Leukocyte concentration and composition in platelet-rich plasma (PRP) influences the growth factor and protease concentrations

Abstract

Background:

Platelet-rich plasma (PRP) therapy has become an increasingly popular treatment for orthopaedics and sports-related injuries, and various clinically available PRP preparation methods exist. However, the differences in PRP quality among numerous preparation methods remain unclear. Specifically, the benefit of including leukocytes in the PRP product remains controversial, and few studies have been conducted to evaluate the effects of the interaction between platelets and leukocytes on the growth factor concentrations. The aim of the present study was to compare the biological characteristics of PRPs focusing on the leukocyte concentration and composition.

Methods:

Leucocyte rich (LR)-PRP, leucocyte poor (LP)-PRP, and pure-PRP were prepared from the peripheral blood of 6 healthy male volunteers (mean age: 31.3 years). The concentrations of platelets, leukocytes, erythrocytes, growth factors (transforming growth factor-beta 1: TGF-β1; fibroblast growth factor-basic: FGF-b; platelet-derived growth factor-BB: PDGF-BB; vascular endothelial growth factor: VEGF) and matrix metalloproteinase-9 (MMP-9) from each of the PRP samples were measured. Considering the interaction between platelets and leukocytes,

correlations between platelets/leukocytes and growth factors/MMP-9 were analyzed using partial correlation coefficients.

Results:

The platelet concentration did not differ among the three PRP preparation methods. Conversely, the leukocyte concentration was dramatically different: 14.9±4.5 (10^3/μl) in LR-PRP, 2.4±1.3 (10^3/μl) in LP-PRP, 0.2±0.2 (10^3/μl) in pure-PRP. The platelet concentration positively correlated with all growth factors. On the other hand, the leukocyte concentration positively correlated with PDGF-BB and the VEGF concentration, while it negatively correlated with FGF-b. Regarding catabolic factors, the MMP-9 concentration strongly correlated with the leukocyte concentration, while there was no correlation between the platelet and MMP-9 concentrations.

Conclusions:

These findings demonstrate that leukocytes strongly influence the quality of PRPs. Therefore, modifying the PRP preparation method according to the pathology is essential to achieve better clinical results with PRP therapy.

Introduction

Platelet-rich plasma (PRP) is an autologous platelet concentrate that contains diverse growth factors, such as platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF-β), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), insulin-like growth factor 1 (IGF-1), as well as other cytokines that possess not only anabolic effects, but also catabolic effects on the tissue healing process[1, 2]. PRP therapy is promising as a simple, safe (because of its autologous origin), low-cost, and minimally invasive technique that can promote tissue repair processes[2, 3]. PRP therapy was initially introduced in maxillofacial and plastic surgery in the 1990s[4] and subsequently in many other fields[5-7]. Recently, PRP therapy has been used as one of the therapeutic applications for orthopaedics and sports-related injuries[2, 8, 9].

Despite an increasing number of clinical studies, some studies have shown less favorable results[10, 11]. Numerous PRP preparation methods make comparisons between the studies difficult and interfere with the ability to explain conflicting biologic results[12]. Some classification systems based on the presence or absence of leukocytes, activation status and platelet concentration have been proposed[13, 14, 9], however, the differences in PRP quality among these numerous preparation methods remain unclear. Specifically, the benefit of including leukocytes in the PRP product remains controversial[15, 16], and few studies have thus far been conducted to evaluate the effects of the interaction between platelets and leukocytes on the growth factor concentrations.

Therefore, the aim of the present study was to analyze the influence of leukocytes on the levels of various growth factor and protease concentrations by the comparison of three different PRP preparation methods focusing on the leukocyte concentration and composition.

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Methods

All procedures in this study were approved by the Institutional Review Board of Juntendo University. Prior to drawing peripheral blood, informed consent was obtained from each donor.

Blood collection and PRP preparation

The peripheral blood was collected from six healthy male volunteers (mean age: 31.3 years, range: 27-39 years). A single technician collected 22 ml of venous blood from each participant using a 21-gauge needle. Immediately following collection, the blood was divided and 10 ml of each sample were aliquoted for the three different preparation methods (two different anticoagulated tubes). The remaining 2 ml of blood was collected in an EDTA-coated tube for a whole blood analysis. The three different PRP preparation methods were performed in order to compare the growth factor and protease concentrations in different leukocyte concentrations. The preparation methods are briefly described as follows: a double-spin method was used to obtain both high amounts of platelets and leukocytes (LR-PRP), a single-spin method was used to obtain a high number of platelets and lower number of leukocytes (LP-PRP), and a single-spin method was used to obtain platelet concentrates with a minimum number of leukocytes (pure-PRP) (**Table 1**); the detailed methods of these three PRP preparation methods are described below.

Preparation of LR-PRP: Approximately 10 ml of blood were collected in a 15 ml tube (BD Biosciences, Bedford, MA, USA) containing 1 ml of 3.8% sodium citrate as an anticoagulant. After an initial centrifugation step of 400g for 10 minutes at room temperature, the upper layer and buffy-coat were transferred to another tube. After a second centrifugation step of 2,000g for

3 minutes at room temperature, the supernatant (platelet-poor plasma, PPP) was collected and used as a control. The platelet pellet was resuspended in the remaining 1 ml of plasma volume to produce LR-PRP.

Preparation of LP-PRP and pure-PRP: Approximately 10 ml of blood were collected in a 15 ml tube (BD Biosciences, Bedford, MA, USA) containing 1 ml of 3.8% sodium citrate as an anticoagulant. The anticoagulated blood was carefully transferred to a new 15 ml tube containing 2.5 ml of lymphocyte separation medium (LSM, MP Biomedicals, Santa Ana, CA, USA). After a centrifugation step of 800g for 10 minutes at room temperature, the supernatant was removed and discarded. Of the remaining 1 ml of plasma just above the buffy-coat layer, the upper half (500 μl) was isolated as pure-PRP and the lower half (500 μl) was collected as LP-PRP.

Hematological analysis

The platelet, leukocyte, and erythrocyte concentrations and leukocyte compositions from the whole blood and from each of the PRP and PPP samples were determined using an automated hematology analyzer (Ac-T diff, Beckman Coulter, Brea, CA, USA) immediately after preparation. After this analysis, all samples were stored at -80℃ until further analysis.

