Growth Factor and Catabolic Cytokine Concentrations Are Influenced by the Cellular Composition of Platelet-Rich Plasma

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Background: Previous studies of bioactive molecules in platelet-rich plasma (PRP) have documented growth factor concentrations that promote tissue healing. However, the effects of leukocytes and inflammatory molecules in PRP have not been defined.

Hypothesis: The hypothesis for this study was that the concentration of growth factors and catabolic cytokines would be dependent on the cellular composition of PRP.

Study Design: Controlled laboratory study.

Methods: Platelet-rich plasma was made from 11 human volunteers using 2 commercial systems: Arthrex ACP (Autologous Conditioned Plasma) Double Syringe System (PRP-1), which concentrates platelets and minimizes leukocytes, and Biomet GPS III Mini Platelet Concentrate System (PRP-2), which concentrates both platelets and leukocytes. Transforming growth factor-β1 (TGF-β1), platelet-derived growth factor–AB (PDGF-AB), matrix metalloproteinase-9 (MMP-9), and interleukin-1β (IL-1β) were measured with enzyme-linked immunosorbent assay (ELISA).

Results: The PRP-1 system consisted of concentrated platelets (1.99$\times$) and diminished leukocytes (0.13$\times$) compared with blood, while PRP-2 contained concentrated platelets (4.69$\times$) and leukocytes (4.26$\times$) compared with blood. Growth factors were significantly increased in PRP-2 compared with PRP-1 (TGF-β1: PRP-2 = 89 ng/mL, PRP-1 = 20 ng/mL, $P < .05$; PDGF-AB: PRP-2 = 22 ng/mL, PRP-1 = 6.4 ng/mL, $P < .05$). The PRP-1 system did not have a higher concentration of PDGF-AB compared with whole blood. Catabolic cytokines were significantly increased in PRP-2 compared with PRP-1 (MMP-9: PRP-2 = 222 ng/mL, PRP-1 = 40 ng/mL, $P < .05$; IL-1β: PRP-2 = 3.67 pg/mL, PRP-1 = 0.31 pg/mL, $P < .05$). Significant, positive correlations were found between TGF-β1 and platelets ($r^2 = .75$, $P < .001$), PDGF-AB and platelets ($r^2 = .60$, $P < .001$), MMP-9 and neutrophils ($r^2 = .37$, $P < .001$), IL-1β and neutrophils ($r^2 = .73$, $P < .001$), and IL-1β and monocytes ($r^2 = .75$, $P < .001$).

Conclusion: Growth factor and catabolic cytokine concentrations were influenced by the cellular composition of PRP. Platelets increased anabolic signaling and, in contrast, leukocytes increased catabolic signaling molecules. Platelet-rich plasma products should be analyzed for content of platelets and leukocytes as both can influence the biologic effects of PRP.

Clinical Relevance: Depending on the clinical application, preparations of PRP should be considered based on their ability to concentrate platelets and leukocytes with sensitivity to pathologic conditions that will benefit most from increased platelet or reduced leukocyte concentration.

Keywords: platelet-rich plasma; PRP; IL-1β; MMP-9; TGF-β; PDGF; growth factors

Platelet-rich plasma (PRP) is an autologous therapy increasingly used in human and veterinary medicine. Platelet-rich plasma treatment to promote tissue healing is well documented in dental, maxillofacial, orthopaedic, and diabetic injuries.[14,19,24] The basis for treatment has historically

centered on maximizing the growth factors found in platelet α-granules to promote an anabolic environment at the site of injury. Numerous studies have examined the effects of PRP in vitro and in vivo, demonstrating benefits including improved cellular remodeling and decreased time to healing.[1] Platelets and PRP have been shown to contain and release growth factors including transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and basic fibroblast growth factor (bFGF). There are numerous ways to generate PRP, and the molecular characterization of PRP is typically limited to platelet and growth factor analysis despite concerns for the effects of concentrated leukocytes in the production of PRP.[24,31] Leukocytes contain and produce biologically active cytokines that are primarily catabolic or inflammatory in nature and might influence the clinical outcome of PRP application.

