Time-dependent release of growth factors from implant surfaces treated with plasma rich in growth factors

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Abstract: Plasma rich in growth factors (PRGFs) technology is an autologous platelet-rich plasma approach that provides a pool of growth factors and cytokines that have been shown to increase tissue regeneration and accelerate dental implant osseointegration. In this framework, the spatiotemporal release of growth factors and the establishment of a provisional fibrin matrix are likely to be key aspects governing the stimulation of the early phases of tissue regeneration around implants. We investigated the kinetics of growth factor release at implant surfaces functionalized either with PRGFs or platelet-poor plasma and correlated the results obtained with the morphology of the resulting interfaces. Our main finding is that activation and clot formation favors longer residence times of the growth factors at the interfaces studied, probably due to their retention in the adsorbed fibrin matrix. The concentration of the platelet-derived growth factors above the interfaces becomes negligible after 2–4 days and is significantly higher in the case of activated interfaces than in the case of nonactivated ones, whereas that of the plasmatic hepatocyte growth factor is independent of platelet concentration and activation, and remains significant for up to 9 days. Platelet-rich plasma preparations should be activated to permit growth factor release and thereby facilitate implant surface osseointegration. © 2012 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 00A:000–000, 2012.

Key Words: growth factors, platelet-rich plasma, PRGF-Endoret, activation, calcium, thrombin


INTRODUCTION
Platelets play a crucial role in several stages of the wound-healing process, starting with coagulation.1–5 Activated at the wound site, they aggregate and form a primary platelet plug. Phosphatidyl serine, a phospholipid exported to the platelet surface on activation, catalyzes various steps in the coagulation cascade6 that culminate in the conversion of fibrinogen to fibrin. The latter polymerizes, forming a clot, in which platelets serve as anchor-points. In addition to the hemostatic properties, the fibrin matrix of the clot serves as natural scaffolding for the regeneration of the healthy tissue at the site of the wound.

In recent years, it became clear that platelets’ role in the wound-healing process is not limited to hemostasis.6 They orchestrate both the inflammatory response and the subsequent tissue remodeling that follow the coagulation phase7 by releasing, on activation, an array of growth factors and immune system mediators that are normally stored in the granules inside the quiescent platelets.4,6–8 The roles that some of them play in the wound-healing processes were recently reviewed by Eppley7 and by Anitua.8 The recognition of the role played by growth factors in the wound-healing process leads to studies where they are delivered directly to the wound site.9–20 Their application does indeed speed up the wound-healing process and improves tissue regeneration around implants. However, a more efficient and controlled way to deliver growth factors to the wound is to use patients’ own platelets.21 First attempts to use platelet-rich plasma products in maxillofacial surgery steamed from the use of fibrin glues as hemostatic agents.22 By now, several procedures have been developed to obtain and apply platelet-rich plasma.7,23–28 These procedures vary in terms of the final platelet concentration in the preparation that is applied.
to the wound and in terms of other parameters, such as presence or absence of leukocytes or method to achieve platelet activation among others. Logically, these different biological characteristics may affect their clinical results. An effort should therefore be made to clearly differentiate and characterize the type of plasma preparation used.

Accordingly, in this study, we have employed a well-established technology, the plasma rich in growth factors (PRGF-Endoret) protocol developed by Anitua et al., which pioneered the activation of implant surfaces. It consists in one-step centrifugation of citrate-anticoagulated whole blood to obtain a leukocyte-free platelet-rich plasma, followed by activation with CaCl₂ solution and application to the surface of the implant as well as to the implantation site immediately before implant placement. The ability of PRGF-Endoret to choreograph wound-healing has been harnessed in various areas of surgical wound repair, implant integration, sports, and regenerative medicine. Treatment of the wound sites and implant surfaces with activated PRGF-Endoret creates a platelet-containing fibrin matrix, thus mimicking the first step of healing.

Our goal is to investigate the kinetics of growth factor release from the resulting implant interface, and how changes in the preparation protocol affect this kinetics. The variables include platelet count in the plasma and the use of thrombin with CaCl₂ (CaT) or CaCl₂ (Ca) alone in its activation. The interface that is formed on the implant surface is preheated for 30 min with 2% sodium dodecyl sulfate solution (Fluka, St. Louis) that was filtered through 0.2 μm pore diameter syringe filter (BD, Franklin Lakes, N.J.) and rinsed under a stream of nanopure water. Water was blown off with a filtered nitrogen stream. Dry surfaces were further treated with UV-Ozone for 30 min in a UV/Ozone cleaner (BioForce Nanosciences) that was preheated for 30 min immediately before use.