Quantification of the growth factors and protease

The concentrations of the growth factors and protease (TGF-β1, VEGF, PDGF-BB, FGF-b, and MMP-9) from each of the PRP and PPP samples were measured. A single freeze-thaw cycle was used to induce platelet activation and the release of growth factors and cytokine. Wasterlain et al. proposed this method as one of the PRP activation procedures especially for *in vitro* laboratory

experiments[16]. The concentrations of PDGF-BB before and after freeze-thaw process were measured to confirm whether this procedure accurately activates PRP (**Supplemental figure 1**). The samples were thawed and then incubated for 1 h at 37℃. After incubation, the samples were centrifuged for 5 minutes at 13,000g at room temperature, and the supernatants were tested. TGF-β1 and MMP-9 quantification assays were performed using an enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN, USA). FGF-b, PDGF-BB, and VEGF concentrations were determined using a Bio-Rad Multiplex analysis kit (Bio-Plex, Bio-Rad, Hercules, CA, USA). All procedures were carried out according to the manufacturer's instructions. For the assay of the inhibition of platelet-neutrophil interaction, one of the activated LR-PRP samples was incubated in the absence or presence of anti-human P-selectin antibody (BD Biosciences, Bedford, MA, USA) 20μg/ml for 20 minutes at room temperature. TGF-β1 concentrations were measured by ELISA assay.

Statistical analysis

All data are presented as the mean ± SD. Comparisons of all pairs in each group were assessed using a one-way nonparametric analysis of variance (ANOVA) followed by the Tukey post-hoc test. Considering the interaction between platelets and leukocytes, linear correlations between the platelet and the growth factor/protease concentrations, as well as between the leukocyte and the growth factor/protease concentrations, were analyzed by partial correlation coefficients adjusted for leukocytes and platelets respectively. All p-values were two-sided and p-values of less than 0.05 were considered to be statistically significant. Statistical analyses were performed using the SPSS Statistics version 20.0 software package (IBM Company, Chicago, IL, USA).

Results

First, we confirmed the cell types contained in the PRPs prepared by the three different methods. The complete blood counts (CBCs) of the whole blood, LR-PRP, LP-PRP, pure-PRP, and PPP are summarized in **Table 2**.

Platelet concentration

The platelet counts in the LR-PRP and LP-PRP were nearly the same (approximately 4-fold whole blood), while those in pure-PRP resulted in relatively lower platelet concentrations (2.2-fold whole blood). However, there were no significant differences with regard to the platelet concentrations among the three preparation methods (**Fig. 1A**).

Leukocyte concentration

The LR-PRP contained the highest number of leukocytes (approximately 2.7-fold whole blood). The LP-PRP contained fewer leukocytes (approximately 0.5-fold whole blood), whereas the pure-PRP contained very few leukocytes, nearly the same amount as the PPP. There were significant differences in the concentration of leukocytes among the three preparation methods compared with one another ($p<0.01$) (**Fig. 1B**).

Leukocyte composition

The composition of the leukocytes was predominantly neutrophils in the peripheral blood, whereas lymphocytes were predominant in the LP-PRP and LR-PRP (**Fig. 2**). According to the results of the concentration and composition of the leukocytes, the LR-PRP abundantly contained all leukocyte cell types, whereas the LP-PRP contained a small quantity of lymphocytes and a few neutrophils (**Figs. 1, 2**).

151 **Erythrocyte concentration**

152 The LR-PRP contained approximately 0.7-fold whole blood of erythrocytes, whereas the LP-
153 PRP and pure-PRP contained very few erythrocytes, nearly the same as the PPP (**Fig. 1C**).

154

155 **Quantification of the growth factors and protease in three different PRP preparations**

156 The concentrations of both PDGF-BB and VEGF were the highest in the LR-PRP, followed by
157 the LP-PRP, pure-PRP, and PPP (**Fig. 3**). The concentration of PDGF-BB was significantly
158 different between the LR-PRP and pure-PRP and between the LP-PRP and pure-PRP ($p=0.015$
159 and $p=0.045$, respectively). The concentration of VEGF was significantly different between the
160 LP-PRP and pure-PRP ($p=0.036$). The TGF-β1 concentration was the highest in the LP-PRP, and
161 there was a significant difference between the LP-PRP and pure-PRP ($p=0.040$). The FGF-b
162 concentration was not significantly different among the three preparation methods. Finally, we
163 evaluated one of the major catabolic protease, MMP-9. The concentration of MMP-9 was
164 significantly higher in the LR-PRP than the LP-PRP ($p=0.031$), pure-PRP ($p=0.024$), and PPP
165 ($p=0.026$) (**Fig. 3**).

166

167 **Correlations between growth factor/protease concentrations and platelet/leukocyte**
168 **concentrations**

169 The distributions of the platelet/leukocyte concentrations and growth factor/protease
170 concentrations are shown in **Fig. 4A and B**. The platelet concentration positively correlated with
171 all growth factors (TGF-β1: $r=0.58$, $p=0.004$; FGF-b: $r=0.90$, $p<0.001$; PDGF-BB: $r=0.87$,
172 $p<0.001$; VEGF: $r=0.62$, $p<0.001$). On the other hand, the leukocyte concentration positively

correlated with the PDGF-BB and VEGF concentrations (PDGF-BB: r=0.56, p=0.006; VEGF: r=0.65, p=0.001), while it negatively correlated with the FGF-b concentration (r=-0.44, p=0.036) (**Table 3**). With regard to the TGF-β1 concentration, there tended to be a negative correlation between the leukocyte and TGF-β1 concentrations (r=-0.20, p=0.357) (**Table 3**). Regarding catabolic factors, the MMP-9 concentration strongly correlated with the leukocyte concentration (r=0.81, p<0.001), while there was no correlation between the platelet and MMP-9 concentrations (r=0.33, p=0.125) (**Table 3**).

Inhibition of platelet-neutrophil interaction increases the level of TGF-β1 concentration

To confirm if the inhibition of platelet-neutrophil interaction could alter the concentration of TGF-β1, we cultured LR-PRP in the presence of anti-human P-selectin antibody which inhibits the platelet-neutrophil adhesion. We found that the TGF-β1 concentration was significantly increased by inhibiting platelet-neutrophil interaction in LR-PRP (p<0.001) (**Fig. 5**).

Discussion

PRP therapy has become an increasingly popular treatment and various clinically available PRP preparation methods exist. However, to the best of our knowledge, this is the first report comparing the quality of LR-PRP, LP-PRP and pure-PRP in the same concentration of platelet. We revealed that the growth factor concentrations to be dependent on the leukocyte concentrations, and a catabolic protease (MMP-9) was expressed at a considerably high concentration in the LR-PRP. Our data demonstrated that the PRP contents are quite different from one another; therefore, the efficacy of PRP therapy must be discussed for each PRP preparation method.

