

Scientific Foundations

Platelet Rich Plasma: Biology and New Technology

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Platelets play a central role in hemostasis and wound healing. The latter is mediated by release of secretory proteins on platelet activation, which directly or indirectly influences virtually all aspects of the wound healing cascade. Studies in basic science have shown a dose-response relationship between the platelet concentration and levels of secretory proteins, as well as between platelet concentration and certain proliferative events of significance to the healing wound. Technologies to provide autologous platelet rich plasma to the repair site are now being used in a wide variety of clinical applications, with the majority of such studies suggesting a role in the surgeon's armamentarium. Little standardization in the field exists, which has made it difficult to fully evaluate the literature on the subject and unequivocally establish applications for which the technology truly has merit. This article presents fundamental background on platelet biology and the role of platelets in both hemostasis and wound healing, as well as methods of preparing, characterizing, and using platelet rich plasma, to provide the reader a foundation on which to critically evaluate prior studies and plan future work.

Key Words: Platelets, platelet rich plasma, wound healing, growth factors, secretory proteins

Healing of tissue, both soft and hard, is mediated by a complex array of intra- and extracellular events that are regulated by signaling proteins, a process that is incompletely understood.¹⁻⁵ What is certain is that

platelets play a prominent, if not deciding, role.^{3,6} Platelet activation, in response to tissue damage and cardiovascular rent, results in the formation of a platelet plug and blood clot to provide hemostasis and the secretion of biologically active proteins. These proteins, in turn, set the stage for tissue healing, which includes cellular chemotaxis, proliferation, and differentiation; removal of tissue debris; angiogenesis; the laying down of extracellular matrix; and regeneration of the appropriate type of tissue.^{2-4,6} In vitro, there is a dose-response relationship between platelet concentration and the proliferation of human adult mesenchymal stem cells (MSCs), the proliferation of fibroblasts, and the production of Type I collagen.^{7,8} This suggests that the application of autogenous platelet rich plasma, or PRP, can enhance wound healing, as has been demonstrated in controlled animal studies for both soft and hard tissue.^{9,10}

Gandhi et al¹¹, measured the amount of platelet-derived growth factor (PDGF) and transforming growth factor β (TGF- β), two of the key proteins secreted from activated platelets, in the fracture hematoma of patients with and without diabetes mellitus. There were significantly lower amounts of these growth factors in the patients with diabetes, suggesting a partial mechanism for the poorer healing response typical of such patients. Gandhi et al¹² also measured levels of these two growth factors in the fracture hematoma of 24 patients with fresh fractures (within 20 days of injury) but were unable to detect these proteins in the nonunion tissue of 7 patients with nonunion (>4 months after injury) of fractures of the foot and ankle. Autologous platelet rich plasma (PRP) from the nonunion cohort was prepared with high, measured levels of PDGF and TGF- β . The PRP was applied to the nonunion during revision surgery, which resulted in radiographic union by an average of 8.5 weeks. In a controlled clinical study, Marx¹³ found that mandibular bone grafts containing PRP showed an increase in radiographic density during a 6-month period, a greater trabecular bone area, and a higher graft maturity index, compared with grafts that did not receive PRP.

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The clinical use of PRP for a wide variety of applications has been reported, most prevalently in the periodontal, craniofacial, and spine literature.^{2,13-30} Collectively, these studies provide strong evidence to support the clinical use of PRP; however, many are anecdotal, and few include controls to definitively determine the role of PRP. In addition, there is little consensus regarding PRP production and characterization, which can impede the establishment of standards that are necessary to integrate the vast literature in basic and clinical science on the subject.³¹⁻³⁵ The goal of this article is to describe platelet biology and its role in hemostasis and wound healing, as well as the preparation, characterization, and use of PRP, to provide the reader with a foundation that is essential for the critical evaluation of this subject.

PLATELET ORIGIN, MORPHOLOGY, AND DISTRIBUTION

Platelets are cytoplasmic fragments of megakaryocytes, a type of white blood cell, and are formed in the marrow.³⁶⁻³⁸ They are the smallest of the blood cells, round or oval in shape, and approximately 2 μm in diameter. The cell membrane is trilaminar with a glycoprotein receptor surface overlying and partially interspersed with and penetrating a bilayer of phospholipids and cholesterol.²⁸ They lack nuclei but contain organelles and structures such as mitochondria, microtubules, and granules (alpha, delta, and lambda).^{13,28,38,39} The α granules, bound by a unit membrane, are formed during megakaryocyte maturation, are about 200 to 500 nm in diameter, and number approximately 50 to 80 per formed platelet.⁴⁰ They contain more than 30 bioactive proteins, many of which have a fundamental role in hemostasis or tissue healing.^{3,40}

The platelet cytoplasm contains an open, canalicular system that increases the effective surface area for intake of stimulatory agonists and the discharge of effector secretions.²⁸ The submembrane region contains microfilaments of actin and myosin that mediate morphologic alterations.²⁸ Metabolically, these cells possess a tricarboxylic acid cycle and use glucose via the glycolytic and hexose monophosphate shunt pathways.³⁸ Their function is closely linked to their metabolic activity.