TABLE 1
Platelet, White Blood Cell, and Hematocrit Concentrations in Venous Blood, PRP-1, and PRP-2*

	Platelets $\times 10^3/\mu L$		White Blood Cells $\times 10^3/\mu L$		Hematocrit	
	Mean $\pm$ SD	Range	Mean $\pm$ SD	Range	Mean $\pm$ SD	Range
Venous blood	$183 \pm 39.6^{a\dagger}$	114-232	5.66 ± 1.41^a	2.8-7.7	35.1 ± 2.51^a	31-39
PRP-1	$361 \pm 87.0^{a\dagger}$	245-493	0.68 ± 0.42^c	0.2-1.4	0.46 ± 0.52^c	0-1.0
PRP-2	701 ± 473^b	114-1520	23.7 ± 5.91^b	14.3-30.1	4.82 ± 1.83^b	3.0-8.0

*N = 11. Superscript letters indicate significant difference between groups, but within hematocrit, white blood cell, or platelet-rich plasma (PRP), by analysis of variance with Tukey post hoc test, $P < .05$.
†Indicates significant difference between venous blood and PRP-1 using 2-sample t test, $P < .05$.

Commercial preparations vary dramatically in their ability to concentrate platelets and leukocytes. The majority of PRP research does not investigate the effects of leukocytes and catabolism. However, delivery of concentrated leukocytes to a site of injury might not provide a favorable environment for tissue repair or healing.[7,23] In support of this, our laboratory demonstrated that leukocyte concentration is positively correlated with catabolic gene expression in tendon and ligament and negatively correlated with tendon and ligament matrix gene expression.[13]

The objective of this study was to compare 2 commercial systems that generate PRP: Arthrex ACP (Autologous Conditioned Plasma) Double Syringe System (hereafter called PRP-1) (Arthrex Inc, Naples, Florida) and Biomet GPS III Mini Platelet Concentrate Separation Kit (hereafter called PRP-2) (Biomet Inc, Warsaw, Indiana). Comparisons of cellular composition of each formulation of PRP and venous blood were followed with evaluation of growth factors and catabolic mediators to characterize the signaling environment provided by these disparate products with the knowledge that PRP-1 concentrates platelets and minimizes leukocytes and PRP-2 concentrates both platelets and leukocytes compared with blood. We hypothesized that the anabolic growth factors TGF-β1 and PDGF-AB would positively correlate to platelet concentration, and the catabolic cytokines, matrix-metalloproteinase 9 (MMP-9), and interleukin-1β (IL-1β) would positively correlate to leukocyte concentration.

METHODS

All procedures were approved by university institutional regulatory bodies.

PRP Generation

Venous blood was collected from 11 healthy human volunteers into acid-citrate dextrose A (ACD-A) anticoagulant (1 mL ACD-A/10 mL blood). Each blood sample was divided and used to generate PRP using both systems. Neither PRP was buffered or activated after processing. Platelet, leukocyte (white blood cell [WBC]) concentrations, and hematocrit were determined in single 500-μL samples of venous blood, PRP-1, and PRP-2 using an ADVIA 2120 automated cell counter (Siemens Healthcare Diagnostics, Deerfield, Illinois). Samples were snap-frozen in individual aliquots for each enzyme-linked immunosorbent assay (ELISA) and stored at –80°C for analyses.

Growth Factor and Catabolic Cytokine Quantification

Growth factors and catabolic cytokines were measured in duplicate aliquots using ELISAs after a freeze-thaw process. Total TGF-β1 concentrations were determined using the TGFβ1 E$_{max}$ ImmunoAssay System (Promega Corporation, Madison, Wisconsin) and PDGF-AB was measured using the PDGF-AB Quantikine ELISA Kit (R&D Systems, Minneapolis, Minnesota). Total MMP-9 concentration was determined using the MMP-9 Biotrak Activity Assay (GE Healthcare Biosciences, Piscataway, New Jersey) and IL-1β concentrations were measured with the IL-1 beta/IL-1F2 Quantikine HS ELISA Kit (R&D Systems). Samples were analyzed using a multiple detection plate reader (Tecan SAFIRE, Durham, North Carolina).

