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Platelet-Rich Plasma Contains High Concentrations of DKK1, A Potent Inhibitor of Wnt Signaling that Limits Bone Regeneration and Hair Growth

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Abstract

Platelet-rich plasma (PRP) is a popular autologous therapy in the tissue regenerative technology space based on its enriched milieu of platelet-released growth factors (GFs), including platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF). PRP is frequently used for hair restoration, wound healing, tendon and ligament repairs, and other soft-tissue settings, but PRP did not fulfill its original promise for enhancing bone grafts and bone augmentation in dentistry or orthopedics because it does not consistently promote osteogenesis. The effects of PRP on bone regeneration are often described as controversial, contradictory, and confusing, including evidence that greater platelet enrichment often leads to lesser osteogenic responses. The latter paradox suggests that limited PRP efficacy in bone regeneration may relate to platelet-derived factors that inhibit osteoblasts rather than inadequate GF concentrations. One candidate platelet-derived anti-osteogenic factor is Dickkopf-1 (DKK1), a potent soluble inhibitor of Wnt signaling that limits bone regeneration after bone damage. Recognizing that platelets store DKK1 in their alpha granules and rapidly release it upon platelet activation, we evaluated levels of DKK1, PDGF, and VEGF in human serum, plasma, platelet-poor plasma (PPP), and PRP. Sclerostin, another inhibitor of Wnt signaling that limits bone formation in undamaged bone, was also evaluated because platelets are not known to secrete sclerostin. We show that DKK1, PDGF, and VEGF concentrations are much higher in serum versus plasma, probably due to platelet activation during serum preparation. DKK1, PDGF, and VEGF concentrations were also higher in PRP versus plasma, and in plasma versus PPP. Activation of plasma or PRP with thrombin resulted in marked increases in DKK1, PDGF, and VEGF concentrations, with DKK1 levels in activated PRP being over 40-fold higher than the minimum concentration reported to inhibit osteogenesis in cell culture models. Conversely, sclerostin levels in plasma and PRP were unaffected by platelet activation and were significantly lower in serum versus standard plasma, providing new evidence that platelets do not secrete sclerostin. These results suggest that the beneficial effects of high GF levels in PRP may be limited by the presence of high levels of DKK1 released from the same platelet alpha granules.

Keywords: Platelet-Rich Plasma; DKK1; Wnt Signaling; Bone Regeneration; Hair Growth

Abbreviations: PRP: Platelet-Rich Plasma; PDGF: Platelet-Derived Growth Factor; VEGF: Vascular Endothelial Growth Factor; PPP: Platelet-Poor Plasma; GFs: Growth Factors; FGF: Fibroblast Growth Factor; BMI: Body Mass Index.

Introduction

Platelet-rich plasma (PRP) is used as a tissue regeneration therapy in a variety of clinical settings, including bone defects [1], spinal fusion [2], fracture healing [3,4], hair regeneration [5], dermal wound healing [6], and tendon and ligament repairs [7,8]. PRP was originally developed to enhance bone grafting based on its enriched concentrations of growth factors (GFs) that are stored in platelets and released upon platelet activation[9]. PRP contains over 20 growth factors, including platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and transforming growth factor beta (TGF-β) [10]. These and several other platelet-released factors have osteogenic and bone regenerative properties, but early experiments indicated that PRP did not enhance the efficacy of bone grafts [11]. Subsequent review articles indicate that the efficacy of PRP for bone grafting and bone healing is inconsistent and often lacking, with many findings described as controversial, contradictory, and confusing[4,12-16]. Suboptimal bone regeneration with PRP could potentially reflect insufficient growth factor concentrations, but PRP with the greatest concentrations of growth factors and platelets often shows relatively lesser osteogenic effects [12,13,17-22]. This paradox suggests that suboptimal bone regeneration with PRP could reflect the presence of platelet-derived anti-osteogenic factors that limit osteoblast responses to platelet-released growth factors. One candidate factor is Dickkopf-1 (DKK1), a secreted factor that limits bone regeneration by inhibiting Wnt signaling [23-31]. DKK1 is expressed by osteocytes primarily during growth and in response to skeletal injury [30,32]. DKK1 is also stored in platelet alpha granules [33] and rapidly released upon platelet activation [34]. The clinical relevance of activated platelets as a source of DKK1 is supported by evidence that circulating DKK1 levels are lower in aspirin users [35] and higher in patients with recent hip fracture [36]. Moreover, rodent and non-human primate data indicate that anti-DKK1 antibodies (DKK1-Ab) enhance bone regeneration while having minimal osteogenic effects on uninjured bones in adult animals [26,27,30,31]. DKK1 also promotes hair loss [23,37-41] and inhibits dermal wound healing [42] suggesting that platelet-released DKK1 may also limit PRP benefits for hair restoration and wound healing.

