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White cell and platelet content affects the release of bioactive factors in different blood-derived scaffolds

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Abstract

Platelet-derived factors are biomaterials that might accelerate healing process in oral, maxillofacial, and several other applications. Release of specific factors by platelet concentrates is critical to achieving a successful outcome. Here, we have shown that platelet-rich fibrin (PRF) clots were beneficial sources of leukocytes, which may directly affect the release of chemokines and growth factors. When compared with the standard leukocyte-PRF (L-PRF), the experimental low-force modified procedure [defined as advanced-PRF (A-PRF)] entrapped the same content of viable leukocytes, released a similar amount of inflammatory cytokines, but secreted 3-, 1.6-, 3-, and 1.2-fold higher levels of Eotaxin, CCL5, platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF), respectively. A leukocyte-free scaffold, such as plasma rich in growth factors (PRGF), released only platelet-specific factors and, in particular, the F3 fraction, the richest in growth factors, secreted higher amount of CCL5 and PDGF compared to F1 and F2 fractions. In conclusion, different procedures and leukocyte content affect cytokine, chemokines, and growth factor release from platelet derivatives, which may be helpful in different clinical settings.

Keywords

Chemokines, growth factors, leukocytes, platelet products, tissue regeneration

History

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Introduction

Platelet products are currently used for clinical and surgical applications that require tissue regeneration [1,2]. Their potential beneficial outcomes, including hard and soft tissue regeneration [3], local hemostasis [4], acceleration of wound healing [5], make them suitable as therapeutic options, in different medical fields [6–8]. The driving concept is to collect and gather the most active components from the blood sample (platelets, fibrin, and eventually leukocytes) and to prepare them in a clinically usable form [9]. During normal tissue repair *in vivo*, platelets release high concentrations of biologically active proteins, such as growth factors (GFs), and other substances [10]. Primary factors associated with platelets include platelet-derived growth factor (PDGF) and transforming growth factor β (TGF-β), which have been mostly involved in cell proliferation, chemotaxis, and extracellular matrix production/angiogenesis [1,11]. Other GFs discharged from the platelets are fibroblast growth factors (FGF) 1 and 2 and vascular endothelial growth factor (VEGF) which play critical roles in the hemostasis, proliferative, and remodeling phases of wound healing [12–15]. Platelet degranulation also leads to the release of cytokine and chemokines, such as C-C motif ligand 5 (CCL5), interleukin (IL)-1β, IL-8, and macrophage inflammatory protein (MIP)-1α, which contribute to the healing process [16]. Several technical procedures have been developed to obtain different platelet concentrates with variable yield of platelets and cellular components [17–20]. Consistently, they also differ for the qualitative and quantitative release of GFs, cytokines, and chemokines and may find different indications [11,16,21,22]. For instance, plasma rich in growth factors (PRGF) provides supraphysiological concentrations of GFs at the injury environment, which could be used therapeutically to accelerate natural healing [23]. PRGF does not contain leukocytes and requires a specific centrifugation to separate the whole blood into two or three fractions, from the poorest in GFs (the upper fraction) to the richest in GFs (the lower fraction) [24,25]. In a new protocol, PRGF is obtained from the whole plasma column with a reduction in the amount of anticoagulant and activator [26].

Other conceptually different platelet-derived biomaterials include leukocyte-platelet-rich fibrin (L-PRF), characterized by strong fibrin polymerization obtained during the centrifugation process [27,28]. This procedure requires blood collection without anticoagulant and immediate centrifugation for the formation of a fibrin clot, which includes not only platelets but also leukocytes [4,28]. Leukocytes are also a significant source of cytokines and GFs which may synergistically interact with those released by platelets [29]. Together with many cell types, including blood cells, fibroblasts, and endothelial cells, leukocytes participate to the final healing process and, with an intricate crosstalk, each cell type may specifically affect the function of the other cell types

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[21,22]. Most recently, a modified product of the same L-PRF family has emerged, named A-PRF, which requires glass tubes, a lower g-force and an increased time of centrifugation compared to standard L-PRF [30]. However, little is known about the leukocyte composition and the factors released by A-PRF.

Therefore, the aim of this study was to gain insight into the leukocyte subpopulations of L- and A-PRF and investigate cytokines and GFs released by these biomaterials.

