Platelet-rich fibrin (PRF): A second-generation platelet concentrate. Part III: Leucocyte activation: A new feature for platelet concentrates?

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Platelet-rich fibrin (PRF) belongs to a new generation of platelet concentrates, with simplified processing and without biochemical blood handling. In this third article, we investigate the immune features of this biomaterial. During PRF processing, leucocytes could also secrete cytokines in reaction to the hemostatic and inflammatory phenomena artificially induced in the centrifuged tube. We therefore undertook to quantify 5 significant cell mediators within platelet poor plasma supernatant and PRF clot exudate serum: 3 proinflammatory cytokines (IL-1β, IL-6, and TNF-α), an antiinflammatory cytokine (IL-4), and a key growth promoter of angiogenesis (VEGF). Our data are correlated with that obtained in plasma (nonactivated blood) and in sera (activated blood). These initial analyses revealed that PRF could be an immune regulation node with inflammation retrocontrol abilities. This concept could explain the reduction of postoperative infections when PRF is used as surgical additive. **(Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2006;101:E51-5)**

Platelet-rich fibrin (PRF) represents a new step in the platelet gel therapeutic concept.[1-4] Clinical data reveal that this biomaterial would be a favorable matrix for the development of a coherent healing without inflammatory excess.[5,6] It is therefore very important to identify in the PRF clot all the powerful homeostatic regulation molecules capable of controlling postsurgical inflammation. Although platelet secretions obviously play a significant role,[7-10] many other blood elements are able to release specific cytokines, the properties of which could intervene in the regulation of inflammatory reactions.

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CYTOKINES, HOMEOSTASIS, AND INFLAMMATION

What is inflammation?

Inflammation is defined by all reaction phenomena initiated in response to a specific aggression. The inflammatory process proceeds in 3 successive phases: vascular phase, cellular phase, and cicatrization phase.

The vascular phase is characterized by the development of hemostasis (ie, the constitution of a fibrin-based cicatricial matrix) and the installation of a leucocytic node (i.e., the arrival on the injured site of inflammatory cells able to coordinate all concerned cellular forces leading them to the antiinfectious cover of initial healing steps). Finally, all the hemostatic processes lead to coagulation along the vascular wound and to the formation of a fibrin clot.

The initial vasoexudative phenomena allow leucocyte migration to the inflammatory site. The first in place are polymorphonuclear leucocytes; they are replaced by monocytes/macrophages with their high phagocytosis ability. Lymphocytes and plasmocytes take part in the specific antigenic reaction.

All these cells are activated on inflammatory sites and secrete many cytokines and growth promoters. The inflammation mediators take part in the fibroblast recruitment, induce proliferation, and stimulate biosynthetic activity, leading to the secretion of proteases (matrix

metalloproteases, plasmin) as well as the neosynthesis of the matricial macromolecules.

As inflammatory processes are inherent to the surgical act itself, the PRF addition might decrease many harmful effects, mainly by correcting certain destructive and noxious excesses during the healing process of wounded tissues.

Inflammatory cytokines

The number of mediators implied in inflammatory processes is highly significant. We thus specifically focused on 3 principal inflammatory cytokines: IL-1β, IL-6 and TNF-α. Note that during an inflammatory phenomenon, the secretion peaks from these 3 cytokines are synchronized in space and time. In fact, these 3 molecules together constitute a key link in inflammation.

Interleukin-1β (IL-1β). IL-1 is produced by the activated macrophages, neutrophils, endothelial cells, fibroblasts, keratinocytes, and Langerhans cells. It is a key mediator of inflammation control.[11-13] It exists in 2 isoforms, α and β, corresponding to 2 different genes, but conserves 27% proteinic homology. IL-1β remains the prevalent form.

The IL-1 synthesis is mediated by TNF-α, Interferons (IFN) α, β, and γ, and bacterial endotoxins. Its main activity is the stimulation of T helper lymphocytes.

Note that in combination with TNF-α, IL-1 would be implied in osteolysis: Indeed, it activates osteoclasts and inhibits bone formation.[14]

Interleukin 6 (IL-6). IL-6 is an inflammatory cytokine associated to the IL-1β and TNF-α[15] circuit. Its principal sources in vivo are stimulated monocytes, fibroblasts, and endothelial cells. Macrophages, T and B lymphocytes, granulocytes, mastocytes, chondrocytes, and osteoblasts also produce IL-6 after stimulation. And physiologically, IL-6 secretion is stimulated by IL-1, bacterial endotoxins, TNF-α, and PDGF. IL-6 can also stimulate or inhibit its own synthesis.[16]

IL-6 is a differentiation factor for B lymphocytes and an activator for T lymphocytes. In the presence of IL-2, it induces the differentiation of mature and immature T lymphocytes into cytotoxic T lymphocytes. Moreover, after cell pretreatment and activation by IL-4, IL-6 is able to induce the final differentiation of B lymphocytes into secreting plasmocytes. Indeed, within the B lymphocytes populations, IL-6 significantly stimulates the secretion of antibodies. This rate can increase 120 to 400 times. Lastly, note that IL-6 and IL-3 act in a synergistic way to promote hematopoietic stem cell proliferation in vitro.