Most of the controversy regarding PRP therapy is based on the absence of a clear definition of PRP therapy[17]. There are numerous PRP preparation methods, however, the differences in PRP quality among them remain unclear. Recently, some classification systems according to the presence or absence of leukocytes, activation status and platelet concentration have been proposed[13, 14, 9]. Dohan et al. proposed a classification system dividing many products into 4 main families according to their fibrin architecture and the presence of leukocytes[14]. Mishra et al. proposed a classification system according to the presence or absence of leukocytes, activation status and platelet concentration[9]. DeLong et al. proposed the PAW classification system according to the platelet quantity, activation mode of the platelets and the presence of leukocytes (in particular neutrophils)[13]; the PAW classification system is the most subdivided classification system regarding the leukocyte concentration and cell types. Notably, although the LP-PRP and pure-PRP can be categorized in the same group in PAW classification, the concentrations of growth factors are significantly different between the LP-PRP and pure-PRP (Fig. 3). Therefore, we should be aware that the PRP preparation methods are quite different, and

physicians should perform quality estimations (e.g., cell counts of the PRP) for each occurrence of PRP therapy in order to determine the efficacy of PRP therapy.

In the present study, the platelet concentrations positively correlated with the PDGF-BB, VEGF, TGF-β1 and FGF-b concentrations and showed no negative correlations with any growth factor. On the other hand, the leukocyte concentrations showed positive correlations with the PDGF-BB and VEGF concentrations, while it negatively correlated with the FGF-b concentration and had the tendency of a negative correlation with the TGF-β1 concentration. Regarding catabolic factors, the MMP-9 concentration strongly correlated with the leukocyte concentration, while there was no correlation between the platelet and MMP-9 concentrations (**Table 3**). These results indicated that the leukocyte concentrations have a strong influence on the growth factor and protease concentrations, and they differed depending on the type of growth factor or protease. Wasterlain et al. reported the association between the cell type and growth factor in the PRP[16]. PDGF and VEGF are derived from both platelets and leukocytes. MMP-9 is primarily derived from neutrophils, while TGF-β is primarily derived from platelets. Therefore, our results from the correlation analysis could be partly explained by cell-cell interactions. From this viewpoint, we checked if the inhibition of platelet-neutrophil interaction could alter the concentration of TGF-β1, and found that the TGF-β1 concentration was significantly increased by inhibiting platelet-neutrophil interaction in LR-PRP (**Fig. 5**). This is a similar observation reported in the field of blood transfusion that neutrophils had downregulatory effects on the platelet activity[18-20].

In clinically, the effects of leukocytes on PRP therapy[15, 16] remain debatable. Some investigators have reported a beneficial effect of leukocytes on increased antibacterial and immunological resistance[21]. Additionally, leukocytes have been reported to correlate with

increased growth factor and cytokine release[21]. Other investigators have recommended avoiding tissue exposure to leukocytes as an inflammatory reaction may occur[22, 23]. Sundman et al. and Browning et al. demonstrated that high levels of catabolic mediators, such as MMPs, were observed in the LR-PRP compared with the LP-PRP or PPP[24, 25]. Indeed, our data showed considerably high levels of MMP-9 in the LR-PRP which contained high concentrations of leukocytes (**Fig. 3E**). Therefore, we should note that the LR-PRP possesses both anabolic and catabolic effects that can lead to the degradation of tendon, ligament, muscle, and chondrocytes, which are frequently involved in the motor organ diseases, and chose an appropriate PRP preparation method according to the pathology or the purpose of the therapy.

There are several limitations associated with this study. First, the sample size was small. This may have affected the results, although our findings were consistent with other PRP-related studies[21, 12, 25]. Second, we only performed a quantitative assessment of the PRP contents in this study. We did not demonstrate whether the differences in the PRP contents would lead to differences in the biological effects in *in vitro* or *in vivo* systems. Third, although we evaluated the MMP-9 concentration as a catabolic factor, we did not evaluate the concentrations of any other inflammatory cytokines such as IL-1β, IL-6, or TNF-α. Fourth, we used a freeze-thaw cycle as the PRP activation method instead of using calcium chloride or thrombin. This method would not reflect the physiological release kinetics of growth factors, however, it would be useful for *in vitro* laboratory experiments because it does not use a chemical mechanism of platelet activation.

In conclusion, the quality of PRP varies according to the preparation method used. Our data demonstrated that the leukocyte concentration and composition strongly influence the quality of PRPs. Therefore, modifying the PRP preparation method according to the pathology or the

purpose of the therapy is essential to achieve better clinical results with PRP therapy. Further investigations are necessary to optimize PRP therapy for the treatment of sports-related injuries.

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330

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Figure Legends

Fig. 1

(A) Platelet concentrations, (B) leukocyte concentrations, and (C) erythrocyte concentrations of LR-PRP, LP-PRP, pure-PRP and PPP. Data are shown as the ratio of the concentration of each PRP or PPP to the concentration of whole blood. Data are presented as mean ± SD (*$p<0.05$, **$p<0.01$).

Fig. 2

Leukocyte composition in whole blood, LR-PRP and LP-PRP. (A) neutrophil concentrations, (B) lymphocyte concentrations, and (C) monocyte concentrations. Composition in pure-PRP and PPP could not be measured because of too small leukocyte counts.

Fig. 3

Growth factor and catabolic cytokine concentrations in LR-PRP, LP-PRP, pure-PRP and PPP. (A) Transforming growth factor-beta 1 (TGF-β1), (B) fibroblast growth factor-b (FGF-b), (C) platelet-derived growth factor-BB (PDGF-BB), (D) vascular endothelial growth factor (VEGF), (E) matrix metalloproteinase-9 (MMP-9). Data are presented as the mean ± SD (*$p<0.05$, **$p<0.01$).

Fig. 4

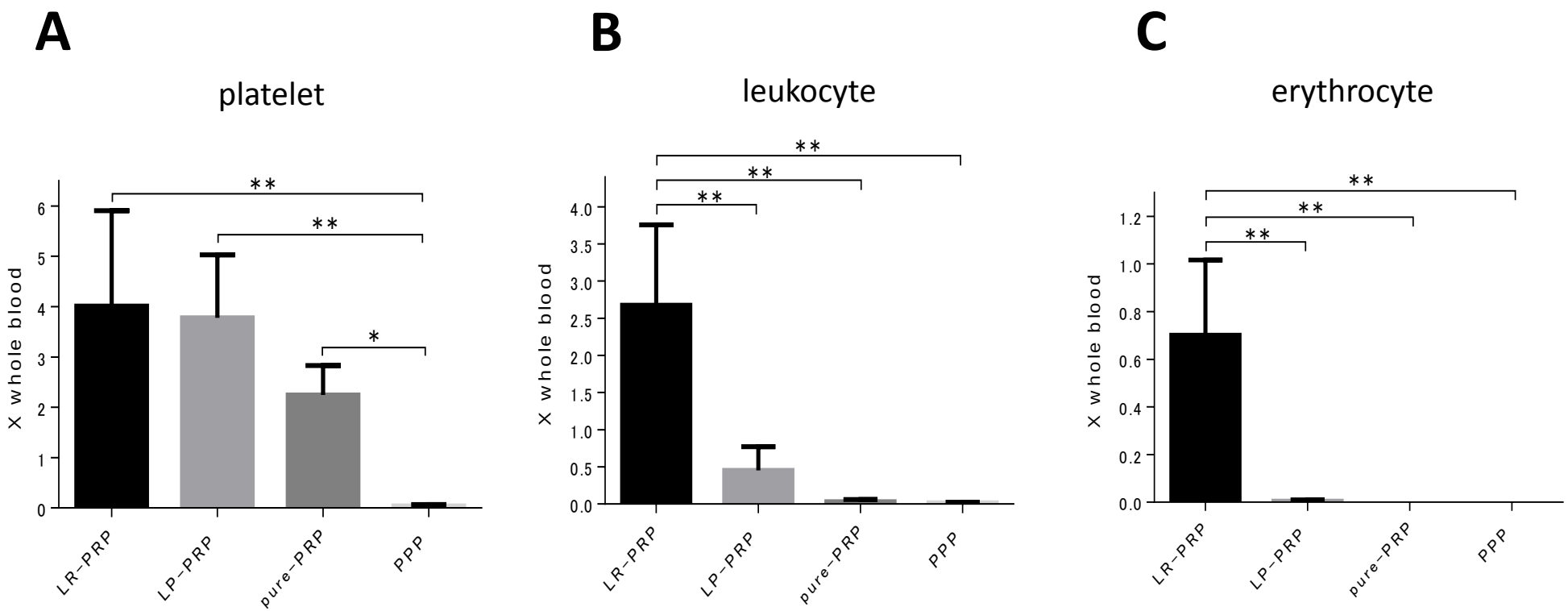
(A) Distribution of the platelet concentration and TGF-β1 concentration, FGF-b concentration, PDGF-BB concentration, VEGF concentration, and MMP-9 concentration.