Platelets reside intravascularly, with high concentration in the spleen.³⁸ Normal blood contains approximately 140,000 to 400,000 platelets/ mm^3 which remain in the circulation for an average of about 10 days before removal by macrophages of the reticuloendothelial system.^{28,38} The condition of depressed platelet level is called thrombocytopenia, which is

characterized by persistent bleeding from cuts and wounds, by petechiae and ecchymoses, and oozing of blood from vascular beds.³⁸

PLATELET FUNCTION

The two functional roles of platelets are hemostasis and the initiation of wound healing, a somewhat arbitrary division because hemostasis can be considered to be the first stage of healing.⁴¹ Nevertheless, for convenience, the physiological role of platelets is described in two parts.

Hemostasis

At sites of tissue injury, platelets aggregate and rapidly change from a rounded shape to one that includes large sticky protuberances, or pseudopodia, a process called *activation*.^{28,39} They adhere to elements that become exposed on damage to blood vessels, such as collagen, the basement membranes of capillaries, and subendothelial microfibrils.³⁸ Release of adenosine diphosphate (ADP) by platelets on contact with these surfaces causes further aggregation. Other factors that mediate these changes include thrombin and adrenalin.^{38,39} If the vascular defect is small, this platelet plug may be sufficient to stop blood loss. However, if it is large, a blood clot is also required to stop the bleeding.

The blood clotting mechanism is initiated by one of two pathways: intrinsic and extrinsic.³⁸ In both cases, this is a cascaded reaction sequence in which inactive factors become activated and catalyze the formation of products from precursors, which in turn activate more factors until the final products are formed. Both pathways share many of the latter steps in the sequence, with the intrinsic pathway including additional initial steps. As such, the extrinsic pathway is shorter and faster than the intrinsic pathway. Both pathways converge with the activation of factor X. The intrinsic pathway is initiated by damage, or alteration, to blood independent of contact with damaged tissue, whereas the extrinsic pathway is initiated by exposure to factors derived from damaged tissue.³⁸

Secretion of factor V by the α granules of activated platelets (more will be said about α granule secretion later) binds to activated factor X to produce prothrombin activator, which in the presence of calcium, catalyzes the formation of thrombin from prothrombin.^{28,38} Thrombin then catalyzes the production of fibrin monomer from fibrinogen, which in the presence of calcium and fibrin stabilizing factor (factor XIII), forms fibrin threads. Thrombin also

binds to platelet surface receptors and activates serum factor VIII, also contained in the α granules, which complexes with factor IX on the platelet surface. Activated factors VIII and IX participate in the activation of factor X via the intrinsic pathway. The blood clot consists of the fibrin mesh containing the platelet aggregate, as well as entrapped red and white blood cells. Contraction of the platelet actin myosin fibers is responsible for retraction of the clot, which occurs within 20 minutes to 1 hour, further closing the vessel.^{28,36} During clot retraction, the platelet releasate is expressed. Thromboxane and serotonin, released from the platelet aggregate, causes vasoconstriction, which further aids hemostasis.² Figure 1 shows a schematic that illustrates the pathways by which platelets affect clot formation.

For blood to be maintained in the liquid state, *ex vivo*, for transfusion, storage, or further processing, the clotting mechanism must be rendered ineffective. The addition of citrate ions to blood forms calcium citrate, a soluble but un-ionized substance. Because the calcium ion is required at several steps in the coagulation cascade, this forms the basis for the anticoagulant effect of acid-citrate-dextrose (ACD) and citrate-phosphate-dextrose (CPD) blood preservative solutions, which include other substances to maintain cellular viability.^{33,36}

Wound Healing

General Concepts

Wound healing can be divided into three overlapping stages: inflammatory, proliferative, and remodeling.^{2,4,5,41}

The initial response to tissue injury is inflammation, whereby the goal is to provide rapid hemostasis and begin the sequence of events that leads to regeneration of tissue. As blood escapes from the damaged vessels, a hematoma forms, filling the tissue space, with platelets playing a key role, as described. Activated platelets and other cells release various growth factors and cytokines that result in cell migration, proliferation, differentiation, and matrix synthesis.^{4,5} The fibrin mesh of the hematoma becomes a provisional matrix that maintains the regenerative space and provides a scaffold for cell migration and proliferation.^{5,18} Neutrophils are the first inflammatory cells to invade the wound site, providing rapid protection against infection and removal of tissue debris, with lifetimes measured in hours and days.^{2,4-6,41} This is followed by influx of monocytes and T lymphocytes.⁴ The monocytes differentiate to macrophages, which become the predominant cell type. Macrophages have lifetimes measured in days

to months and assist the neutrophils in their function as well as secreting factors that direct succeeding events.^{2,4-6} The overall importance of the T lymphocytes to successful wound repair is unclear.⁴¹ Mesenchymal stem cells (MSCs) migrate into the region to provide the uncommitted cell line that will be responsible for formation of bone, cartilage, fibrous tissue, blood vessels, and other tissues.⁴ Fibroblasts migrate into the region and begin to proliferate, producing extracellular matrix.^{4,42} Blood vessel endothelial cells near the injury proliferate, forming new capillaries that extend into the injured site, beginning the process of angiogenesis.^{2,4} Near the end of the inflammatory phase, granulation tissue, named for its pink, soft granular appearance, forms, which is a transient, well-vascularized tissue devoid of nerves but rich in fibroblasts, capillaries, and chronic inflammatory cells that provides a metabolically rich environment to aid repair.^{5,43}