Sclerostin is another soluble inhibitor of Wnt signaling that inhibits bone formation [43-45]. Similar to DKK1, sclerostin expression by osteocytes increases after skeletal injury [30]. In contrast to DKK1, osteocytes constitutively secrete biologically meaningful levels of sclerostin long after skeletal maturity, and sclerostin is not known to be secreted by platelets. Anti-sclerostin antibodies (Scl-Ab) increase bone mass throughout the intact skeleton of rodents [46-50], non-human primates [31,51] and adult humans [52-55]. Scl-Ab also increases bony callus development in low-hurdle fracture healing models [49,56-58], though fracture healing benefits with Scl-Ab are inconsistent in challenging fracture healing models [30,31,59-62] and were not apparent in clinical fracture healing trials [63,64].

The current study evaluated the levels of DKK1 and sclerostin in human PRP, standard plasma, platelet-poor plasma (PPP), and serum. PDGF and VEGF were also evaluated as representative platelet-released GFs. We also assessed the effects of thrombin-induced platelet activation on levels of these four analytes in standard plasma and PRP. The results indicate that DKK1, PDGF, and VEGF concentrations are significantly higher in serum and PRP versus standard plasma, and markedly higher in activated versus nonactivated PRP and plasma. These findings suggest that plasma is a more appropriate matrix than serum for evaluating bioavailable levels of circulating DKK1, PDGF, and VEGF, and may also suggest that the regenerative effects of PRP on some tissues may be limited by its enriched levels of DKK1.

Materials and Methods

Plasma, PRP, and Serum Preparation

Human serum and heparinized plasma samples were prepared from whole blood collected by venipuncture from four healthy adult volunteers. Donors were non-smoking Caucasian males with an age range of 27-62 years, weight range of 74.8-86.2 kg, and body mass index (BMI) range of 22 [4-25]. Three donors had type O-positive blood and the other donor was type B-negative. Plasma and serum samples were respectively pooled and gently mixed. A 20 mL aliquot of pooled plasma was centrifuged at 2000 g for 10 minutes. After centrifugation, 15 mL of the supernatant was transferred to a separate tube and the remaining plasma, which constituted the PRP, was gently resuspended by pipetting. The aforementioned supernatant was centrifuged again at 2000 g for 10 minutes, and 4 mL of the resulting supernatant, which constituted platelet-poor plasma (PPP), was transferred to a fresh tube. Platelet concentrations in PPP, plasma, and PRP were determined with an automated hematology analyzer.

Platelet activation

Aliquots of plasma and PRP were activated by adding 100 U/mL of bovine thrombin and 50 μL/mL of 10% calcium chloride. Activated and non-activated samples of plasma and PRP were incubated for 30 min at 37° C and 5% CO2. After incubation, aqueous samples of activated plasma and PRP were removed from the fibrin gel by gentle pipetting. The activated aqueous PRP was centrifuged at 2000 g for 10

minutes to remove excess platelets before analyte assays. All samples were stored at -80° C until analysis.

DKK1, Sclerostin, PDGF, and VEGF Analyses

Serum, plasma, PPP, and PRP samples were assayed in triplicate for concentrations of the Wnt inhibitors DKK1 and sclerostin and two platelet-released growth factors, PDGF and VEGF. Samples were diluted 1X and 10X based on pilot studies that narrowed down the expected concentrations of each analyte relative to their standard curves. Concentrations of PDGF (PDGF-BB) and VEGF (VEGF165) were determined with human ELISAs from R&D Systems (PDGF: Cat# DBB00; VEGF: Cat# DVE00) using a SpectraMax 250 microplate reader from Molecular Devices. Human DKK1 and sclerostin were measured with a Luminex multiplex MagPix system using reagents from Millipore (Cat# HBNMAG-51). Reported concentrations of VEGF, DKK1, and sclerostin were based on 1X dilution, and PDGF concentrations were based on 10X dilution.