(B) Distribution of the leukocyte concentration and TGF-β1 concentration, FGF-b concentration, PDGF-BB concentration, VEGF concentration, and MMP-9 concentration.

Fig.5

One of the activated LR-PRP samples was incubated in the absence or presence of 20μg/ml of anti-human P-selectin antibody for 20 minutes at room temperature. TGF-β1 concentrations were measured by ELISA assay. Data are presented as the mean ± SD performed in triplicate. Unpaired t test was used to calculate the p values (*$p<0.05$, **$p<0.01$).

Fig. 1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LR-PRP
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Fig. 2

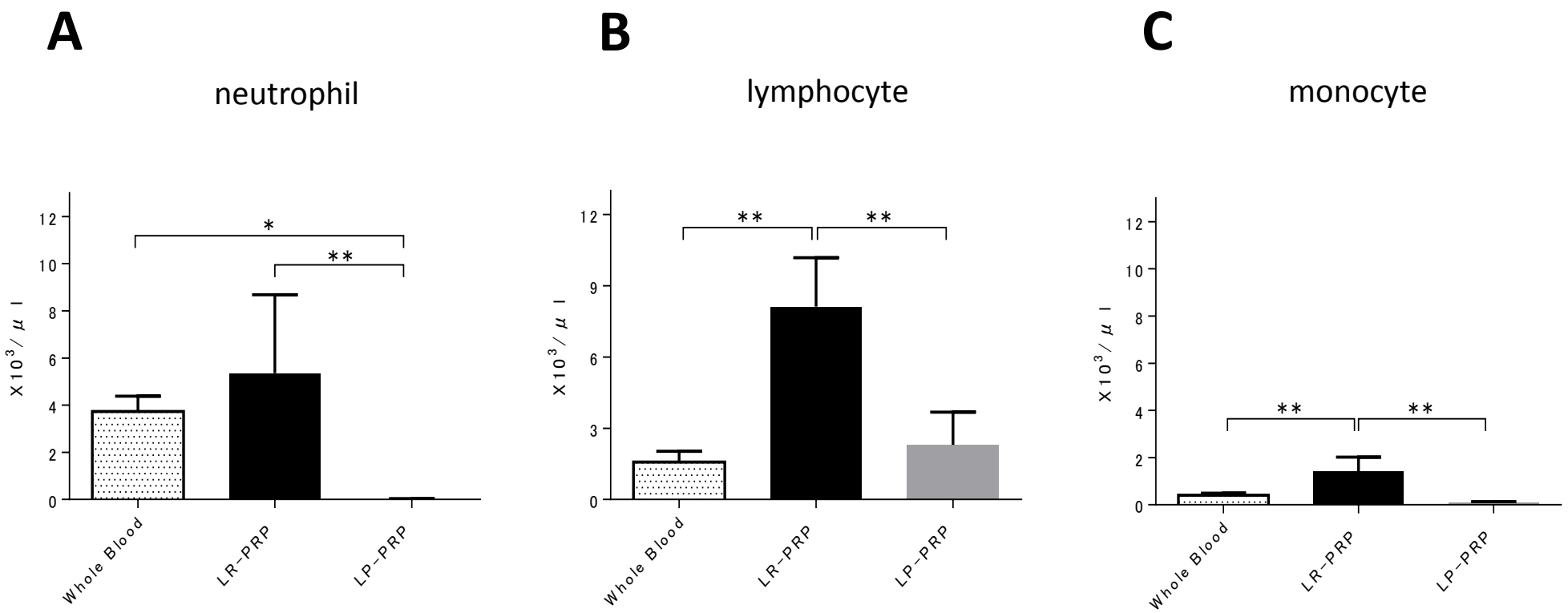
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Fig. 3