During the proliferative phase, the damaged, necrotic tissue is removed and replaced with living tissue that is specific to the local tissue environment (eg, bone, cartilage, fibrous tissue, etc). Local factors, including the growth factor and cytokine profile, hormones, nutrients, pH, oxygen tension, and the electrical and mechanical environment mediate the differentiation of the MSCs into osteoblasts, fibroblasts, chondrocytes, and other cell types as required to generate the appropriate type of tissue.⁴

During remodeling, the newly generated tissue reshapes and reorganizes to more closely resemble the original tissue. Cell density and vascularity decrease, excess repair matrix is removed, and the collagen fibers of the repair matrix become oriented along lines of stress to maximize strength.^{2,4} Bone remodeling generally is described by Wolff's Law.^{4,44} This final stage of healing is protracted, occurring over the course of years.^{2,4}

Scar tissue is regenerated tissue that consists primarily of fibroblasts and matrix and may restore integrity but not form and function.⁴ Soft tissue and skin heals by scar formation.^{4,45} The healed tissue may consist of some components of the original tissue that have re-formed within the scar. Bone is unique in that it typically heals without scar; that is, the healed tissue cannot be distinguished from uninjured bone.⁴ Tissue, patient, and treatment variables affect the rate and quality of the healing response.⁴

Platelet Influence

The α granules of platelets contain numerous proteins that provide powerful influence on wound healing, including platelet derived growth factor (PDGF- $\alpha\alpha$,

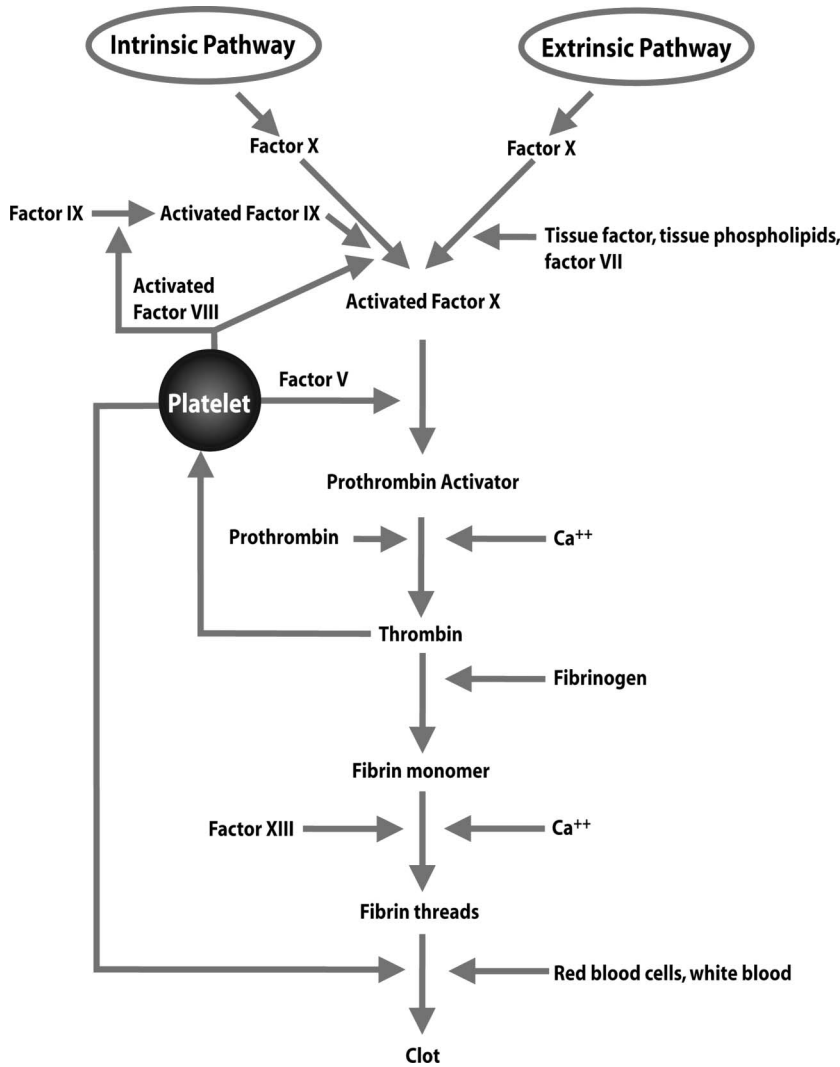


Fig 1 Schematic of the role of platelets in clot formation.

$\beta\beta$, and $\alpha\beta$ isomers), transforming growth factor- β (TGF- β , β 1 and β 2 isomers), platelet factor 4 (PF4), interleukin-1 (IL-1), platelet-derived angiogenesis factor (PDAF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), platelet-derived endothelial growth factor (PDEGF), epithelial cell growth factor (ECGF), insulin-like growth factor (IGF), osteocalcin (Oc), osteonectin (On), fibrinogen (Fg), vitronectin (Vn), fibronectin (Fn), and thrombospondin-1 (TSP-1).^{2,6,18,25,28,36,40,46} Collectively, these proteins are members of the families of growth factors, cytokines, and chemokines. For the purpose of this review, these proteins are broadly referred to as *secretory proteins*.