Statistical Analysis

General analysis of variance with the Tukey post hoc test was performed on platelet, WBC, and hematocrit concentrations, as well as TGF-β1, PDGF-AB, and MMP-9 concentrations. A 2-sample t test was performed on platelet concentrations, cell count fold changes, and IL-1β concentrations. The IL-1β assay used was not validated for strongly hemolyzed samples, and therefore whole blood samples, which were hemolyzed after freeze-thaw, were not assayed and only PRP-1 and PRP-2 were compared. Preliminary studies demonstrated spike and recovery tests of PRP-1 and PRP-2 that were consistent with those published by R&D Systems (data not shown). Pearson correlation analyses were performed on TGF-β1 and PDGF-AB with platelet concentrations, and MMP-9 and IL-1β with WBC concentrations. Cell counts and ELISA values were normally distributed. Statistical analyses were performed on mean values of duplicates using Statistix 9 software (Analytical Software, Tallahassee, Florida) and significance was set at $P < .05$.

RESULTS

Hematology Values

Both systems produced approximately 3 mL PRP from a starting venous blood volume of 10 mL (PRP-1) or 30 mL

TABLE 2
Leukocyte Composition of PRP-1 and PRP-2[a]

| | Leukocytes/μL | | | | Percentage of Total Leukocytes | | | |
| | PRP-1 | | PRP-2 | | PRP-1 | | PRP-2 | |
Leukocyte	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range
Neutrophil	109 ± 202	0-600	8455 ± 7412	800-25 800	5.77 ± 9.2	0-28.6	29.5 ± 17.0	3.6-60.6
Lymphocyte	791 ± 303	100-3700	12 810 ± 3438	7700-17 200	73.9 ± 14.5	50-100	51.9 ± 12.4	28.7-72.3
Monocyte	91 ± 176	0-600	2236 ± 698	1400-3500	5.46 ± 6.6	0-16.7	9.07 ± 2.3	4.9-11.8
Large cell	91 ± 122	0-400	2046 ± 933	900-3800	6.13 ± 6.4	0-16.7	8.40 ± 4.1	3.1-17.4
Basophil	—		173 ± 47	100-200	—		0.71 ± 0.19	0.4-1.0
Eosinophil	—		109 ± 83	0-300	—		0.42 ± 0.26	0-0.7

[a]N = 11. Leukocyte composition in platelet-rich plasma (PRP) was determined using an automated cell counter. The mean count of eosinophils and basophils in PRP-1 was 0.
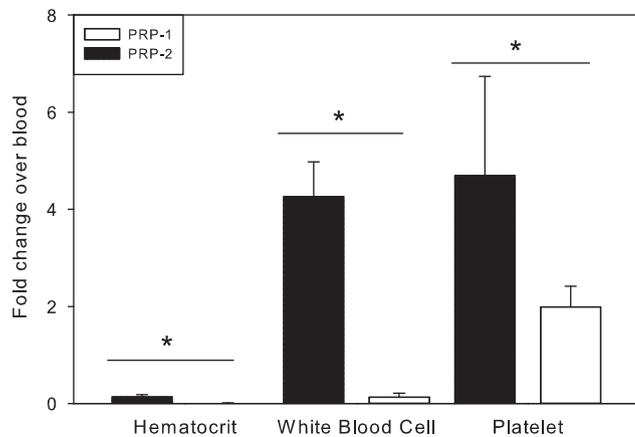
Figure 1. Hematocrit, leukocyte, and platelet concentrations in platelet-rich plasma–1 (PRP-1) or PRP-2 divided by venous blood to generate a fold change. Bars represent mean ± standard deviation of n = 11. Asterisks represent difference between groups, but within hematocrit, white blood cell, or platelet fold changes by 2-sample *t* test, $P < .05$.