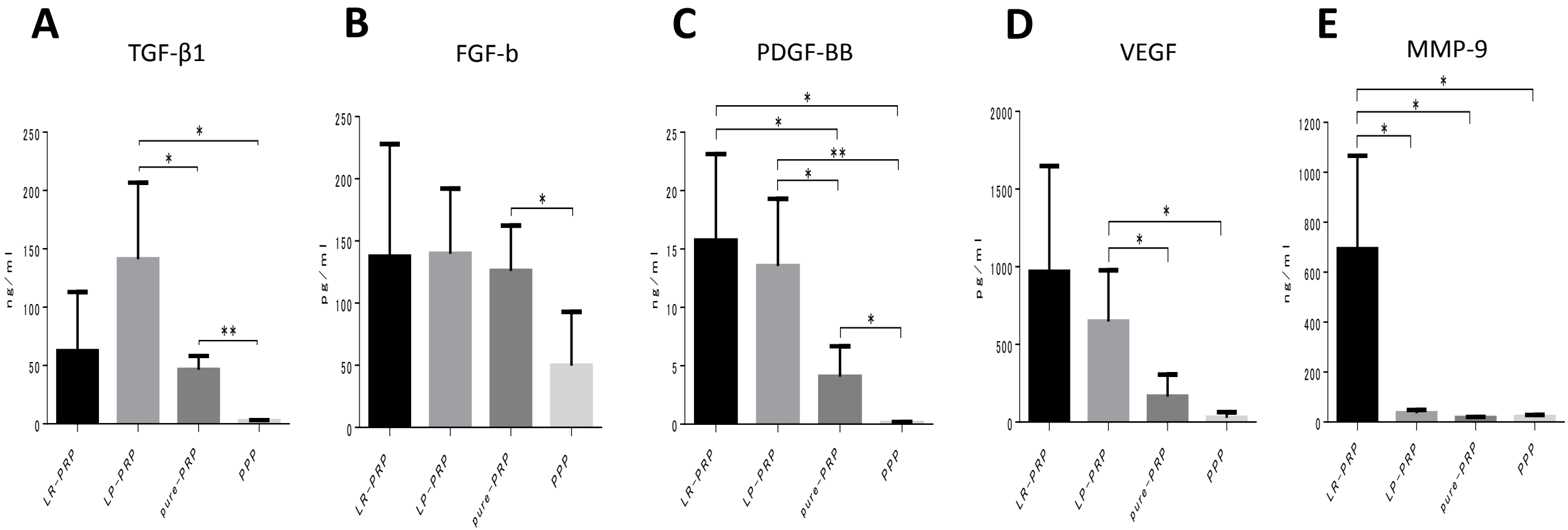
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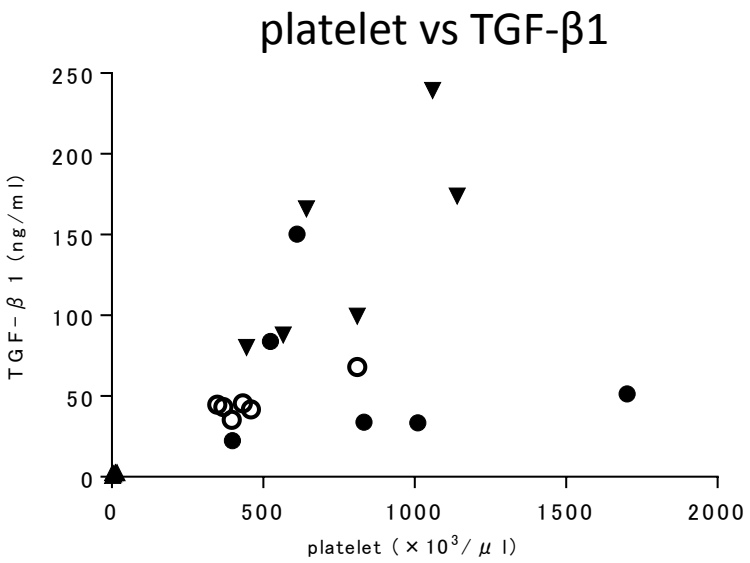
Fig. 4A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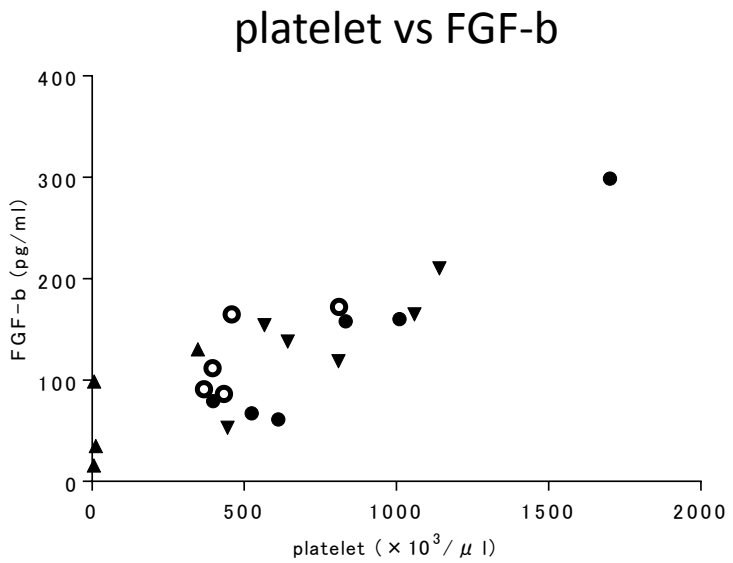
platelet (×10³/μl)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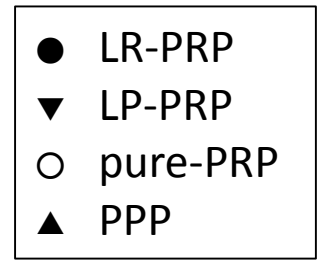
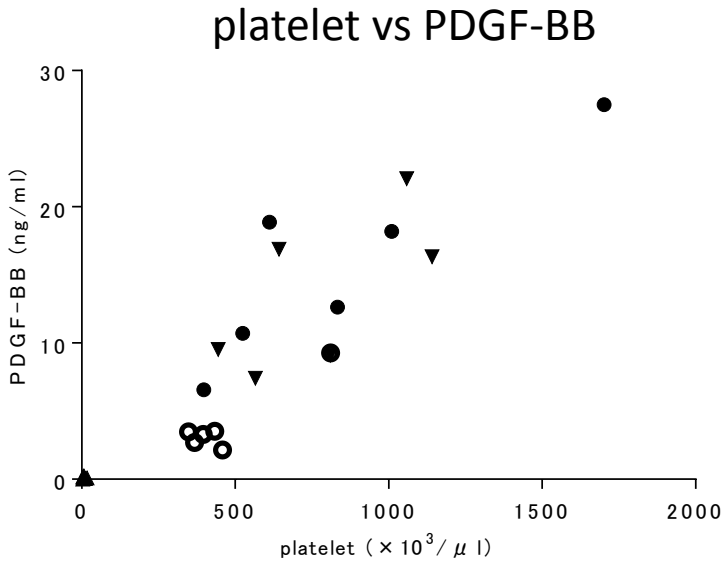
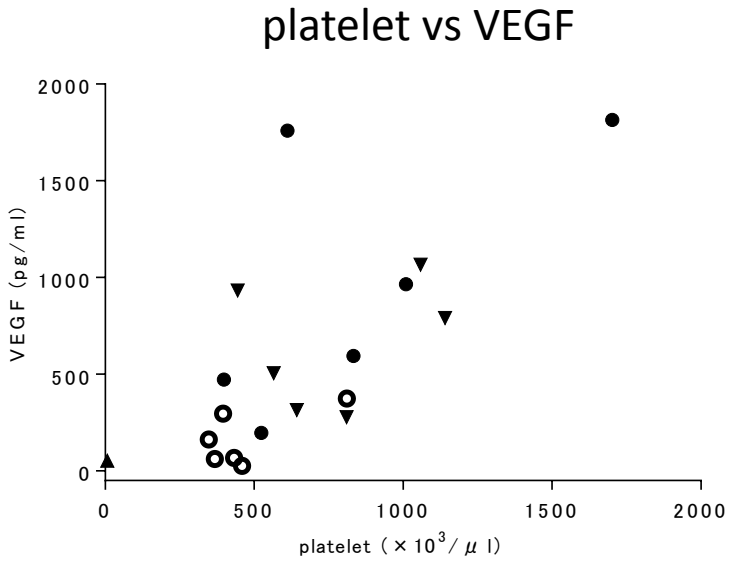