Activation causes the α granules to fuse to the platelet cell membrane (also called degranulation) where at least some of the secretory proteins (eg,

PDGF and TGF- β) are transformed to a bioactive state by the addition of histones and carbohydrate side chains.^{6,13,40} The active proteins are then secreted, binding to transmembrane receptors of target cells (eg, mesenchymal stem cells, osteoblasts, fibroblasts, endothelial cells, and epidermal cells). These agonist-bound transmembrane receptors activate an intracellular signal protein that causes the expression of a gene sequence that directs cellular proliferation, matrix formation, osteoid production, collagen synthesis, among other things.⁶

The active secretion of these proteins by platelets begins within 10 minutes after clotting, with more than 95% of the presynthesized growth factors secreted within 1 hour.⁶ After this initial burst, the platelets synthesize and secrete additional proteins for the balance of their life (5–10 days).^{6,18,37} At this point,

macrophages, which have arrived via vascular ingrowth stimulated by the platelets, assume responsibility for wound healing regulation by secreting factors. Thus, the platelets at the repair site set the pace for wound repair.^{6,37}

The numerous proteins secreted by the activated platelets influence many of the aspects of healing; Anitua et al³ provide a recent, detailed review. For example, PDGF is chemotactic for macrophages, whereas the combined roles of PDGF, TGF- β , and IGF are chemotaxis and mitogenesis of stem cells and osteoblasts, angiogenesis for capillary ingrowth, bone matrix formation, and collagen synthesis.^{3,18,26} TGF- β and PDGF also assist in bone mineralization.¹⁰ As a group, the adhesive proteins Fg, Fn, Vn, and TSP-1 participate in thrombus formation, and some also have mitogenic characteristics.^{3,47,48} Some of the secretory proteins released from platelets are absent in chronic, nonhealing wounds.²⁸ Although it is generally believed that platelets do not contain bone morphogenetic proteins (BMPs),^{6,37} Sipe et al,⁴⁹ recently identified both BMP-2 and BMP-4 within platelet lysate, suggesting that this might contribute to their role in bone formation and repair. Figure 2 shows a schematic of the role of platelets in wound healing.

Despite the prominent role of platelets in the healing cascade, animals rendered thrombocytopenic, while displaying altered wound healing characteristics that may be site-dependent, may go on to heal, perhaps through the involvement of compensatory mechanisms.⁴¹

PLATELET RICH PLASMA

Platelet rich plasma is, by definition, a volume of the plasma fraction of autologous blood having a platelet concentration above baseline.³³ As such, PRP contains not only a high level of platelets, but also the full complement of clotting factors. Other terms in the literature to describe platelet preparations include platelet concentrate, platelet gel, and platelet releasate.^{23,28,33,50} Whereas, ideally there should be universal agreement regarding definitions and terminology, at the very least, the nature of the platelet derivative studied should be precisely and unambiguously described. It is apparent that if a portion of the blood plasma, PRP, is enriched in platelets, the balance will be deficient in platelets. The latter, platelet poor plasma (PPP), has a clinical role as fibrin sealant for hemostasis.^{2,22,25,37,51}

There are at least five important issues, which are discussed here in the following order: (1) platelet concentration ratio, (2) processing methodology, (3)

quantification of secretory protein concentration, (4) handling and application, and (5) clinical use.

Platelet Concentration Ratio

To an extent, the amount of hematoma that forms in response to trauma is proportional to the tissue injury. Thus, delivery of PRP can be thought of as responding with hematoma in excess of that which would have been physiologically produced. The effect of PRP on wound healing is likely a function of many variables, including the platelet concentration, PRP volume delivered, the extent and type of injury, and perhaps the overall medical condition of the patient. Given the number of variables and their potential for interaction, it is not surprising that there is no single recommendation for the fold-increase of platelets in PRP over baseline.

Marx^{6,33} states that a "working definition" of PRP is 1,000,000 platelets/ μ L, with lesser concentrations unable to be relied on to enhance wound healing and greater concentrations not yet shown to provide further enhancement. By contrast, Anitua et al,³ state that the aim is to prepare PRP with a platelet count in excess of 300,000 platelets/ μ L.

The method used to measure platelet concentration can add uncertainty to concentration ratio determination. Manual counts of stained platelet smears, as well as automated machine methods, may be used.^{13,24,50} The manual method counts individual platelets, whereas automated scanning techniques, such as used by the Coulter Counter (Beckman-Coulter, Fullerton, CA), may count clumps of platelets as a single platelet, providing a underestimate.¹³ This stresses the importance of using consistent methods and properly interpreting the results of other investigators whose counting methods may differ.

It has been suggested that PRP should achieve a three- to five-fold increase in platelet concentration over baseline,^{13,32,50} although the dependence of clinical benefit on platelet concentration versus total number of platelets delivered may need to await further investigation.³⁴ Weibrich et al,⁴⁶ suggest that different individuals may require different platelet concentration ratios to achieve comparable biological effect.