(PRP-2). Platelet concentration in PRP-2 was significantly higher than other samples (Table 1). Blood and PRP-1 platelet concentration were statistically different when compared with each other. White blood cells were significantly different between all groups. Platelets and WBC fold changes (PRP value/whole blood value) were significantly higher in PRP-2 compared with PRP-1 (Figure 1). The predominant leukocyte in PRP-1 was lymphocytes (71.9%). The predominant leukocytes in PRP-2 were lymphocytes and neutrophils (82.4%). There was a 73-fold difference in neutrophils between PRP-2 and PRP-1 and a 23-fold difference in monocytes between PRP-2 and PRP-1 (Table 2).

Growth Factor Concentration: PDGF-AB and TGF-β1

The PDGF-AB concentration was significantly higher in PRP-2 than in other groups (Figure 2A). There was no statistically significant difference between blood and PRP-1.

There was a positive correlation between PDGF-AB and platelets that was significant (Figure 3B; $r^2 = .60$, $P < 0.001$). The TGF-β1 concentrations were significantly different for all groups, with PRP-2 as the highest concentration and blood as the lowest concentration (Figure 2A). There was a positive correlation between TGF-β1 and platelets that was significant (Figure 3A; $r^2 = .75$, $P < 0.001$).

Catabolic Mediator Concentration: MMP-9 and IL-1β

The MMP-9 concentration was significantly different between the PRP groups, with PRP-2 having the highest concentration and PRP-1 the lowest concentration (Figure 2B). There was a positive correlation between MMP-9 and neutrophils (Figure 4; $r^2 = .37$, $P < .001$). The IL-1β concentration was significantly higher in PRP-2 compared with PRP-1 (Figure 2B). There was a positive correlation between IL-1β and neutrophils (Figure 5A; $r^2 = .73$, $P < .001$), and a positive correlation between IL-1β and monocytes (Figure 5B; $r^2 = .75$, $P < .001$) that were both significant.

DISCUSSION

The findings of this study support our hypothesis that platelet and leukocyte composition of PRP reflects the concentration of growth factors and catabolic cytokines. The PRP-2 system had the highest concentration of both platelets and leukocytes, while PRP-1 had concentrated platelets compared with blood, but diluted leukocytes compared with blood. Correlation statistics between platelets and growth factors, and specific leukocytes and catabolic mediators, confirmed an association between the cellular content of the PRP with signaling molecules.