LR-PRP
LP-PRP
pure-PRP
PPP


platelet vs PDGF-BB
PDGF-BB (ng/ml)
30
20
10
0
0
500
1000
1500
2000
platelet (×10³/μl)


platelet vs VEGF
VEGF (pg/ml)
2000
1500
1000
500
0
0
500
1000
1500
2000
platelet (×10³/μl)

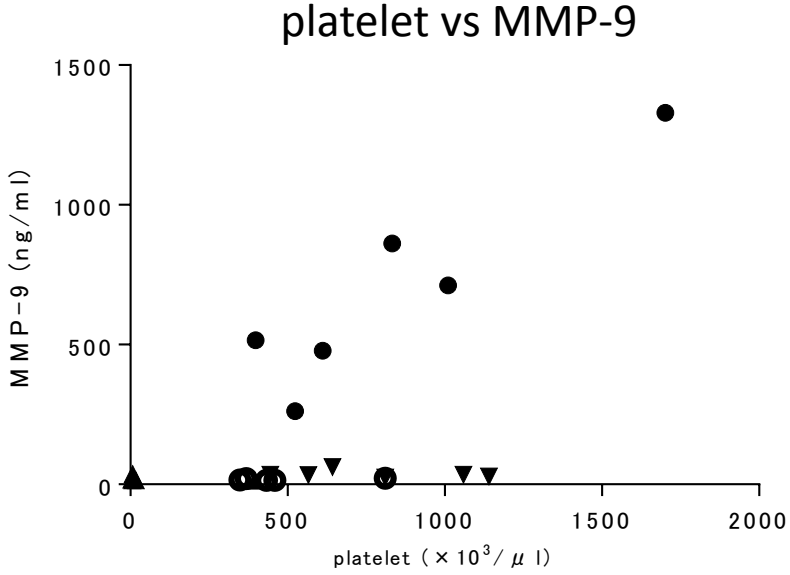
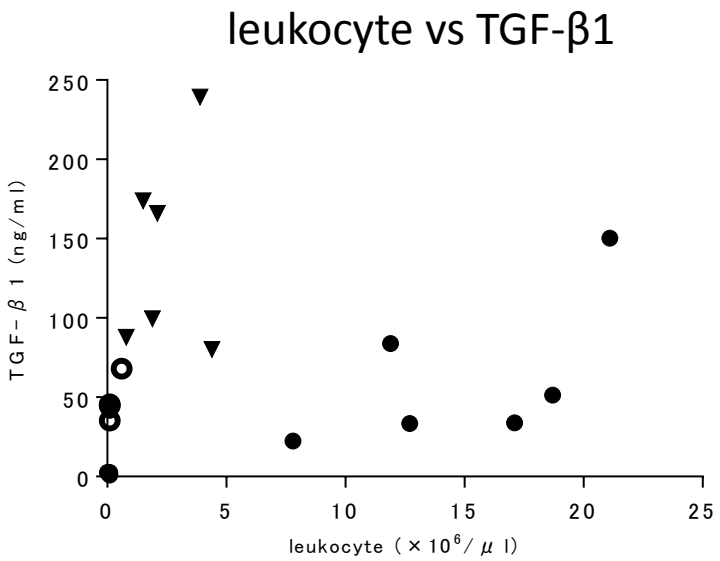
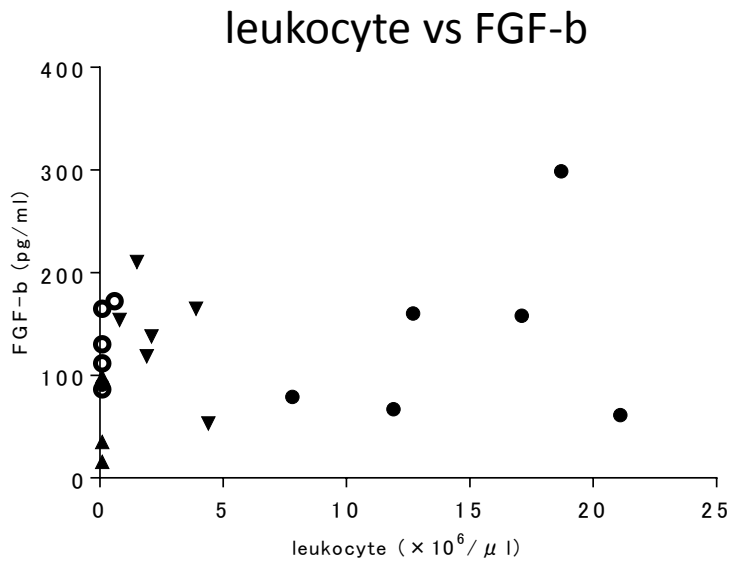
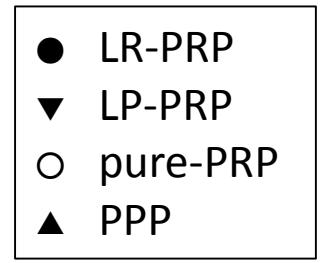
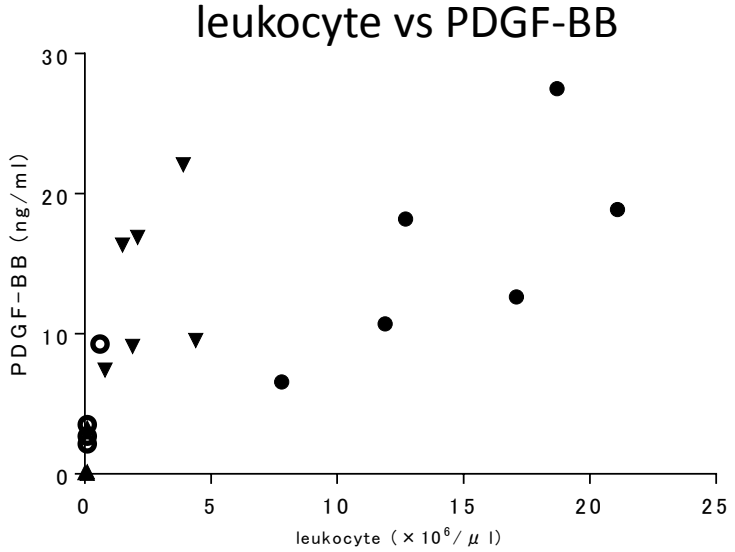

platelet vs MMP-9
MMP-9 (ng/ml)
1500
1000
500
0
0
500
1000
1500
2000
platelet (×10³/μl)