Platelet concentration ratios of less than 2-fold to 8.5-fold have been reported.^{6,13,24,31,32,50,52}

Processing Technique

Ideally, blood should be drawn before surgery commences because the surgery itself will lead to platelet activation that may interfere with preparation.^{22,25}

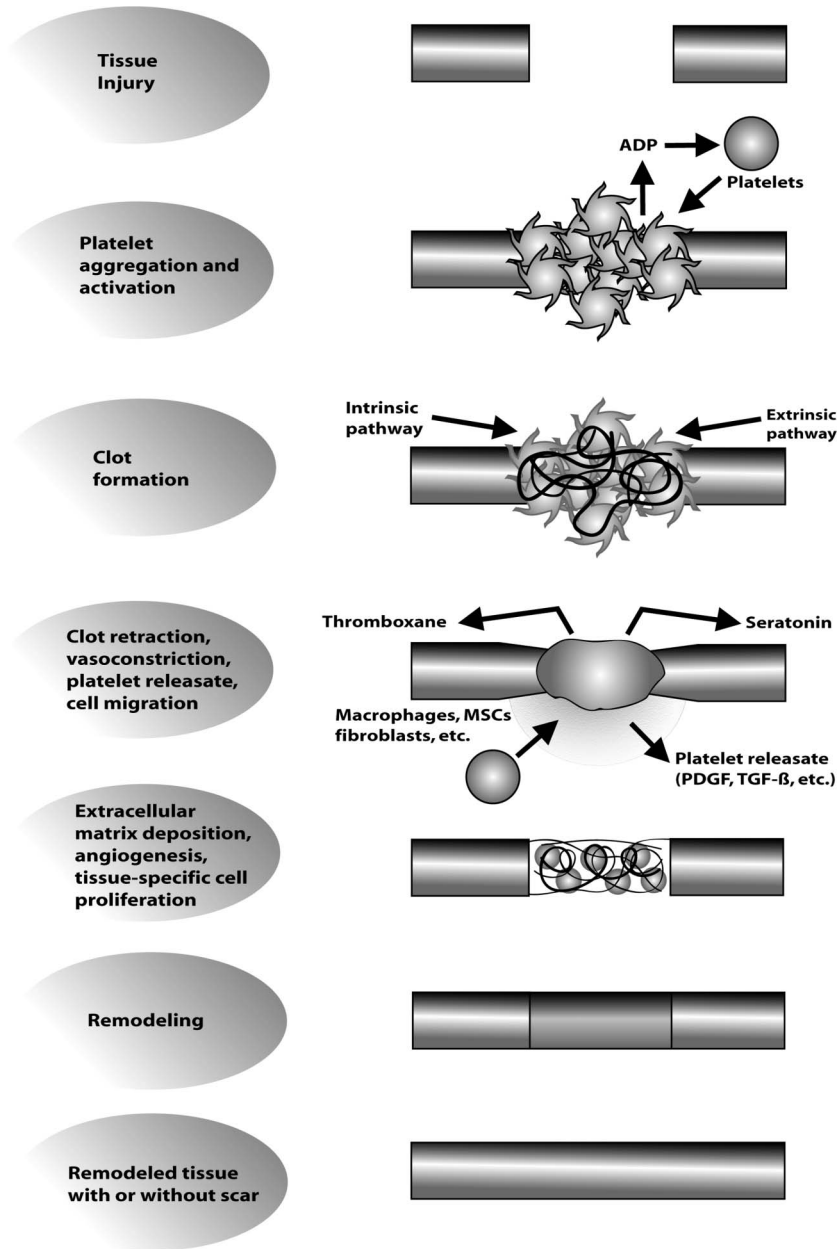


Fig 2 Schematic of the role of platelets in wound healing.

Platelets will collect at the surgical site to initiate clotting and healing, somewhat reducing the whole blood platelet count.³³

When anticoagulated blood is centrifuged, three layers become evident: the bottom layer comprised of red blood cells (specific gravity = 1.09), the middle layer comprised of platelets and white blood cells (buffy coat, specific gravity = 1.06), and the top plasma layer (specific gravity = 1.03).²⁸ This forms the basis of current methods for producing PRP, with

the yield approximately 10% of the volume of whole blood drawn.¹³ It is important that the procedure avoid fragmenting the platelets. Because it is the process of activation that results in the completion of the tertiary structure of some of the secretory proteins, fragmentation could result in the release of high levels but with compromised bioactivity.⁵⁰ The use of ACD-A anticoagulant, as well as low G forces during centrifugation, preserves the integrity of the platelet membrane.^{33,50} Furthermore, platelet activation

occurring during processing should be kept to a minimum. This is because although bioactive secretory proteins would be produced, they might be lost and not transferred to the surgical bed when the clot is implanted, although the magnitude of this may be a function of the mode of delivery.³¹ Platelet activation can be quantified by measuring P-selectin, a protein contained in the inner face of the α granule membrane.⁴⁰ Upon platelet activation, the α granule membrane fuses with the platelet membrane, and this protein becomes expressed on the platelet surface, where it can be measured.^{40,50} Thus, measurement of P-selectin provides valuable information regarding PRP.^{31,32}