Statistics

Analyte concentrations in plasma versus serum were compared by unpaired T-tests. Platelet counts and analytes in PPP, plasma, and PRP were compared by one-way ANOVA and Tukey's multiple comparison test. The effects of platelet activation on analyte levels in plasma and PRP were compared using two-way ANOVA and Tukey's multiple comparison test. All statistical analyses were performed with GraphPad Prism Version 9.5.1.

Results

Wnt Inhibitor and Growth Factor Concentrations Differ in Serum Versus Plasma

DKK1 levels are significantly higher whereas sclerostin levels are significantly lower in serum versus standard plasma (Figure 1). Similar to DKK1, concentrations of platelet-released growth factors PDGF and VEGF are significantly higher in serum versus plasma (Figure 1).

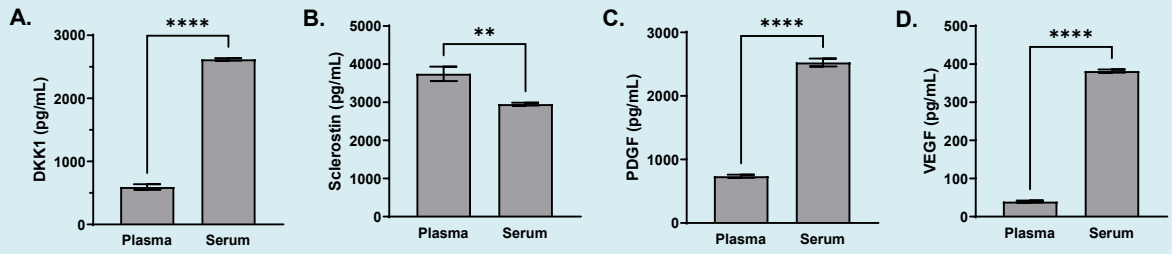

Figure 1: Concentrations of the Wnt inhibitors DKK1 and sclerostin (A-B) and the growth factors PDGF and VEGF (C-D) in normal human serum versus plasma. Data represent means ± SD. **P < 0.01, ****P < 0.0001 by T test.

Concentrations of Platelets, Wnt Inhibitors, and Growth Factors in PPP, Plasma, and PRP

Mean platelet concentrations were 17.6 ± 4.5 (SD) million/mL for PPP, 165.7 ± 27.0 million/mL for standard plasma, and 856.3 ± 79.4 million/mL for PRP (Figure 2).

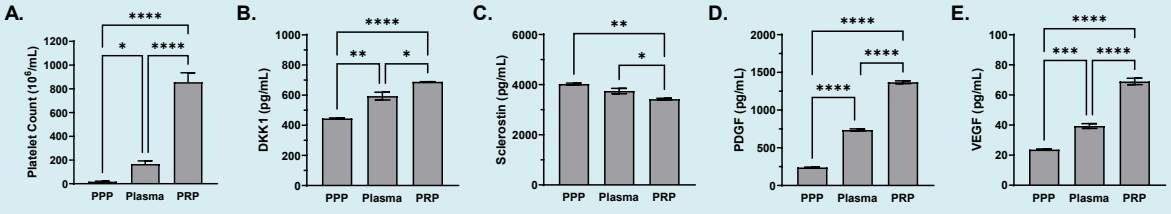

Figure 2: A: Platelet counts in human PPP, standard plasma, and PRP. B-E: DKK1, sclerostin, PDGF-BB, and VEGF concentrations in PPP, plasma, and PRP. Data represent means and standard deviations. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001 by one-way ANOVA and Tukey's multiple comparison test.

Wnt Inhibitor and Growth Factor Levels after Activation of Plasma and PRP

Activation of standard plasma or PRP with thrombin and calcium chloride led to significantly greater concentrations of DKK1, PDGF, and VEGF versus non-activated control samples (Figure 3). DKK1, PDGF, and VEGF concentrations were also significantly higher in activated PRP versus activated standard plasma. Sclerostin concentrations were similar in plasma and PRP with and without activation (Figure 3).