Our findings support other studies demonstrating that PRP concentrates platelets and therefore the growth factors contained in α-granules.[13,25] Both TGF-β1 and PDGF-AB are desirable in wound healing for their biochemical modifications of the local environment. Transforming growth factor–β1 has previously been show to improve collagen synthesis and deposition in vitro.[10,18] Platelet-derived growth factor has been shown to be chemotactic to macrophages
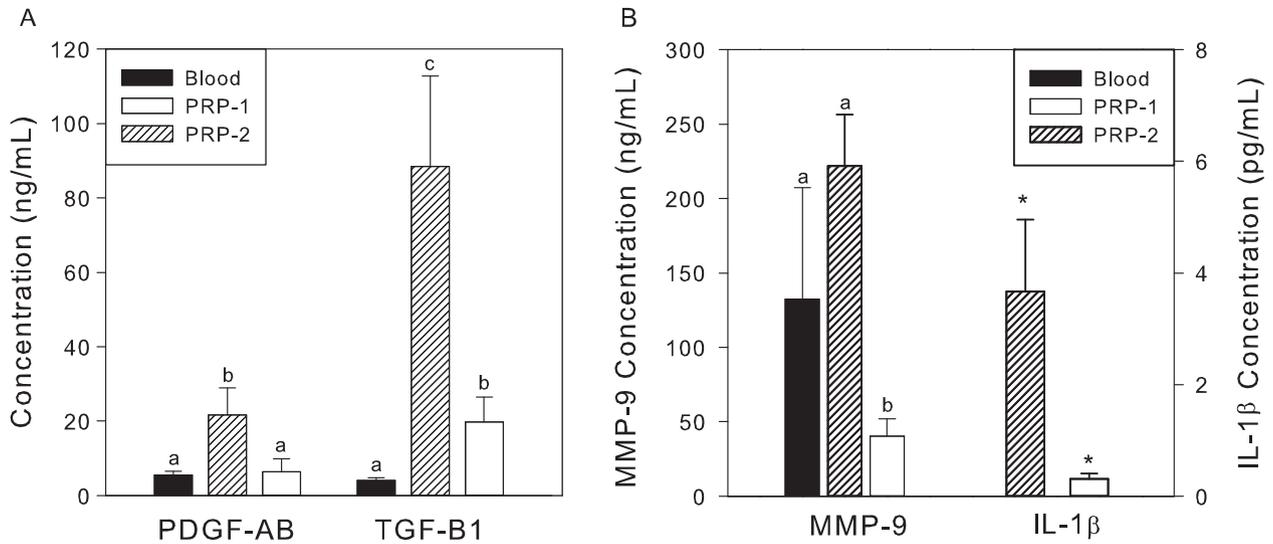
Figure 2. A, concentration of platelet-derived growth factor–AB (PDGF-AB) and transforming growth factor–β1 (TGF-β1) in venous whole blood, platelet-rich plasma–1 (PRP-1), and PRP-2. B, concentration of matrix metalloproteinase-9 (MMP-9) in blood, PRP-1, and PRP-2 and concentration of interleukin-1β (IL-1β) in PRP-1 and PRP-2. There are no blood samples of IL-1β because the assay is not validated for strongly hemolyzed samples. Bars represent mean of x ± standard deviation. Superscript letters indicate significant difference between groups, but within PDGF-AB, TGF-β1, or MMP-9 by analysis of variance with Tukey post hoc test, $P < .05$. Asterisks indicate significant difference between PRP-1 and PRP-2 using 2-sample t test, $P < .05$.
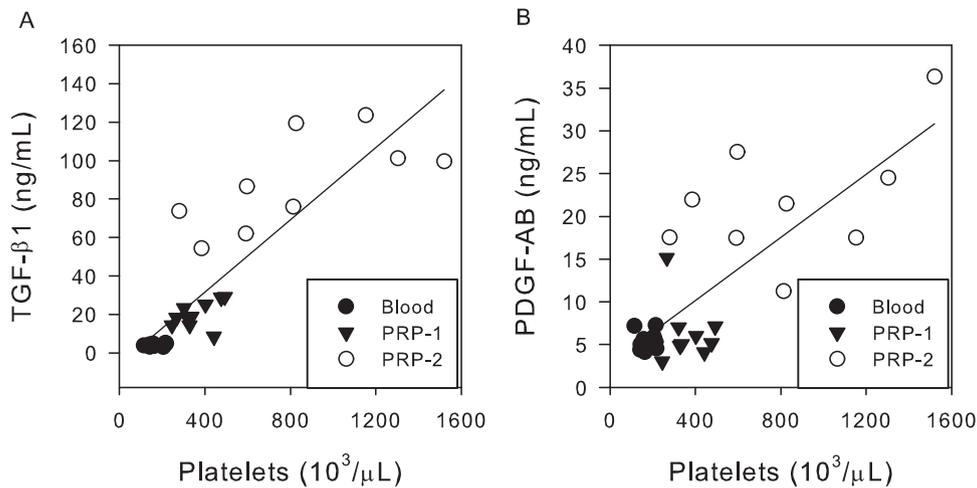
Figure 3. Correlations between (A) transforming growth factor–β1 (TGF-β1) and platelets and (B), platelet-derived growth factor–AB (PDGF-AB) and platelets in venous blood, platelet-rich plasma–1 (PRP-1), and PRP-2. Linear regression is shown with solid line.