Methods

Subject characteristics and biomaterial preparation

For the preparation of L-PRF, A-PRF, and PRGF, blood collection was carried out on nine healthy volunteer donors (M/F: 4/5; age: 26,4 ± 1.6). All were nonsmokers, nonobese (body mass index [BMI] range: 20.4–26.3) and with no history of recent medication neither disease correlated with the coagulation process. Platelet, red blood cell, and lymphocyte counts were determined in anticoagulated blood from the donors. Informed consent was obtained from every subject before blood drawing. The procedure was approved by the ethical committee of the University of Naples (prot no. 148_2015).

Four tubes of blood without anticoagulant were obtained from the antecubital vein of each volunteer to obtain two L-PRF and two A-PRF clots.

L-PRF clots were prepared from 9 ml of peripheral blood collected in plastic tubes (Intra-Spin by Intra-Lock, Boca-Raton, USA) without anticoagulant and immediately centrifuged for 12-min at 400 g using a dedicated centrifuge (PC-02, Process for PRF, Nice, France), as recommended by the manufacturer. For a better comparison of the results, A-PRF clots were obtained by 9 ml of whole blood, instead of commonly used 10 ml, from a single 14-min step of centrifugation at 100 g using glass tubes (Process, Nice, France) according to manufacturer's instruction.

PRGF preparations were obtained as described by Anitua et al. [24]. Briefly, blood trough antecubital vein puncture was collected into 9-ml tubes with 3.8% sodium citrate and was separated in a centrifuge (BTI System IV, Victoria, Spain) at 580 g for 8 min at room temperature. After centrifugation, PPGF (plasma poor in growth factors) was discarded and the remaining plasma was drawn off in three consecutive fractions. The first fraction (F1) made up about 2 ml located at the top of the tube, the second fraction (F2) formed the next 1 ml, and the third fraction (F3) formed the bottom ml just above the red cell layer. Each fraction was gently poured in a sterile glass container and was activated by adding 10% calcium chloride and waiting a dense polymerization at 37°C for 15 mins.

Sample preparation

For the immunophenotypic analysis of cells isolated from PRF, a single-cell leukocyte suspension was prepared by pressing the clots through a 120 μm nylon screen mesh. Then, cell suspensions were centrifuged (300 × *g* for 5 mins, 4°C) and washed twice with phosphate-buffered saline (PBS). To remove erythrocytes, cell pellets were resuspended in 1 ml of ammonium-chloride-potassium (ACK) lysing buffer (Lonza, Basel, Switzerland), incubated for 10 minutes on ice and washed twice with PBS.

For the evaluation of platelet preparation release, L- and A-PRF clots and PRGF gels were incubated for 24 hrs in serum-free Dulbecco modified Eagle medium (DMEM)-F12 (1:1) containing 0.25% bovine serum albumin (BSA). Then, conditioned media were collected and centrifuged at 14 000 × *g* to remove cellular debris and analyzed for cytokine and GF content, as described below.

Flow cytometry

Single-cell leukocyte suspensions were stained (20 mins, 4°C) in PBS + 10% human AB serum (Lonza, Basel, Switzerland) + 0.05% NaN_3 (Staining buffer, SB). The following antibodies were used: anti-CD45 APC (clone 5B1, dilution 1:20), anti-CD25 PE (clone 4E3, dilution 1:20), anti-CD3 PerCP (clone BW264/56, dilution 1:10), anti-CD14 FITC (clone TÜK4, dilution 1:20) (Miltenyi Biotec, Bergisch Gladbach, Germany), anti-CD4 FITC (clone RPA-T4, dilution 1:10), anti-CD4 PE-Cy7 (clone SK3, dilution 1:40), anti-CD8 FITC (clone SK1, dilution 1:20), anti-CD45RA FITC (clone L48, dilution 1:20), anti-CD45RO PE (clone UCHL1, dilution 1:20), anti-HLA-DR PE (clone L243, dilution 1:20), and anti-CD56 FITC (clone NCAM16.2, dilution 1:20) (BD Biosciences, Franklin Lakes, NJ, USA). Samples were acquired on a BD LSRFortessa and analyzed using FACSDiva Software (BD Biosciences, Franklin Lakes, NJ, USA). Data are expressed as percentage of positive cells and median fluorescence intensity (MFI) of positive cells.

Determination of cytokines and growth factors released by platelet derivatives

Conditioned media collected from L-PRF, A-PRF, and F1, F2, and F3 gels were screened for the concentration of IL-2, IL-4, IL-6, IL-8, IL-10, IL-17, Eotaxin, interferon-gamma (IFNγ) MIP-1α, MIP-1β, CCL5, tumor necrosis factor-alpha (TNFα), PDGF, and VEGF using the Bioplex Multiplex human cytokine and GF assay (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions.