### TABLE I. Cell Counts Obtained for the Plasmas Used

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Cell Type</th>
<th>Multiplier and Units</th>
<th>Instrument Error</th>
<th>ELISA</th>
<th>SEM</th>
<th>± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRGF</td>
<td>wBC</td>
<td>×10³/μL</td>
<td>±0.8</td>
<td>0.1</td>
<td>0.0</td>
<td>0.1</td>
</tr>
<tr>
<td>PpP</td>
<td>wBC</td>
<td>×10³/μL</td>
<td>±0.8</td>
<td>0.1</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>rBC</td>
<td>×10³/μL</td>
<td>±0.18</td>
<td>0.02</td>
<td>0.01</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>Plt</td>
<td>×10³/μL</td>
<td>±40</td>
<td>244 (3.01)</td>
<td>520 (2.89)</td>
<td>34 (1.56)</td>
</tr>
</tbody>
</table>

Platelet enrichment factor with respect to whole blood is indicated in parentheses.

PRGF-Endoret and platelet-poor plasma preparation

Blood from healthy donors was collected into 3.8% (wt/vol) sodium citrate-containing tubes (Biotechnology Institute S.L., Spain). Blood samples were centrifuged at 580 g for 8 min in a PRGF System IV centrifuge (Biotechnology Institute S.L., Spain) to obtain PRGFs in accordance with the PRGF-Endoret procedure. Platelet-poor plasma (PpP) was obtained by the second centrifugation of PRGF-Endoret at 4500 g for 6 min at room temperature in a Sigma 3K30 centrifuge (Sartorius AG, Germany). Cell counts (Table I) were obtained with an ABC Micros60 hemocytometer counter (Horiba, Japan).

Experimental procedure

The experiments were carried out with different interface models: calcium activated (Ca), calcium/thrombin activated (CaT), and nonactivated (NA) PRGF and PpP. For Ca samples, 1.25 μL of a 456 mM calcium chloride solution (Sigma-Aldrich, St. Louis) was incubated on the surfaces of the disks for 1 h. For thrombin-activated samples, 5 μL of a 0.4 mg/mL thrombin solution (Sigma-Aldrich, St. Louis), diluted in water, were mixed with 25 μL of PRGF or PpP immediately before application to the surface of the disk. Implants were incubated with 25 μL of plasma preparations

Materials and methods

Surface preparation and cleaning

Rough, commercially pure grade IV titanium discs (6 mm diameter and 1 mm height) were produced by Biotechnology Institute S.L., Spain, after their normal industrial procedures for dental implant fabrication. The discs were cleaned for 30 min with 2% sodium dodecyl sulfate solution (Fluka, St. Louis) that was filtered through 0.2 μm pore diameter syringe filter (BD, Franklin Lakes, N.J.) and rinsed under a stream of nanopure water. Water was blown off with a filtered nitrogen stream. Dry surfaces were further treated with UV-Ozone for 30 min in a UV/Ozone cleaner (BioForce Nanosciences) that was preheated for 30 min immediately before use.
for 1 h, at which point the solution above the clot (supernatant) was removed with a pipette. This is the D0 sample. A 200 µL of fresh medium was added on top of the clot and the samples were incubated at 37°C in an incubator for 2 days. After 2 days, 200 µL of the medium was removed (the D2 sample) and replaced with fresh medium. The samples were incubated, and the procedure was repeated after 4 days (D4) and 9 days (D9). Collected samples were frozen at −20°C and stored until the end of the experiment. The medium used was DMEM/F-12 (Ham) Dubelcco’s modified Eagle’s medium without phenol red (Gibco, Invitrogen, NY) complemented with 0.3 mg/mL of genetecin (Gibco, Invitrogen, NY) and 1% of penicillin/streptomycin (Gibco, Invitrogen, NY). This procedure is schematically depicted in Figure 1. All the assays were carried out in triplicate.

**ELISA analysis**
Frozen samples were thawed by placing them at room temperature and the concentrations of HGF, PDGF-AB, TGF-β1, and VEGF were measured using Quantikine kits (R&D Systems, Minneapolis) after the manufacturer’s instructions.

**Sample characterization**
The surface chemistry was analyzed by X-ray photoelectronic spectroscopy to ensure that the implants did not
contain more than 30% of carbon. The spectra were obtained with a SPECS SAGE HR 100 instrument with non-monochromatized Mg Kα radiation. Wide scan (1100–0 eV Binding Energy, BE) spectra were acquired with an electron take-off angle of 90°. The hydrocarbon peak component in the C1 spectra was set at 285.0 eV to correct for sample charging. The roughness of the bare surfaces was measured by optical profilometry (3D Sensofar Plu, Terrasa, Spain). We employed a Gaussian filter of 20 μm × 20 μm to separate roughness from waviness. The main roughness values were Sa = 0.71 ± 0.05 μm, Sq = 0.94 ± 0.09 μm, and Sdr = 77.51 ± 13.07%. SEM (JEOL JSM-6490LV, Tokyo, Japan) was used to study sample morphology on groups described before and on bare surfaces. For this analysis, plasma samples were fixed by incubation with 2.5% of glutaraldehyde (Sigma-Aldrich, St. Louis, MO) solution in phosphate-buffered saline (Sigma-Aldrich, St. Louis, MO) for 12 h. Fixed samples were dehydrated by sequential immersion in serial diluted solutions of 0, 10, 30, 50, 70, 90, and 100% (v/v) of ethanol in water. Dehydrated samples were then air-dried, coated with gold (or gold-palladium) in a sample preparation chamber with sputtering system (Gatan Alto 1000E,