Although a standard laboratory centrifuge can be used to produce PRP, the process is labor intensive, generally requiring two spins and multiple transfers; consequently, sterility may be difficult to maintain.^{33,50,53} Furthermore, such techniques may not reliably produce the desired increase in platelet concentration or in the levels of key secretory proteins.⁶

Standard cell separators and salvage devices, such as CATS (Fresenius, Wilmington, DE), Sequestra (Medtronic, Minneapolis, MN), Haemonetics Cell Saver 5 (Haemonetics Corp., Braintree, MA), and others, generally operate on a full unit of blood.^{32,34,54} In general, they use continuous flow centrifuge bowl or continuous flow disk separation technology and both a hard (fast) and a soft (slow) spin, yielding platelet concentrations from two to four times baseline.^{32,54,55} Weibrich et al⁴⁶ described a discontinuous technique with a cell separator that produces a five-fold increase in platelet count. The red blood cells, and some, or all, of the PPP can be returned to the patient to maintain volume stasis.^{24,54}

There are many surgical procedures, such as periodontal, cosmetic, and others, in which relatively small volumes of PRP are required.^{22,25} Some of these procedures may be performed in an office setting, which makes drawing a full unit of blood undesirable and would legally preclude the reintroduction of the unused portion of the blood to the patient.³³ Consequently, small, compact office systems have been developed that produce approximately 6 mL of PRP from 45 to 60 mL of blood, obviating the need for reinfusion.^{6,22,33,56,57} Such systems include the GPS (Cell Factor Technologies, Inc., Warsaw, IN), the PCCS (Implant Innovations, Inc., Palm Beach Gardens, FL), the Symphony II (DePuy, Warsaw, IN), the SmartPReP (Harvest Technologies Corp, Norwell, MA), and the Magellan (Medtronic, Minneapolis, MN).^{6,31,32,34,37,54,56} As a group, these systems differ widely in their ability to collect and concentrate

platelets, with approximately 30% to 85% of the available platelets collected and from a less than a two-fold to about an eight-fold increase in the platelet concentration over baseline.^{6,31,32,54} However, it is imperative that investigators operate the equipment per the manufacturer's instructions and have confidence in the measurement technique for such characterization to have meaning. Some of the units permit the processing of two sets of disposables at once or performing multiple sequential processes using the same disposable set so that multiples of the 6-mL standard volume of PRP can be produced, if required.

In general, most systems do not concentrate the plasma proteins of the coagulation cascade.^{32,34,58} Plasma protein concentrations above baseline can be achieved through secondary ultrafiltration, as is done with the UltraConcentrator (Interpore Cross, Irvine, CA) and the Access System (Interpore Cross), in which the buffy coat collected from a centrifugation stage is passed through hollow fibers with an effective pore size of 30 kDa. As much as two-thirds of the aqueous phase is removed by filtration, thus the concentrations of the retained plasma proteins and formed elements are correspondingly increased.^{58,59} Hood and Arm⁵⁸ suggest that higher fibrinogen levels are associated with denser gels, which set up more reliably and can enhance the sustained release profile of the platelet factors, whereas Waters and Roberts³⁴ believe that further investigation of this is warranted.

There are numerous other variables that can influence the quality of the PRP, with Waters and Roberts³⁴ listing several. Using two cell-salvage devices and two tabletop devices over the course of 260 clinical cases, they found that PRP platelet counts were significantly higher when blood was drawn from a peripheral or central vein versus an arterial line; there was a downward trend in platelet counts with longer draw time; and there was no correlation between the patient's baseline platelet count and the PRP platelet count. Although there was no apparent relationship between PRP platelet count or fibrinogen concentration and a semiquantitative assessment of gel consistency, the study did not include the means by which to increase fibrinogen and other plasma protein levels above baseline.

PRP Secretory Protein Levels

The regenerative potential of PRP depends, to large extent, on the levels of secretory proteins that are released on activation.^{31,46} This will depend on several factors, including (1) the levels of these proteins contained in the platelets—a patient variable; (2) the processing technique, which will influence platelet

concentration and whether platelets are activated or fragmented during preparation; and (3) the completeness of platelet activation before measurement.^{31,33,46,60}

Levels of secretory proteins are commonly quantified by enzyme-linked immunosorbent assay (ELISA).^{31,46} Briefly, the technique involves the adsorption of antibodies specific to a single secretory protein onto the walls of a microplate. The platelet releasate is introduced to the microplate, and the single type of secretory protein specific for that adsorbed antibody binds to the antibody. After a rinse to remove unbound material, a similar antibody, this time linked to the enzyme horseradish peroxidase, is introduced, which binds to the bound secretory protein, leaving the enzyme exposed. After another rinse, the amount of bound peroxidase is proportional to the amount of the given secretory protein in the specimen. Next, a substrate solution (tetramethylbenzene and hydrogen peroxide) is added, which forms a color at a rate that is proportional to the amount of bound peroxidase. After a predetermined time is allowed for color development, the reaction is stopped by addition of sulfuric acid, and the optical density is measured at 450 nm. The concentration of the protein is read from a standard curve.