Statistical analysis

Data were analyzed with GraphPad Prism 6.0 software (GraphPad Software Inc., La Jolla, CA) by unpaired two-tailed *t*-test and one-way ANOVA followed by Sidak's multiple comparison tests. *p*-values of <0.05 were considered statistically significant.

Results

Analysis of leukocyte subpopulations isolated from L-PRF and A-PRF

We selected nine blood donors whose mean leukocyte count was 5.64 ± 0.5 × 10^3/μl (neutrophils 57.4 ± 3.3%; lymphocytes 33.75 ± 2.8%; monocytes 6.7 ± 0.8%; eosinophils 1.17 ± 0.3%; basophils 0.87 ± 0.3%). The mean platelet count in the whole blood samples was 234.5 ± 29.6 × 10^3/μl, while red blood cells were 4.8 ± 0.2 × 10^6/μl. Next, we performed an immunophenotypic analysis of leukocytes isolated from PRF. The majority of leukocytes ($CD45^+$ cells) isolated from PRF were $CD3^+$ T cells, with a prevalence of $CD4^+$ over $CD8^+$ cells. We also found small percentages of $CD56^+$ NK cells and $CD14^+$ monocytes (Figure 1A). Since $CD4^+$ T cells represented the most frequent cell subset among $CD45^+$ leukocytes, we sought to characterize their phenotype. $CD4^+$ T cells isolated from PRF comprised both naïve ($CD45RA^+$) and memory ($CD45RO^+$) cells, with a prevalence of the former over the latter. When we assessed the expression of the activation markers CD25 and human leukocytes antigen-antigen D related (HLA-DR) we found that only a small percentage of $CD4^+$ T cells expressed CD25, while no expression of HLA-DR could be observed (Figure 1B). Importantly, there was no statistically significant difference between L-PRF and A-PRF in terms of frequency on each leukocyte subpopulation and expression of CD25 on $CD4^+$ T cells (Figure 1C).

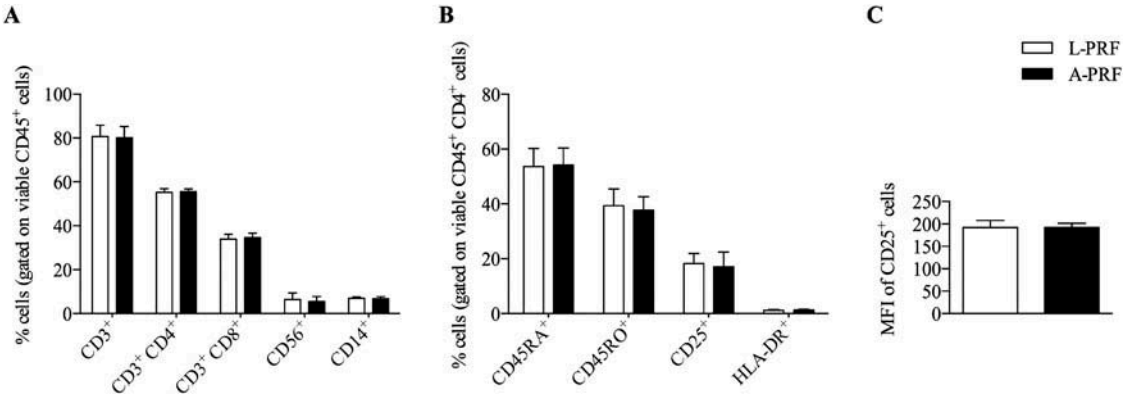

Figure 1. Analysis of leukocyte subpopulations and activation markers in cells isolated from L-PRF (white bars) and A-PRF (black bars). (A) The percentages of the following subpopulations were assessed among total leukocytes (viable $CD45^+$ cells): T lymphocytes ($CD3^+$), $CD4^+$ T lymphocytes ($CD3^+$ $CD4^+$), $CD8^+$ T lymphocytes ($CD3^+$ $CD8^+$), NK cells ($CD56^+$), $CD14^+$ monocytes ($CD14^+$); (B) the percentages of the following subpopulations and of cells expressing activation markers were assessed among $CD4^+$ T lymphocytes (viable $CD45^+$ FSclo SSclo $CD4^+$ cells): naïve ($CD45RA^+$) and memory ($CD45RO^+$) cells, $CD25^+$ and HLA-DR^+ (activation markers) cells; and (C) the expression of CD25 was quantified on viable $CD45^+$ FSclo SSclo $CD4^+$ $CD25^+$ cells as MFI.