IL-6 thus constitutes a major amplification pathway for signals transmitted to immune cells.[17,18] Thus, IL-6 will support the reaction chains leading to inflammation, destruction, and remodeling.

Tumor necrosis factor α (TNF-α). TNF-α is one of the cytokines first released during the inflammatory response to bacterial endotoxin aggression. After stimulation by bacterial antigens, TNF-α is secreted by monocytes/macrophages, neutrophils, polymorphonuclear leucocytes, and T lymphocytes. Its production is under-regulated by IL-6 and TGF-β.[19]

TNF-α activates monocytes and stimulates the remodelling capacities of fibroblasts. In addition, it increases phagocytosis and neutrophil cytotoxicity and modulates the expression of key mediators such as IL-1 and IL-6.[20,21]

Healing cytokines

A cicatricial property can be defined in relation to 2 aspects:

— Either it inhibits the inflammatory signal pathways and neutralizes their amplification. This is the case of IL-4.
— Or it supports and coordinates the development of initial cicatricial structures such as vascular tubes. This is the case of VEGF.

Interleukin 4 (IL-4). IL-4 is produced mainly by a subpopulation of activated T cells (TH2, CD4+) which also secrete IL-6.[22] This cytokine conventionally supports proliferation and differentiation of the activated B cells; however, its effects are completely dependent on the cytokine environment.[23,24]

During inflammatory phenomena, its principal function appears to support healing by moderating inflammation. For example, it increases fibrillary collagen synthesis by fibroblast[25] and inhibits stimulation of MMP-1 and MMP-3 by IL-1β. In fact, it neutralizes all transduction pathways from IL-1β−mediated signals, such as stimulation of PGE2 synthesis.[26] This role of inflammation regulator is thus very clear. Macrophage treatment by IL-4 prevents the production of IL-1β, TNF-α, and prostaglandins in response to cell activation by bacterial endotoxins or IFN-γ.[27,28]

Vascular endothelial growth factor (VEGF). VEGF is the most powerful and ubiquitous of known vascular growth promoters.[29] It plays a direct role in the control of endothelial cell behaviors, such as proliferation, migration, specialization, or quite simply survival.[30,31] In fact, the simple presence of this cytokine will be enough to start angiogenesis and the combination of its different isoforms will make it possible to direct and refine the development plan of the network growth.[32]

Study questions

With the 5 cytokines previously described, a rather complete description of the main immune links is given, in their inflammatory (IL-1β, IL-6, TNF-α) and

cicatricial (IL-4, VEGF) features. It was therefore of interest to quantify their presence within the PRF clot, whose properties of immune stimulation and inflammation control are clinically established.

Therefore, investigations were carried out on the secretion profile of these cytokines in the different parts of the PRF collection tube, to determine leucocyte behavior during PRF processing.

MATERIAL AND METHODS

Blood collection was carried out on 15 healthy volunteers, nonsmoker males from 20 to 28 years of age. In accordance with French law no. 88-1138 of December 20, 1988, concerning the protection of people participating in biomedical research, and the World Medical Association Helsinki Declaration, the volunteers were given clear and honest information beforehand on the nature and the objectives of our study.

Blood samples were treated according to the PRF protocol with a PC-02 table centrifuge and collection kits provided by Process (Nice, France).[33] They were therefore taken without anticoagulant in 10-mL glass-coated plastic tubes that were immediately centrifuged at 3000 rpm (approximately 400*g*) for 10 minutes. After PRF processing, 2 distinct samples were collected (Fig. 1):

— Supernatant represents acellular plasma, or platelet-poor plasma (PPP).
— Exudate resulting from PRF clot corresponds to the solution trapped in the fibrin meshes. To collect it, it was necessary to leave the PRF clots in a sterile metal cup for approximately 10 minutes to let them slowly release the serum contained therein.

The 2 samples types were stored in Eppendorf's 2 mL collection tubes at −80°C. The rates of IL-1β, IL-4, IL-6, TNF-α, and VEGF were quantified in these samples by ELISA (Quantikine; R&D Systems, Minneapolis, Minn). Average values were deferred on histograms and analyzed statistically.

