay technique also before and after concentration per patient. An increase was found in platelet concentration, PDGF-BB, TGF-β1, VEGF, and EGF, but not with IGF-1 or P selectin.

platelet number and growth factor concentration. Results are given in Figure 2. The best correlation was with TGF-β1, which was still quite low at only 60.41 percent. Results suggest little correlation between platelet number and growth factor concentration and that content of the platelets varies from patient to patient.

DISCUSSION

Platelet-rich plasma achieves hemostasis through the formation of a fibrin clot that is initiated by the activation and aggregation of platelets. The clot is generated by the polymerization of fibrin from the monomer fibrinogen in the presence of calcium and thrombin. Platelet aggregation results in a platelet plug that is held in place by the clot and inhibits blood flow.[8] Beyond maintaining hemostasis, the fibrin clot then provides a matrix for the migration of tissue-forming cells, including fibroblasts that are responsible for collagen synthesis and endothelial cells involved in angiogenesis.[9] Tractional forces generated by these migrating cells on the fibrin clot can aid in wound contraction. These same migrating cells are also responsible for remodeling the clot into repair tissue.[10]

During normal wound healing, trapped platelets become activated and degranulate, resulting in the release of the α-granule content. Platelet α-granules have been shown to contain mitogenic and chemotactic growth factors important in wound healing such as PDGF, TGF-β, VEGF, EGF, and IGF.[11–13]

The effects of these growth factors on cell behavior and on wound healing have been thoroughly studied. The growth factor that has been investigated the most extensively is PDGF. Release of PDGF into a wound bed can have a chemotactic effect on monocytes, neutrophils, fibroblasts, mesenchymal stem cells, and osteoblasts. PDGF is also a powerful mitogen for fibroblasts and smooth muscle cells and is involved in all three phases of wound healing, including angiogenesis, formation of fibrous tissue, and reepithelilization.[14] In a clinical study, pressure ulcers were treated daily (100 μg/g or 300 μg/g) with a PDGF-BB wound-healing gel, becaplermin, and showed significantly decreased ulcer volume compared with ulcers treated with a gel lacking PDGF-BB.[15]

TGF-β is another growth factor released from platelet α-granules. TGF-β is a mitogen for fibroblasts, smooth muscle cells, and osteo-