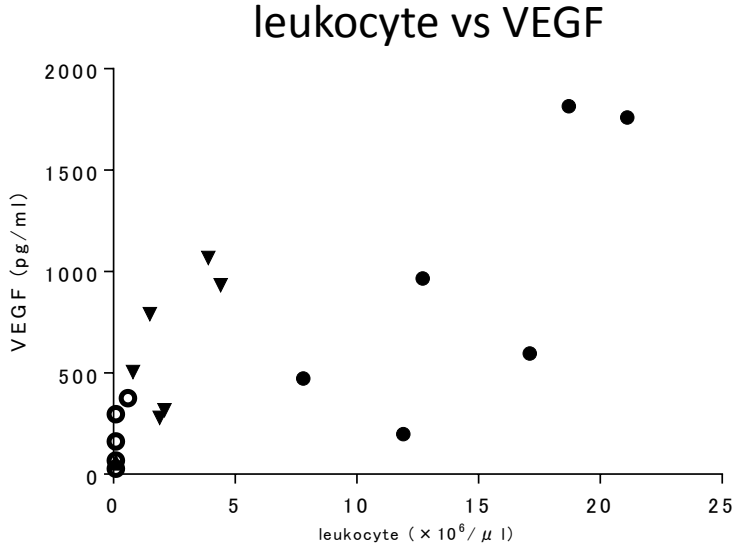
Fig. 4B


leukocyte vs TGF-β1
TGF-β1 (ng/ml)
250
200
150
100
50
0
0
5
10
15
20
25
leukocyte (×10^6/μl)


leukocyte vs FGF-b
FGF-b (pg/ml)
400
300
200
100
0
0
5
10
15
20
25
leukocyte (×10^6/μl)


LR-PRP
LP-PRP
pure-PRP
PPP


leukocyte vs PDGF-BB
PDGF-BB (ng/ml)
30
20
10
0
0
5
10
15
20
25
leukocyte (×10^6/μl)


leukocyte vs VEGF
VEGF (pg/ml)
2000
1500
1000
500
0
0
5
10
15
20
25
leukocyte (×10^6/μl)

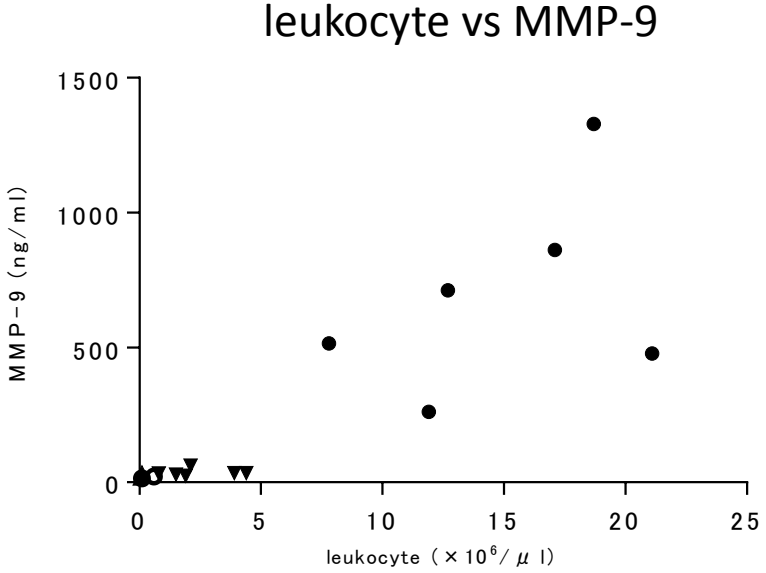
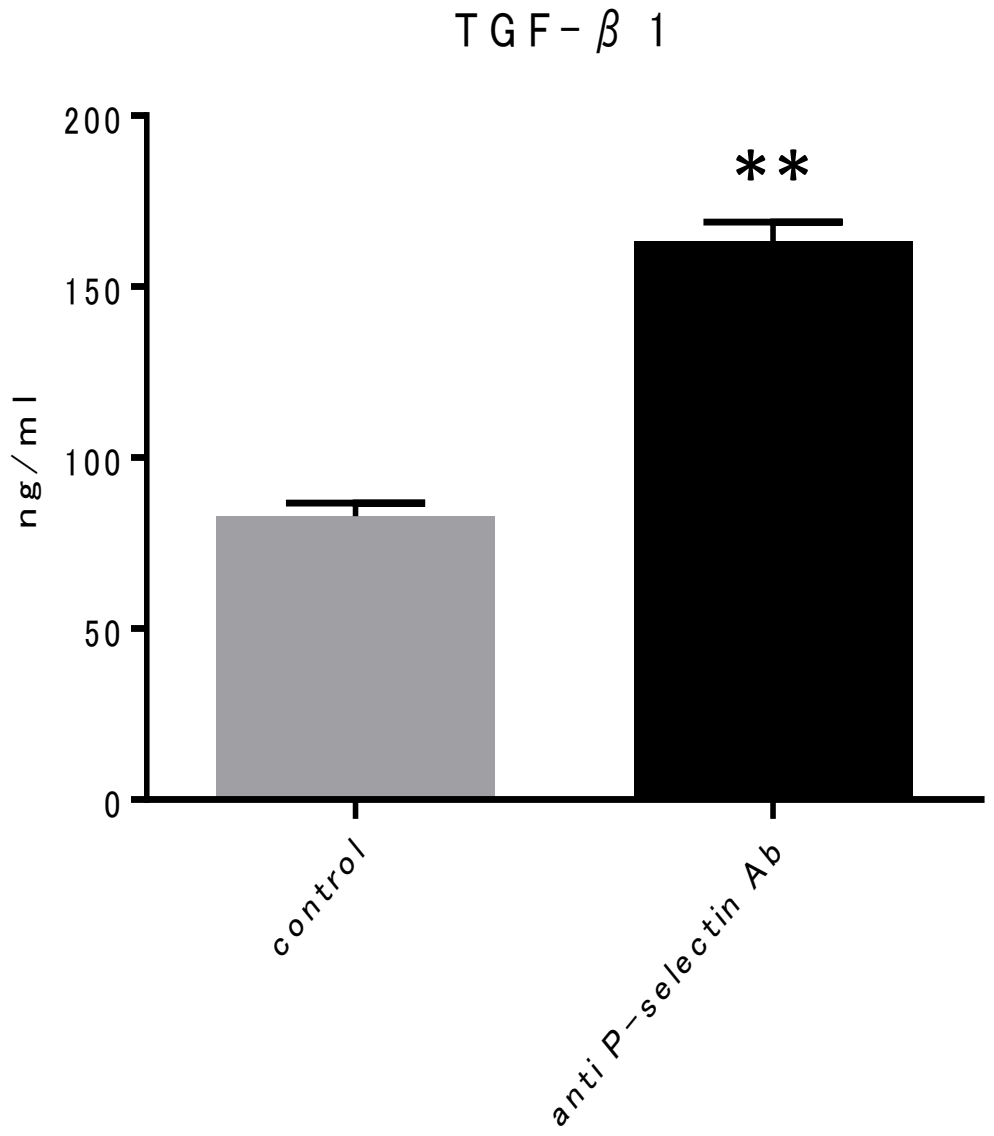

leukocyte vs MMP-9
MMP-9 (ng/ml)
1500
1000
500
0
0
5
10
15
20
25
leukocyte (×10^6/μl)