and fibroblasts, improve glycosaminoglycan and fibronectin deposition, and increase cell activity early in healing.[12,17,27] Transforming growth factor–β1 and PDGF-BB have been shown to decrease healing time in experimentally induced wounds; however, PDGF-AB is the primary isoform of PDGF in humans, and will bind to both the α and β receptors.[3,18] Additional studies showed a positive correlation between platelet concentration and anabolic gene expression, and between WBCs and catabolic gene expression, furthering interest in a PRP formulation that promotes anabolism over catabolism, which is best achieved by an increased ratio of platelets to WBCs.[13]

The PRP-1 system decreases the concentration of leukocytes compared with whole blood while PRP-2 concentrates leukocytes. Concentrated leukocytes deliver increased catabolic cytokines to the site of injection, facilitating extracellular matrix breakdown and leukocyte activation. Neutrophils contain several types of granules that contain numerous cytokines including collagenases, gelatinases, lysozymes, elastases, serprocidins, and myeloperoxidase.[6] T-lymphocytes contain various interleukins, including IL-2, 4, 5, 6, 13, 17, 21, and 22; interferon-γ (IFN-γ), and tumor necrosis factor–α (TNF-α).[2] B-lymphocytes contain fewer but similar cytokines, including IL-6, IL-8, TNF-α,
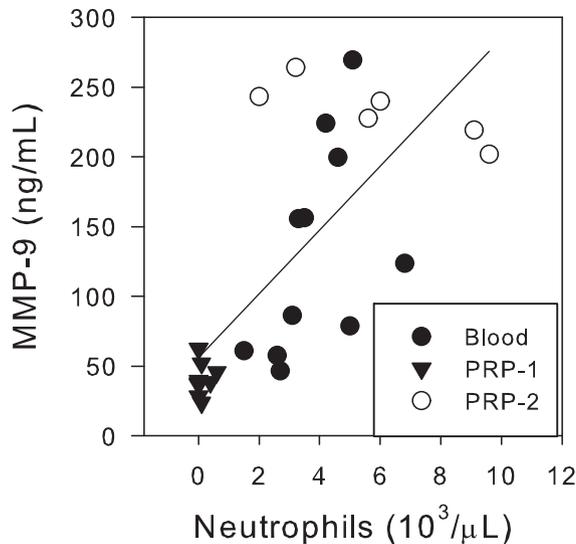
Figure 4. Correlation between matrix metalloproteinase-9 (MMP-9) and neutrophils in venous blood, platelet-rich plasma–1 (PRP-1), and PRP-2. Linear regression is shown with solid line.

and IL-1β.[16] Circulating monocytes have 2 phenotypes in similar concentrations, one of which is clearly proinflammatory and contains numerous cytokines including cathepsin B, L, and S; MMP-2, 3, 9, 13; and TNF-α, while the other phenotype is more balanced between being proinflammatory and proangiogenic.[15] While growth factor treatment has been shown to modulate the effects of catabolic cytokines, including TGF-β1's ability to decrease IL-1β receptor transcription and binding ability while promoting synthesis of IL-1 receptor antagonist (IL-1ra), this ability might be overwhelmed by an excess of WBCs.[20,22]

Evaluation of potential extracellular matrix degradation was based on MMP-9 concentration, as neutrophil-derived MMP-9 is known to degrade collagen and other extracellular matrix molecules and has been implicated as a predicator of poor healing.[4,30] In our study, MMP-9 concentration was positively correlated with neutrophils, with the highest concentration in PRP-2. Matrix metalloproteinase–9 has been shown in several studies to be a component of nonhealing, or poorly healing wounds.[11,29,32] While this study only measured total MMP-9, it has been demonstrated that inactive MMP-9 can be converted to active MMP-9 during inflammation and that measuring higher total MMP-9 can accurately reflect lower active MMP-9 levels.[8,21]