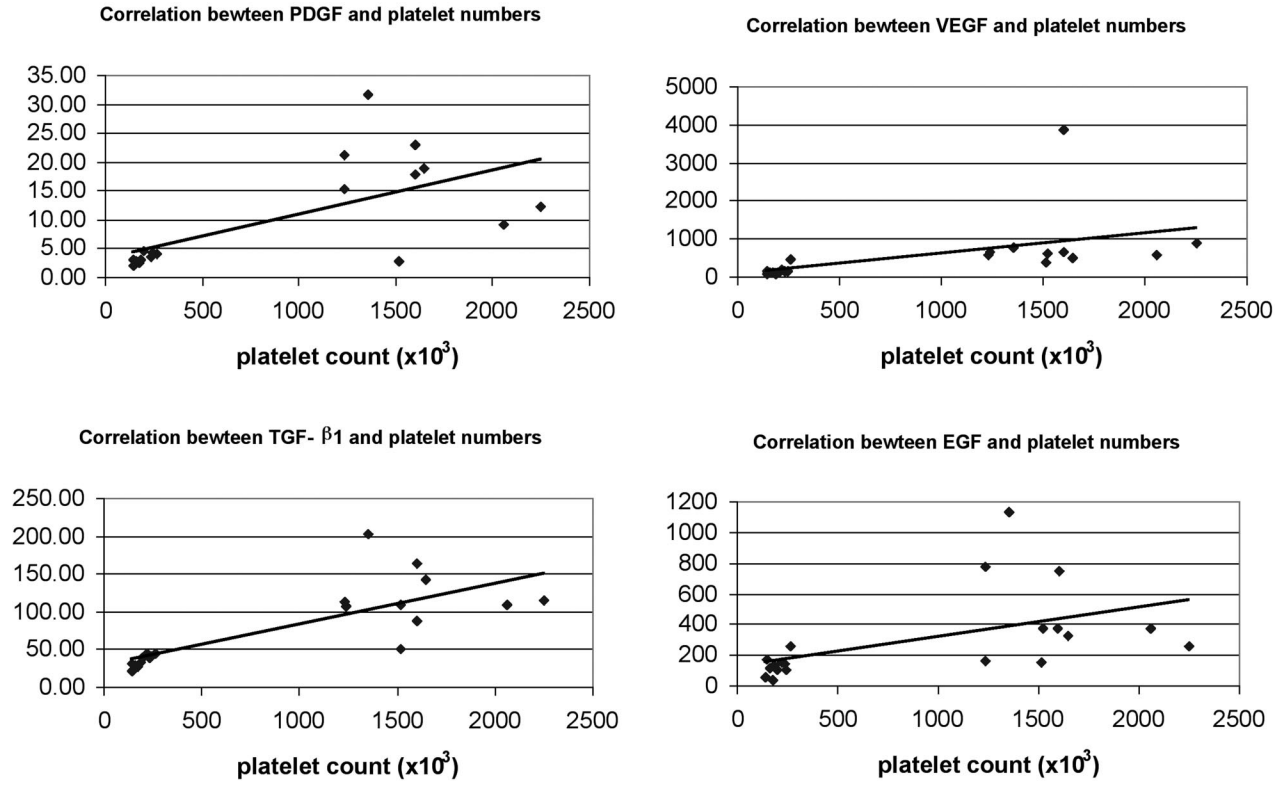

FIG. 2. A linear regression analysis was performed to evaluate the linearity of the growth factor increase with platelet number.

blasts. In addition, it promotes angiogenesis and extracellular matrix production.[14,16] In a rat tibial fracture model, injections of TGF-β (4 and 40 ng) every other day for 40 days resulted in a dose-dependent increase in bone thickness. Furthermore, the 40-ng dose increased mechanical strength.[17] However, Broderick et al. injected a much higher dose (335 μg of TGF-β) in a humeral canine model and found a decrease in bone mineralization.[18]

In addition, actions of other growth factors present in platelet releasate have been described. VEGF promotes angiogenesis and can promote healing of chronic wounds and aid in endochondral ossification.[16,19] However, as seen with high doses of TGF-β, high doses of VEGF (0.5 μg into rat segmental defect) inhibited bone formation.[21] EGF, another platelet-contained growth factor, is a mitogen for fibroblasts, endothelial cells, and keratinocytes, and also is useful in healing chronic wounds.[16] IGF, another platelet-contained growth factor, regulates bone maintenance and is also an important modulator of cell apoptosis, and in combination with PDGF, can promote bone regeneration.[13,20]

Platelet concentrates are potentially useful in wound-healing applications because they function as both a tissue sealant and a drug delivery system that contains a host of powerful mitogenic and chemotactic growth factors. However, the method of platelet-rich plasma preparation has a potentially significant impact on the different levels of platelet recovery and activation. Platelet activation during preparation of the platelet concentrate can result in early α-granule release and loss of the growth factors during the collection process. It is therefore critical to recognize that each platelet-rich plasma preparation method may differ in regard to platelet number, platelet activation rates, and growth factor profiles. Sufficient characterization of the effects of differing platelet-rich plasma preparation methods has not been thoroughly performed. Therefore, it can be largely theoretical that the application of platelet-rich plasma to a wound is really providing a sufficient dose of these useful agents.