The secretory proteins must first be released from the platelets before their levels can be measured. This can be done through platelet activation or through physical disruption of the platelet/ α granule structure. The most common activation method is to add CaCl_2 and thrombin to the PRP.^{25,31,37,50} The thrombin directly activates platelets while the calcium ion replenishes that which was bound by the ACD-A anticoagulant. Although platelet activation using thrombin/ CaCl_2 represents the clinical method of initiating release, the activation that occurs during clot formation does not necessarily lead to complete release.³⁵ Another activation method uses ADP.^{61,62} Zimmerman et al³⁵ measured the levels of released PDGF- $\alpha\beta$, PDGF- $\beta\beta$, and TGF- β 1, following six methods that involved various combinations of freeze/thaw, hypotonic Triton X-100, calcium-thrombin solution, and centrifugation. The method that consistently yielded the greatest measured levels involved Triton X-100 treatment of 15 minutes, followed by freezing at -70°C . Although this method may yield useful information about the total amount of such proteins contained by the platelets at the time of PRP preparation, its clinical relevance is unclear because (1) this is not the method by which platelet releasate is expressed clinically; (2) without true activation, the released proteins may not all be in active form; and (3) platelets continue to synthesize and release proteins for several days, in situ, after

placement. Ultimately, the research question to be answered should dictate the method used.

Secretory protein levels typically are expressed in concentration units (eg, measured amount per milliliter of releasate or per 100,000 platelets). Using a freeze/thaw cycle to release proteins, Weibrich et al,⁴⁶ measured the levels of PDGF- $\alpha\beta$, PDGF- $\beta\beta$, TGF- β 1, TGF- β 2, and IGF-1 in specimens of PRP derived from 115 patients. Minimum and maximum values for each typically spanned one to two orders of magnitude, with means and standard deviations of 117.5 ± 63.4 ng/mL, 9.9 ± 7.5 ng/mL, 169.4 ± 84.5 ng/mL, 0.4 ± 0.3 ng/mL, and 84.2 ± 23.6 ng/mL, respectively. Correlations in the following pairs of growth factors were found: PDGF- $\alpha\beta$ /PDGF- $\beta\beta$, PDGF- $\alpha\beta$ /TGF- β 1, and PDGF- $\beta\beta$ /TGF- β 1. There was little, or no, correlation between the levels of these individual proteins and donor age and gender attributes. Eppley et al³¹ produced PRP from 10 healthy volunteers and, following thrombin/ CaCl_2 -induced activation, measured 17 ± 8 ng/mL (PDGF- $\beta\beta$), 120 ± 42 ng/mL (TGF- β 1), 955 ± 1030 ng/mL (VEGF), 129 ± 61 ng/mL (EGF), and 72 ± 25 ng/mL (IGF-1). Zimmerman et al,³⁵ using various methods to initiate platelet release, measured levels of PDGF- $\alpha\beta$, PDGF- $\beta\beta$, and TGF- β 1 in PRP preparations both rich and deficient in white blood cells, expressing levels both on a per milliliter and a per 100,000 platelet basis. In general, for a given protein, there was a three- to four-fold range in measured level versus release method, and the authors concluded that the release of each growth factor by a given sample preparation method must be investigated and interpreted separately. Several other investigators also have published values for the quantities of secretory proteins released by platelets.^{6,21,63,64}

It would appear reasonable that the concentration of released secretory proteins would be linearly proportional to the platelet concentration ratio. Although such a relationship between PDGF- $\alpha\beta$, TGF- β , VEGF, and EGF and platelet count has been reported,⁶¹ an additional study by the same principal author confirmed this relationship for PDGF, TGF- β , and EGF, but not for VEGF and IGF.⁶² Although a general trend of increasing protein content and platelet count for a variety of secretory proteins (PDGF- $\alpha\beta$, PDGF- $\beta\beta$, TGF- β 1, TGF- β 2, VEGF, EGF, IGF-1) was demonstrated, Eppley et al³¹ and Weibrich et al⁴⁶ found little value in using platelet concentration ratio to predict resultant PRP secretory protein levels. Using thrombin/ CaCl_2 to activate the platelets, Eppley et al,³¹ found variable concentration ratios for several secretory proteins, all lower than the platelet concentration ratio. It is possible that

incomplete platelet activation and variable binding of the expressed proteins to the clot, which would not have been measured in the PRP supernatant, could be a partial explanation.

Whereas the measurement of secretory protein levels in PRP on a routine clinical basis may be difficult to justify, measurement may have value during the course of research studies in which it is desired to gain greater insight into the molecular basis of the mechanism of the effect of PRP.