Fig. 5



Tables

Table 1

Protocol for each preparation method used in this study

Method	Blood volume (ml)	Anticoagulant	No. of centrifuge times	Centrifuge force and time	Final PRP volume (ml)
LR-PRP	10	3.8% sodium citrate, 1 ml	2	1st 400xg 10 min 2nd 2,000xg 3 min	1
LP-PRP	10	3.8% sodium citrate, 1 ml	1	800xg 10 min	0.5
Pure-PRP	10	3.8% sodium citrate, 1 ml	1	800xg 10 min	0.5

Table 2

CBC data of the whole blood, LR-PRP, LP-PRP, pure-PRP, and PPP

	Platelet ($X10^3$/μl)	Leukocyte ($X10^3$/μl)	Neutrophil ($X10^3$/μl)	Lymphocyte ($X10^3$/μl)	Monocyte ($X10^3$/μl)	Erythrocyte ($X10^6$/μl)
Whole blood	206.8±27.1	5.7±0.9	3.7±0.6	1.6±0.4	0.4±0.1	4.7±0.7
LR-PRP	846.5±431.8	14.9±4.5	5.4±3.0	8.1±1.9	1.4±0.6	3.2±1.2
LP-PRP	777.3±253.6	2.4±1.3	0.0±0.0	2.3±0.3	0.1±0.0	0.0±0.0
Pure-PRP	469.2±157.3	0.2±0.2	−	−	−	0.0±0.0
PPP	8.0±5.0	0.1±0.0	−	−	−	0.0±0.0

Data are presented as the mean ± SD.

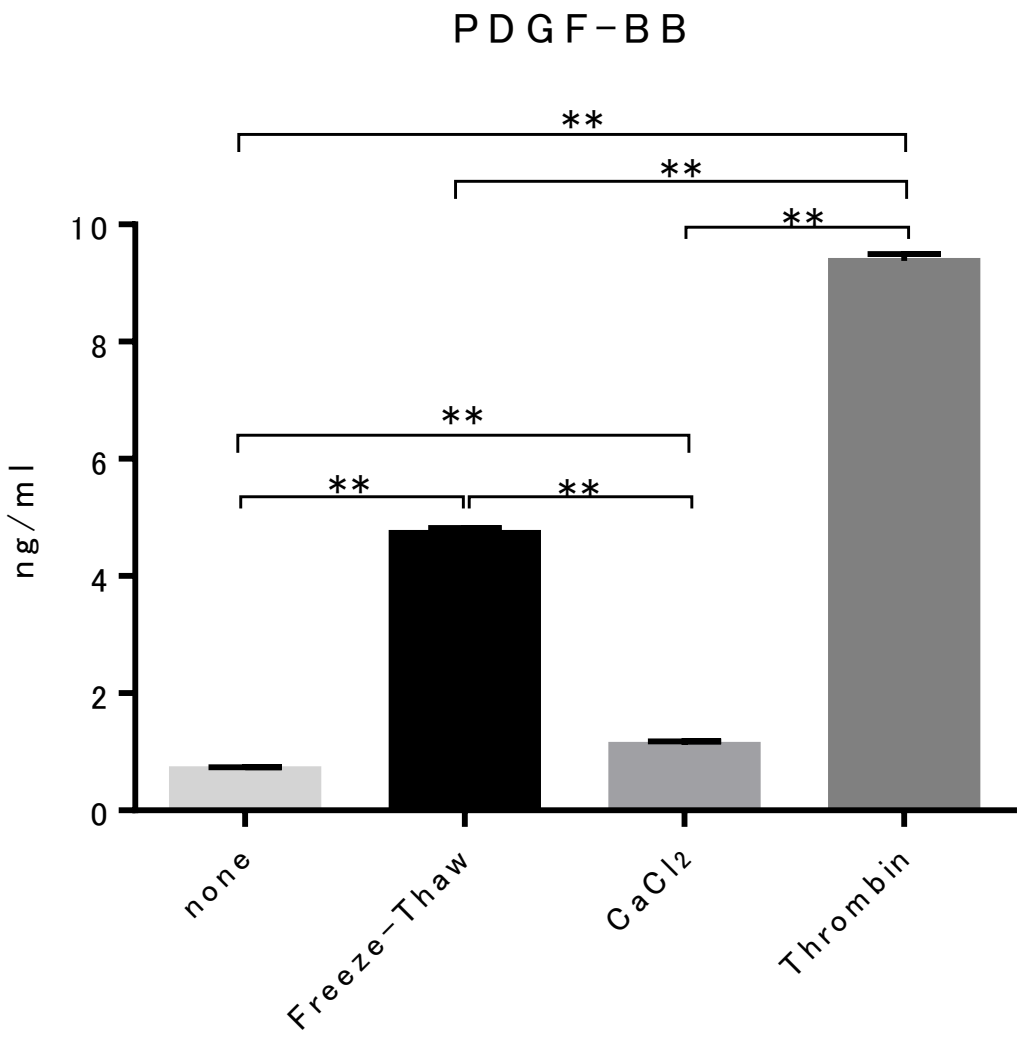
Table 3

Correlations between the platelet/leukocyte concentration and each growth factor/protease concentration

	vs platelet		vs leukocyte	
	r	p-value	r	p-value
TGF-β1	0.58*	0.004	-0.20	0.36
FGF-b	0.90*	<0.001	-0.44*	0.036
PDGF-BB	0.87*	<0.001	0.56*	0.006
VEGF	0.62*	<0.001	0.65*	0.001
MMP-9	0.33	0.125	0.81*	<0.001

Partial correlation coefficient (vs platelet: adjusted for the leukocyte concentration; vs leukocyte: adjusted for the platelet concentration).

Supplemental figure



Supplemental Figure Legends

Effects of different PRP activation methods on the growth factor concentration.

One of the pure-PRP sample used in this study was activated by three different methods: a single freeze-thaw cycle, application of $CaCl_2$ 11.1mg/ml (Sigma-Aldrich, St. Louis, MO, USA), and autologous thrombin (added to PRP at a ratio of 1:10). No activation sample was used as a negative control. PDGF-BB concentrations were measured using a Bio-Rad Multiplex analysis kit. Data are presented as the mean ± SD performed in triplicate. Comparisons of all pairs in each group were assessed using a one-way nonparametric analysis of variance (ANOVA) followed by the Tukey post-hoc test (*$p<0.05$, **$p<0.01$).