These values were then compared to those obtained on total blood according to 2 protocols:

— The blood sample is taken in a dry tube and kept motionless for 30 minutes, to leave it enough time to coagulate completely. Then the tube undergoes a 15-minute centrifugation at 1000*g*, making it possible to recover a representative serum for cytokine rates from completely activated blood.
— The blood sample is taken with anticoagulant (EDTA, citrate, or heparin) then immediately centrifuged for 15 minutes at 1000*g*. Plasma analysis will then indicate the free circulating cytokine rates in nonactivated blood.

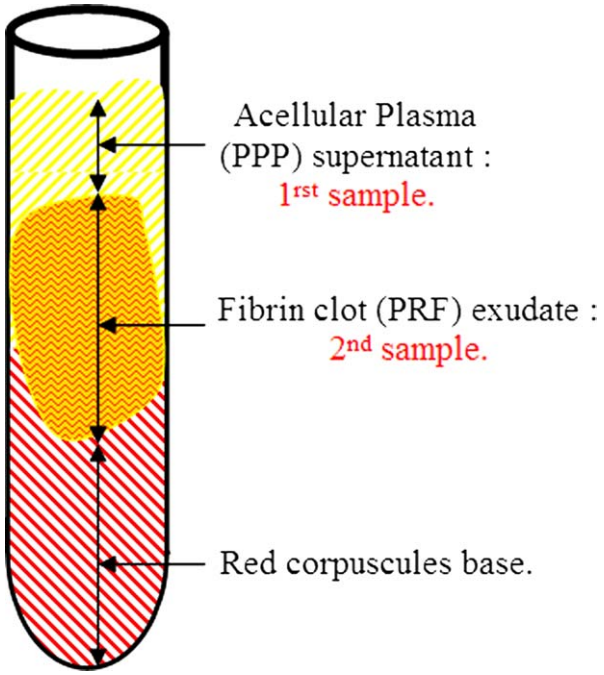

Fig. 1. Schematic representation of the 3 centrifugation strata obtained after PRF processing according to the official Process protocol.

The experimental results were correlated by using Student unilateral test, with a 5% significance threshold. These tests were carried out in 2 different ways:

— between the PPP supernatants and the PRF clot exudates
— between the PRF clot exudates and the average plasmatic and serologic average rates established by the laboratory.

RESULTS

Two major items of data were highlighted by statistical analysis of the results obtained.

First, there are no significant differences ($P < 5\%$) between the cytokine concentrations measured in PPP supernatant and those in the actual PRF clot.

Second, the values obtained in PRF clot exudates (or PPP supernatant) are all significantly higher than those measured in plasma and sera samples.

These results are identical whatever the tested cytokine, from proinflammatory mediators, such as IL-1β (Fig. 2), IL-6 (Fig. 3) and TNF-α (Fig. 4), to antiinflammatory cytokines, such as IL-4 (Fig. 5). Only the VEGF (Fig. 6) was an exception, with particularly high serologic concentrations.

DISCUSSION: IS PRF AN IMMUNE NODE?

Our results highlight an increased secretion of all inflammatory or cicatricial tested interleukins. Their

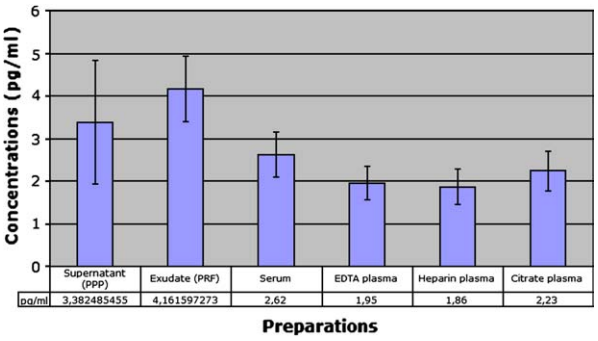

Fig. 2. IL-1β ELISA Quantifications.

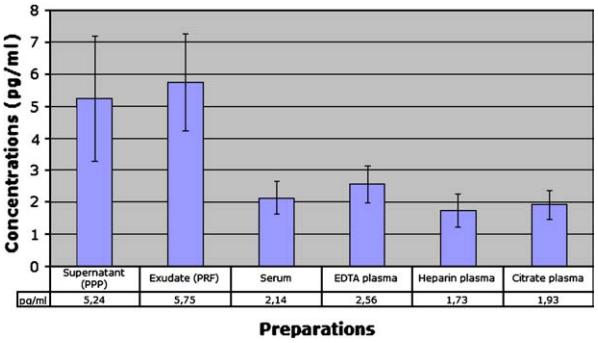

Fig. 3. IL-6 ELISA Quantifications.