Comparison of L-PRF and A-PRF released factors

We compared the release of cytokines/chemokines and GFs by original L-PRF and modified A-PRF clots, incubated in serum-free medium for 24 h. Detectable amount of IL-4, IL-6, IL-8, IL-17, Eotaxin, IFNγ MIP-1α, MIP-1β, CCL5, TNFα, PDGF, and VEGF were released by both platelet concentrates. In contrast, IL-2, IL-10, and bFGF were not detected. Analysis of cytokine and chemokine release revealed that A-PRF secreted Eotaxin and CCL5 at higher levels (increased by 3- and 1.6-fold, respectively) compared to L-PRF (Table I). Moreover, A-PRF clots released a higher amount of IFNγ, albeit without reaching a statistical significance ($p = 0.057$). In contrast, no differences were noted for the concentrations of IL-4, IL-6, IL-8, IL-17, MIP-1α, and MIP-1β released from the two platelet derivatives (Table I). Next, we analyzed PDGF and VEGF secretion, which were, respectively, 3- and 1.7-fold increased in A-PRF compared to L-PRF (Table I).

Table I. Cytokines, chemokines, and growth factors released by L-PRF and A-PRF. L-PRF and A-PRFs clots were incubated with serum-free DMEM-F12 (1:1). After 24 hrs, the media were collected and tested by using the Bioplex multiplex cytokine, chemokines, and growth factors assay kit as described in "Methods" section. Data are shown as mean ± S.E.M. *Denotes statistically differences (*$p < 0.05$; **$p < 0.01$).

	L-PRF (pg/ml)	A-PRF (pg/ml)
IL-2	ND	ND
IL-4	1.16 ± 0.26	0.96 ± 0.23
IL-6	1320.91 ± 477.00	2164.62 ± 713.65
IL-8	6562.96 ± 931.91	7316.73 ± 471.68
IL-10	ND	ND
IL-17	9.17 ± 5.05	2.42 ± 0.56
Eotaxin	4.24 ± 1.49	12.02 ± 1.15 **
IFNγ	30.05 ± 14.75	79.89 ± 17.71
MIP-1α	36.84 ± 7.27	75.35 ± 26.43
MIP-1β	97.73 ± 21.57	117.09 ± 10.11
CCL5	117.08 ± 15.92	191.19 ± 20.27*
TNFα	15.33 ± 3.63	5.87 ± 1.99
bFGF	ND	ND
PDGF	22.74 ± 7.90	69.96 ± 10.53**
VEGF	10.16 ± 1.91	17.93 ± 2.06*

Table II. Cytokines and growth factors released by PRGF. PRGF gels were incubated with serum-free DMEM-F12 (1:1) for 24 hrs; the media were collected and tested by using the Bioplex multiplex cytokines and growth factors assay kit as described in "Methods" section. Data are shown as mean ± S.E.M. *Denotes statistically differences of F2 or F3 versus F1 (*$p < 0.05$).

	PRGF-F1 (pg/ml)	PRGF-F2 (pg/ml)	PRGF-F3 (pg/ml)
Eotaxin	5.36 ± 2.54	1.1 ± 0.13	ND
CCL5	175.86 ± 17.29	322.83 ± 48.71*	387.48 ± 63.74*
PDGF	33.74 ± 8.24	27.34 ± 10.58	49.85 ± 18.15

Platelet-derived factors from PRGF scaffold

Next, the factors released by the three different PRGF fractions were determined. PRGF gels (F1, F2, and F3) were separately collected and activated with calcium chloride. The different fibrin scaffolds were incubated in culture medium for 24 h to obtain conditioned medium (CM). As expected, platelet-derived factors were detected in the medium of PRGF fibrin scaffolds. In contrast, no traces of IL-2, IL-4, IL-6, IL-8, IL-10, IL-17, IFNγ MIP-1α, MIP-1β, TNFα, Bfgf, and VEGF were found (data not shown). Interestingly, we have observed a significant increase of CCL5 release by F2 and F3 compared to F1 fraction (1.8- and 2.2-fold increase, respectively) (Table II). In addition, PDGF content slightly increased in F3 fraction. No differences were noted for the concentration of Eotaxin in F1 and F2, while in F3 it was not detectable (Table II).

Discussion

A large variety of platelet concentrates for surgical use is commercially available and includes a number of unclear products. Under similar names, more than 10 different autologous glues or biomaterials are available. The goal of this research field consists in the use of blood-derived biomaterials to create different therapeutic formulations that adapt to the needs of various biomedical applications, including orthopedic and maxillofacial surgery, sports medicine, bone reconstruction, tissue engineering, and cosmetic and dental implant surgery [4].