In this regard, therefore, it is critical to define the extent of platelet activation that occurs during graft preparation. If platelets become activated and release the contents of the α-granules during the centrifugation process, the growth factors will be diluted and lost into the plasma. To ensure that the platelets are intact until the platelet-rich plasma fraction has been collected, a platelet surface marker for platelet activation, P selectin, was measured. After centrifugation, P selectin measurements for the platelet-rich plasma were not significantly higher than that of the autologous whole blood. This demonstrates that the particular method of platelet-rich plasma preparation used in this study does not prematurely activate the platelets and, as a result, produces the maximum possible growth factor dose in situ.

The enzyme-linked immunosorbent assay technique was used to quantitate the growth factor profile released from the platelet-rich plasma collected. The growth factor profile was compared with the profile of the baseline whole blood. In this study, the growth factors PDGF, TGF-β1, VEGF, and EGF increased as the platelet concentration increased. With the platelet-rich plasma preparation method used in this study, a 6-ml platelet-rich plasma sample contained approximately 9.6×10^9 platelets, releasing approximately 101 ng of PDGF-BB, 720 ng of TGF-β1, 5.7 ng of VEGF, 2.8 ng of EGF, and 0.4 ng of IGF-1.

Only one isoform for each growth factor was measured. For example, PDGF has three isoforms, PDGF-AA, PDGF-AB, and PDGF-BB.[16] Previous studies reported that there is approximately 0.06 ng of PDGF per 1 million platelets.[3] In this study, we found 0.01 ng of the BB isoform of PDGF was released per 1 million platelets. In addition, the amount of TGF-β1 and VEGF detected in this study were well below the levels that were required to inhibit bone formation (335 μg for TGF-β1 and 0.5 μg for VEGF).[18,21]

A linear increase of the growth factors PDGF-AB, TGF-β, VEGF, and EGF with increased platelet number has been previously reported in several recent studies[22] but could not be demonstrated in this study. In another study by the same authors, a direct proportionality was found between cell number and growth factor released only for PDGF, TGF-β, and EGF. A correlation was not found for VEGF and IGF.[23] Similar to the results seen in this study, no correlation between growth factor levels (PDGF-AB, PDGF-BB, and TGF-β1) and platelet concentration has been previously reported.[24] In addition, these findings corroborate previous reports that a significant interpatient variability of growth factor release occurs.

Several parameters influence the relationship between platelet concentration and growth factor levels measured, such as sufficient activation of platelets to initiate α-granule releasate, white blood cell count, and plasma growth factor contamination, and collection of growth factors from the fibrin clot. Platelets have been activated by a variety of methods including adenosine diphosphate[22,23] and freeze-thaw cycles.[24] In our study, thrombin/$CaCl_2$ was used to activate the platelets. Whether this is the most effective method is debatable, but it is clinically convenient and user-friendly for the preparation process.

The concentration of IGF-1 in the platelet-rich plasma was not significantly increased. However, there is very little IGF-1 released from platelets anyway. IGF-1 is a critical growth factor that is required by most cells to proceed through the cell cycle, and it is primarily excreted by the liver into the blood plasma.[25] Because the blood plasma was not concentrated in this platelet-rich plasma preparation system, the levels of IGF-1 released from the concentrated platelets were negligible compared with the values that should be in the plasma, and was therefore not concentrated.

CONCLUSIONS

Results from this study demonstrate that platelets can be sequestered and concentrated 8-fold from whole blood without activating the platelets before desired. These platelets contain a host of growth factors, such as PDGF-BB, TGF-β1, VEGF, and EGF, whose levels are increased when platelets are concentrated into platelet-rich plasma gel preparations. Platelet-rich plasma, and the associated fibrin clot, can potentially aid in wound repair and help achieve and maintain hemostasis, or can be mixed with other tissues as an adjunct to their transplantation healing. Because most platelet-rich plasma gel preparations are applied as a spray, they are fairly easy to administer to the desired anatomic site. Which specific healing applications will benefit from platelet-rich plasma remains to be determined and is an active area of investigation and research in plastic surgery.

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