Handling and Application of PRP

Once the PRP is prepared, it is stable, in the anticoagulated state, for 8 hours, or longer, permitting the blood to be drawn before surgery and used, as needed, during lengthy procedures.^{6,33,65} The PRP must be activated for the platelets to release their α granule contents, with the clot that forms providing a vehicle to contain the secreted proteins and maintain their presence at the wound site. It is most common to accomplish this by adding a solution of 1000 units of topical bovine thrombin per milliliter of 10% CaCl_2 to the PRP.^{2,22,24,31} Marx et al²⁴ described a technique in which 6 mL of PRP, 1 mL of the calcium chloride/thrombin mix, and 1 mL of air are introduced into a 10-mL syringe, with the air acting as a mixing bubble. The syringe is agitated for 6 to 10 seconds to initiate clotting, and then the clot is delivered. Man et al,²² described an alternative technique for delivering the activated PRP. The PRP and calcium chloride/thrombin solution are mixed in a 10:1 (v/v) ratio by use of a dual syringe system. The PRP is drawn into a 10-mL syringe and the activating solution is drawn into a 1-mL syringe. Both syringe plungers are connected to move in concert with both output ports connected to a dual spray applicator tip that allows both solutions to be mixed as they are applied to the surgical bed. PPP can be delivered similarly to function as a fibrin glue or hemostatic agent.^{22,25} Because the alpha granules quickly release their contents on activation, Marx⁶ states that the clotted PRP should be used within 10 minutes of clot initiation. This is not an issue with the dual syringe spray delivery because the PRP is delivered to the wound site immediately after activation. In the case of other mixing techniques, it is important to transfer the clot to the surgical site before clot retraction; otherwise, the clot that is transferred may be deficient in the secretory proteins that were expressed.

In the early to mid-1990s, there were a few reports of issues associated with development of anti-bovine antibodies (antibovine factor V) that cross-reacted with human clotting factors in response to

use of the bovine product to provide hemostasis to open, bleeding vessels.⁶⁶⁻⁶⁸ However, current processing methods remove much more bovine factor V contamination, and its use in PRP gel precludes its exposure to the systemic circulation, possibly explaining why PRP has not produced postoperative bleeding or shown elevation in postoperative prothrombin time or the development of detectable anti-bovine antibodies.¹⁰

Clinical Use of PRP

Advocates think that the benefits of using PRP include an increase in bone and wound healing and a decrease in postoperative infection, pain, and blood loss.³⁴ There have been numerous publications regarding the use of PRP for a diverse range of clinical applications, including periodontal and oral surgery,^{14,16,18,20,24-27,29} maxillofacial surgery,²⁹ cosmetic and plastic surgery,^{2,22,28} spinal fusion,^{15,19,21} heart bypass surgery,¹⁷ and treatment of chronic skin and soft tissue ulcers.^{23,69} The details of the quantity of PRP used and the methods of application are procedure specific. Although most of these studies have yielded excellent outcomes, many had no controls and may have been only small case studies. Consequently, these are of limited usefulness in conclusively demonstrating wound healing enhancement by PRP in the clinical arena. However, a small collection exists of clinical studies with prospective or retrospective controls that have demonstrated a significant enhancement of hard and soft tissue healing with the use of PRP, as summarized in Table 1. Such studies should serve as a model for future investigations so that the merits of PRP use in each application can be unequivocally demonstrated or refuted.

CONCLUSIONS

It makes evolutionary sense that platelets direct wound healing because, by design, they immediately appear at the site of tissue injury in large numbers. Under ordinary circumstances, they will be present exactly where and when needed to create a local environment conducive to tissue regeneration. Through the release of secretory proteins from their α granules on activation, platelets set the pace of wound healing, with their effects manifest long after the clot has been cleared. Basic science studies support the hypothesis of enhancement of healing by the placement of a supraphysiological concentration of autologous platelets at the site of tissue injury. A small number of controlled, clinical studies provide evidence that the use of autologous platelet rich

Table 1. Summary of Controlled, Clinical Studies Using PRP

General Application	Study Design	Key Results	Ref.
Cardiopulmonary bypass	Reinfuse PRP after heparin reversal to examine hemostasis; controlled	65% less banked blood products used in the PRP group	(17)
Mandibular bone augmentation for dental implants	Core biopsies taken for placement of dental implants; 44 patients with grafts using PRP and 44 controls	Grafts matured faster with PRP; trabecular bone area at 6 mo: PRP: 74% ± 11% Non-PRP: 55% ± 6%	(13)
Diabetic foot ulcers	Meta-analysis of more than 25,000 cases with and without (control) platelet therapy	Ulcers treated with platelets were 14%–59% more likely to heal than were those without platelets	(23)
Periodontal	23 patients underwent tooth extraction; 5 received PRP and 5 got PRP/autograft; 10 patients were controls with 3 split-mouth PRP and control	Biopsy at 10 and 16 weeks showed total bone regeneration in 8/10 PRP, with partial regeneration in 2/10; no mature bone in controls; in split-mouth, PRP side had greater bone regeneration	(14)
Lumbar spine fusion	23 patients underwent TLIF using PRP, compared to historical controls; 2-year minimum follow-up	Using PRP, got fusion in 100% of 1-level and 90% of 2-level fusions; PRP group had faster healing, but no significant difference in pseudarthrosis rates between experimental and control groups	(19)
Cutaneous ulcers	Randomized, controlled study of 32 patients with chronic, nonhealing cutaneous wounds of the lower extremity treated with platelets or placebo	81% of platelet-treated patients had epithelialization by 8 weeks compared with 15% of controls; crossover treatment of controls with platelets resulted in epithelialization by an average of 7.1 weeks	(69)

plasma does accelerate soft and hard tissue healing in at least a limited number of applications. Many more controlled clinical studies will be required to confirm these results and to establish under which conditions the application of platelet rich plasma has merit.

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