Evaluation of potential local inflammation was based on IL-1β in PRP. IL-1β concentration was positively correlated with neutrophils and monocytes, with the highest concentration in PRP-2. Interleukin-1β has been shown in numerous studies to be a primary cytokine for inflammation and matrix degradation, including cancers, autoinflammatory diseases, trauma, and tendinitis and it is a common target to decrease inflammation via manipulation of IL-1ra.[5,9,26,29] Specifically, human tendon cells treated with IL-1β demonstrated increased catabolic gene expression including cyclooxygenase-2; MMP-1, 3, and 13; and cytosolic phospholipase A2.
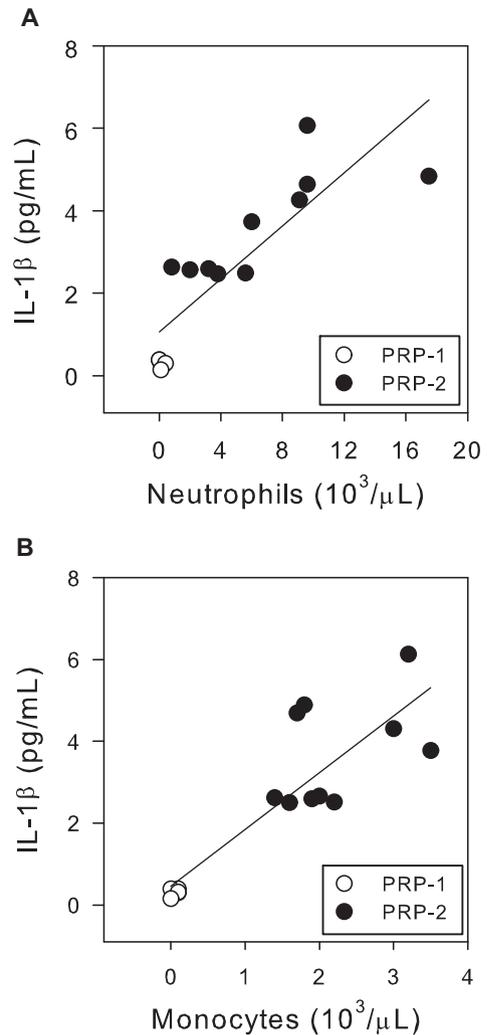
Figure 5. Correlations between interleukin-1β (IL-1β) and (A) neutrophils and (B) monocytes in platelet-rich plasma–1 (PRP-1) and PRP-2. Linear regression is shown with solid line.

In addition, IL-1β treatment caused continued upregulation of IL-1β.[26,28] Injured human rotator cuffs have been shown to have increased IL-1β in the tendon along with other proinflammatory cytokines.[29]

Anabolism requires low levels of leukocytes; concentrated leukocytes might overwhelm the effects of additional growth factors though the numerous and redundant cytokines in peripheral leukocytes. Interleukin-1β treatment of cultured chondrocytes has been shown to antagonize transcription factor binding and induced signaling of TGF-β1.[7,23] The optimal balance between anabolism and catabolism for tissue repair augmentation and the concentration of growth factors and cytokines required to maintain this balance remains unclear. Concentrations of cytokines or growth factors used in laboratory studies are typically greater than those present in PRP, and they are also typically tested in isolation or with 1 other growth factor or cytokine, while PRP represents a biologically active

milieu. It is therefore not accurate to extrapolate potential biologic effects of PRP based on growth factor or cytokine concentrations. The catabolic effects of WBCs in PRP need to be investigated in order to determine the effects on tissue homeostasis. Our results suggest that effects of PRP are not just attributable to concentrated platelets, but to a ratio of platelets to WBCs and, by extension, anabolic signaling molecules to catabolic cytokines. Depending upon the clinical application, commercial preparations of PRP should be considered based upon their ability to concentrate platelets and leukocytes with sensitivity to pathologic conditions that will benefit most from increased platelets or reduced leukocyte concentration. Leukocytes contain active cytokines capable of eliciting an inflammatory response and degrading normal tissue matrix and it is presently unclear which clinical situations, acute or chronic, soft tissue or intra-articular, would benefit from application of PRP rich in WBCs. Additional studies are needed to investigate whether these differences in catabolic signaling molecules have biologic effects in vivo. In addition, future in vivo research will be needed to determine the relative importance of absolute threshold levels of platelets (and growth factors) and leukocytes compared with the ratio of platelets to leukocytes.

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