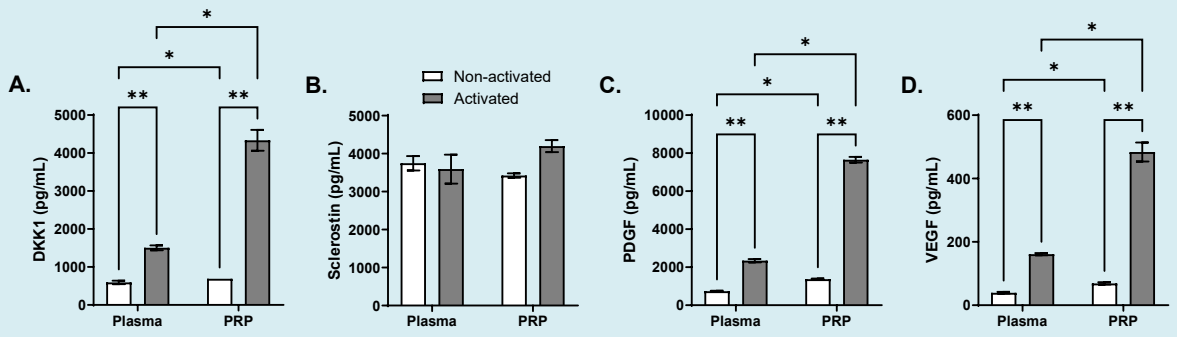

Figure 3: DKK1, sclerostin, PDGF, and VEGF concentrations in human plasma and PRP with and without activation with thrombin and calcium chloride. Data represent means ± SD; note that error bar for DKK1 in non-activated PRP (not visible) is 1.4. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001 by two-way ANOVA and Tukey's multiple comparison test.

Discussion

PRP is used as a regenerative therapy in a variety of injury, disease, and aesthetics settings. PRP was originally developed for bone augmentation, but its osteogenic effects proved to be elusive and inconsistent [4,12,13,15]. Based on evidence that platelets store and secrete DKK1 [33,34], a potent inhibitor of Wnt signaling and osteogenesis [28], we hypothesized and confirmed that DKK1 levels are higher in human PRP versus standard plasma, and that DKK1 levels in plasma and PRP are markedly increased by platelet activation. These new findings could potentially explain why the osteogenic effects of PRP are often inversely related to the degree of platelet and GF enrichment, a frequent observation that has been described as a conundrum [12,13,17-22]. In contrast to DKK1, levels of sclerostin, a different soluble inhibitor of Wnt signaling, are similar in plasma and PRP before and after platelet activation, providing new evidence that human platelets do not secrete sclerostin.

We also show that DKK1, PDGF, and VEGF levels are much higher in serum versus plasma. This difference likely reflects platelet activation during serum preparation, which is supported by our evidence that the treatment of plasma or PRP with platelet-activating thrombin greatly increases the levels of all three factors. These findings suggest that the DKK1 levels measured in prepared serum may be highly exaggerated relative to the bioavailable (i.e., extracellular) fraction of DKK1 in blood, which would position plasma as the preferred matrix for measuring bioavailable DKK1. Numerous articles show significant differences or changes in serum DKK1 levels in various conditions or diseases [36,65-68], but the forced release of substantial amounts of DKK1 from platelets during serum preparation could nonetheless obscure underlying differences in the amounts of DKK1 secreted by other cell types, including osteocytes and myeloma cells [69,70]. We also note that DKK1 released from activated platelets would not be reflected in gene expression analyses, which investigators should consider when relying on and interpreting mRNA-based evaluations of DKK1 expression.

Opposite of DKK1 results, sclerostin levels were significantly lower in serum vs plasma. Similar findings in a previous report were hypothetically attributed to the presence of exogenous heparin in the plasma samples, which may have displaced proteins from sclerostin in a manner that unmasked epitopes to which the assay's anti-sclerostin antibodies bind [71]. We suggest an alternative or additional hypothesis based on the existence of a thrombin cleavage domain that renders sclerostin susceptible to thrombin-mediated degradation and inactivation [72]. This biology suggests that endogenous thrombin activity during serum preparation could reduce sclerostin levels by causing its degradation, which could also explain why the recovery of recombinant sclerostin is reduced upon its exposure to human serum [71]. We note, however, that the activation of human plasma and PRP with bovine thrombin for 30

minutes was not associated with lower sclerostin levels. Nonetheless, evidence that thrombin degrades sclerostin [72] while provoking rapid DKK1 release from platelets [34] suggests that thrombin may act as a molecular 'switch' that transiently shifts the regulation of Wnt signaling away from sclerostin and towards DKK1 at sites of bone damage. Such an arrangement may confer independence in the regulation of osteogenesis, allowing sclerostin to primarily govern bone mass systemically while DKK1 primary governs local bone regeneration [27,28,30,31].