The tissue regeneration or repair process requires harmonious reaction of various types of cells, including immune response cells (neutrophils, macrophages, and lymphocytes), epithelial cells, fibroblasts, and stem cells. The PRF scaffolds represent a

good source of all components necessary for the healing process releasing a large variety of factors [21,27]. Our current data show that A-PRF and L-PRF composition reflects that of peripheral blood in terms of frequency on each leukocyte subpopulation. Both kinds of clots contain living white blood cells, and, possibly, circulating progenitor cells, entrapped in a high-density fibrin network, which also includes platelets.

It is now accepted that platelets have a major role in cell communication, inflammatory, and healing responses [23,31,32]. Platelets are able to synthesize and contain granules with high levels of bioactive molecules, promoting recruitment, and growth and morphogenesis of cells [10]. The inclusion of leukocytes within platelet biomaterials has always been a matter of debate. At variance with platelets, white blood cells are mainly considered to contain and produce inflammatory cytokines. Nevertheless, they play an important role as immune modulators [33]. We have compared *in vitro* behavior of both A-PRF and L-PRF, as platelet/leukocyte scaffolds, through the evaluation of the release of GFs, cytokines, and chemokines.

Our data show that A-PRF releases VEGF at higher levels compared to L-PRF. VEGF is largely produced by leukocytes [34] and is crucially important for the promotion of angiogenesis during tissue repair. Indeed, reduced expression and rapid proteolytic degradation of VEGF are considered partially responsible for poor wound healing, as for example in diabetic wounds, because these effects result in poor angiogenesis during granulation tissue formation [35]. Thus, the addition of VEGF alone or in combination with other GFs may represent a significant step in medical treatment of nonhealing wounds and other ischemic processes [36].

The most abundant factor released by platelets is PDGF, mostly involved in cell proliferation, chemotaxis, and extracellular matrix production/angiogenesis [1,11]. In agreement with other reports [37], we have shown that the release pattern of PDGF was higher in A-PRF compared to L-PRF. The finding that A-PRF releases a greater amount of PDGF compared to L-PRF suggests that the reservoir property of A-PRF fibrin network could enhance platelet content compared to L-PRF; however, this hypothesis requires further investigation. Moreover, A-PRF releases greater amount of both CCL5 and Eotaxin compared to L-PRF. CCL5 and Eotaxin chemokines are small secreted proteins highly involved in chemotaxis and angiogenesis [38]. For instance, Eotaxin (also known as CCL11) induces the formation of blood vessels and stimulates the chemotaxis of human microvascular endothelial cells [39]. However, CCL5, previously called RANTES, promotes angiogenesis both *in vivo* and *in vitro* mainly increasing the expression of VEGF [40]. CCL5 is released at higher levels also by platelet-rich plasma gels [21,22,41,42]. The finding that platelet derivatives secrete CCL5 is particularly interesting for tissue engineering strategies because the local release of this chemokine may intensify cell mobilization, enhance vascularization, and therefore improve the healing process [42].

The mechanism responsible for the differences between L- and A-PRF has not been completely explained, but it may involve activation of the cells embedded in the clot, following condensation and mechanical stress. A-PRF is, in fact, a variation of the original L-PRF, based on lower centrifugation speed and longer centrifugation time [30]. Thus, changing the original protocol could modify the secretory properties of PRF.

Thus, we have reported that L- and A-PRF entrap the same content of viable leukocytes and release a similar amount of inflammatory cytokines. However, A-PRF released higher amount of chemotactic and proangiogenic molecules, like VEGF, PDGF, CCL5, and Eotaxin compared to L-PRF. This finding suggests the use of A-PRF in clinical fields that require tissue repair and new vessel formation. It should be noticed that all our observations on PRFs are still investigational and refer to *in vitro* models, not necessarily intended for use in clinical applications. We have also investigated the release properties of a platelet-derived biomaterial obtained by a completely unrelated procedure, named PRGF. Consistently with the absence of leukocytes in PRGF, we showed an almost complete absence of proinflammatory cytokines in the fractions. However, we have found that PRGF released a significant amount of PDGF and CCL5. Both molecules were found at higher levels in the F3, compared to the other fractions.

Developing therapeutic autologous formulations that control the dose of GFs and their local release into injured tissue is critical to achieving a successful outcome [43].

In conclusion, biomaterials can act as controlled release devices, which will allow for sustained or even on demand delivery of these GF cocktails. Further investigations are needed to consider our experimental approach for clinical use.

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Declaration of interest

The authors report no conflicts of interest.

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