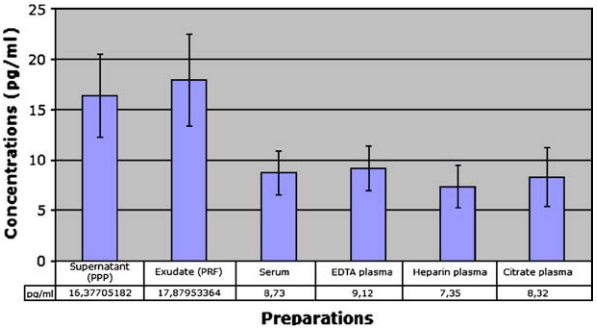

Fig. 4. TNF-α ELISA Quantifications.

origin can only be leucocytic, which means that the PRF slow blood activation process could induce an increased leucocyte degranulation. Indeed, comparisons between PRF clot exudates (or PPP supernatant) and nonactivated (plasma) or activated (serum) blood reveal very clearly an activation phenomenon during PRF processing centrifugation.

Moreover, previous conclusions from platelet cytokine quantifications imply that these 5 tested cytokines would be also progressively trapped in the fibrin networks during polymerization: Like platelet molecules, these leucocytic cytokines will be certainly trapped within the fibrin meshes and slowly released.

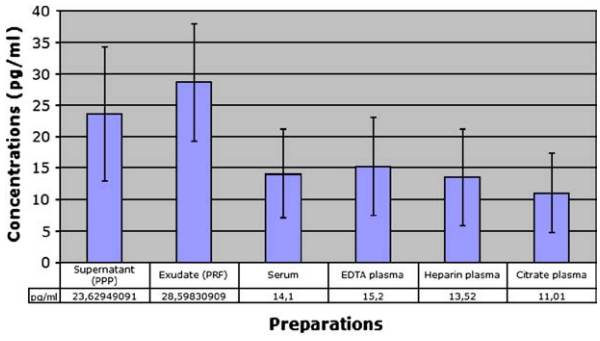

Fig. 5. IL-4 ELISA Quantifications.

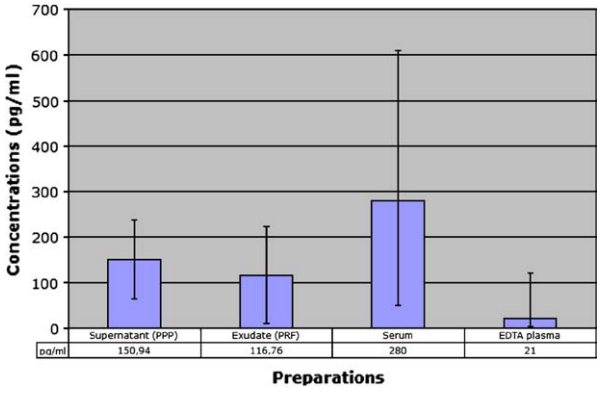

Fig. 6. VEGF ELISA Quantifications.

Only the VEGF presents significantly higher serologic concentrations than exudates, supernatant, or plasmatic rates. Some part of the detected VEGF probably results from platelet activation, which explains its high concentration in activated blood. The leucocytic secretion of VEGF could thus be regarded as negligible. But noted differences between activated blood and supernatant PPP (or PRF exudate) once again point out the importance of the intrinsic trapping of cytokines in the PRF fibrin matrix.

With such a content in immune key cytokines (pro- or anti-inflammatory) and angiogenesis, the PRF clot could be considered as an immune organizing node. Its defense capacities against infections would be quite significant, by the chemotactic properties of these cytokines as well as by their capacity to facilitate the access to the injured site (neovascularization). Lastly, it is very interesting to note the presence of inflammatory retrocontrol cytokines, particularly IL-4.

CONCLUSION

Cytokines play a significant role in the delicate balance of tissue homeostasis. Although cPRP and now PRF platelet features are already well documented,[34-37] this is definitely not the case for inflammatory aspects.

The results obtained therefore open a new research opportunity for the comprehension of these technologies, because PRF is not only a platelet concentrate but also an immune node able to stimulate defense mechanisms. It is even likely that the significant inflammatory regulation noted on surgical sites treated with PRF is the outcome of retrocontrol effects from cytokines trapped in the fibrin network and released during the remodeling of this initial matrix.

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