The gradient of DKK1 levels in non-activated PPP versus plasma versus PRP is comparatively low relative to the degree of platelet and GF enrichment. For example, DKK1 levels were only 55% higher in non-activated PRP versus non-activated PPP ($p < 0.05$), whereas platelet counts were 4765% higher, PDGF was 472% higher, and VEGF was 191% higher. This may suggest that DKK1 is somewhat less susceptible to 'spontaneous' release from resting platelets compared with PDGF and VEGF. Conversely, platelet activation of plasma and PRP by thrombin caused similarly robust increases in the levels of all three factors. Platelet activation at sites of tissue injury and bleeding is likely to provoke DKK1 release, which may explain the transient appearance of DKK1 protein in blood vessels adjacent to skeletal fractures [30]. Experimental fracture healing is impaired by DKK1 [24] and is consistently promoted by DKK1-Ab [25-27,29-31], implying that one key biological role of DKK1 is to provide homeostatic control over Wnt signaling and perhaps other regenerative pathways in damaged bone. This role may have evolutionary value by preventing excessive (over-engineered) healing responses, but DKK1 inhibition may be beneficial for individuals with suboptimal bone healing potential, such as elderly individuals and patients with diabetes. Notably, both of these populations have been reported to have elevated circulating DKK1 levels [35,36,68].

In addition to limiting bone regeneration, DKK1 also inhibits hair growth [23,37-41]. Mouse data show that locally-injected DKK1 promotes hair follicle regression [39] and that transgenic overexpression of DKK1 in skin leads to near-total baldness [37]. DKK1 is also endogenously overexpressed in bald scalp regions of patients with androgenetic alopecia [73], and androgen-induced upregulation of DKK1 expression in cultured human hair follicles causes follicular keratinocyte apoptosis [40]. These findings raise the possibility that platelet-released DKK1 may limit PRP benefits as a hair restoration therapy.

The current work has several limitations, including the lack of proof that high DKK1 levels in PRP limits its ability to stimulate the regeneration of bone or other tissues. Evidence that PRP inhibits Wnt signaling in cultured chondrocytes [74] suggests that it may, as does evidence that the minimum DKK1 concentration required to inhibit osteogenesis [23] is over 40-fold lower than the average DKK1 concentration detected in activated PRP. Another limitation is that levels of the four analytes were not evaluated in activated versus non-activated PPP, which may have had utility as an additional control condition. We also lack details on how long blood samples remained in serum collection tubes prior to removing and refrigerating the serum, which is an uncontrolled variable that may have influenced sclerostin levels via its possible degradation by endogenous thrombin.

In summary, human serum and PRP have higher levels of the Wnt inhibitor DKK1 compared with standard plasma, and DKK1 levels in standard plasma and PRP are greatly increased after platelet activation. Conversely, concentrations of the Wnt inhibitor sclerostin are lower in serum versus plasma, similar in plasma versus PRP, and unaffected by platelet activation. These results confirm that human platelets store and secrete DKK1 but not sclerostin, while also suggesting that plasma may be the preferred matrix over serum for evaluating bioavailable levels of DKK1 in blood. Future research may determine whether inhibiting or removing DKK1 from PRP enhances its regenerative potential. DKK1 can be selectively removed from PRP using DKK1-Ab-coated magnetic beads [75], but this method has less utility for PRP. The reason is that most DKK1 in PRP is sequestered within platelets, and if this DKK1 is made available via platelet activation, much of the PRP converts to fibrin gel that is not compatible with this immunodepletion technique. A more expedient experimental and therapeutic approach might be to mix PRP with a DKK1-Ab or a bispecific antibody that inhibit both DKK1 and sclerostin [30].

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