**FIGURE 2.** SEM images from different samples (A) Ca-activated PrP, (B) Ca-activated PpP, (C) CaT-activated PRGF, (D) CaT-activated PpP, (E) nonactivated PRGF, (F) nonactivated PpP, (G) bare titanium.
Nuffield, UK) and examined by SEM. Images were taken at 10 kV of acceleration voltage.

Statistical analysis
Data are presented as the mean ± standard deviation of at least two replicates of the donor. Differences between the means of each interface were determined by one-way analysis of variance using Origin V7.5 (OriginLab Corporation, Northampton, MA). Statistical significance was accepted for $p < 0.05$.

RESULTS
Six different kinds of interfaces were prepared as shown in Figure 1 by incubating implant surfaces with PRGFs or PpP that was activated with CaCl$_2$, CaCl$_2$ + thrombin, or not activated (Ca, CaT, or NA, respectively). Implant surfaces were prepared according to the standard dental implant surface production process of the manufacturer.

Interface morphology
The morphology of the six interfaces was analyzed by SEM (Figure 2). Platelets and fibrin network can be clearly seen in the calcium-activated samples [Ca, Figure 2(A,B)]. As expected, there were more platelets and thicker fibrils in the PRGF group [Figure 2(A)] than in PpP group [Figure 2(B)] due to the difference in the platelet counts between the two sets of samples (TABLE I). The average fibril thickness (taken from 10 measurements of each of three different samples using the software ImageJ (NIH, Maryland) is 0.42 ± 0.10 µm for Ca-PRGF samples and 0.27 ± 0.07 µm for PpP ones. For CaT-activated samples [Figure 2(C,D)], the fibrin network is denser in PRGF samples comparing with PpP ones. Fiber diameter is expected to decrease with increasing thrombin concentration. This can be clearly seen in PRGF samples, the fibrin network in CaT-activated samples was much thinner than in Ca-activated ones (0.23 ± 0.06 vs. 0.42 ± 0.10 µm), but the effect is not present in PpP samples, showing similar values (0.28 ± 0.09 µm in CaT-activated vs. 0.27 ± 0.07 µm in Ca-activated ones). In the nonactivated samples, for both PRGF [Figure 2(E)] and PpP [Figure 2(F)], platelets presented a round shape and no fibrin network was observed.

Growth factors release kinetics
Plasmatic growth factor. The concentrations of HGF measured at different time points above various plasma-derived interfaces are shown in Figure 3. First, its concentration in the supernatant above the clot after 1 h is nearly independent of the platelet concentration in the samples used to prepare the interfaces (571 and 578 pg/mL for Ca and NA PRGF samples, 453 and 444 pg/mL for the corresponding PpP samples, respectively). This is consistent with its plasmatic localization. Slightly lower amounts were found in the supernatants of the CaT-activated samples (364 and 371 pg/mL, for PRGF and PpP, respectively), but these differences are not statistically significant ($p < 0.05$).

Interestingly, considerable amount of this growth factor was retained by the interface, 30% of the total amount was detected after 2 days in Ca-activated samples. Moreover, its concentration remained non-negligible even after 9 days of incubation. There was no statistically significant difference in the HGF levels between different types of interfaces (PRGF or PpP, Ca, CaT, or NA).
Platelet-derived growth factors. The analysis of platelet-derived growth factor release—PDGF, TGF-β1, and VEGF—is shown in Figure 4–Figure 6. As expected, their concentrations were significantly higher in PRGF samples than in PpP samples ($p < 0.05$).

Activation had a profound effect on the distribution of the growth factors between the surface-adsorbed material and the supernatant above it (panels A in Figs. 4–6), as well as on the kinetics of growth factor release from the interfaces (panels B in Figs. 4–6). Specifically, the concentrations of all three growth factors were always higher in the supernatants above the nonactivated PRGF samples than the Ca-activated ones: 5202 vs. 3031 pg/mL for PDGF, Figure 4(A); 54,684 pg/mL vs. 22,160 pg/mL for TGF-β1, Figure 5(A);
423 pg/mL vs. 312 pg/mL for VEGF, Figure 6(A)]. For VEGF, the difference was much smaller than for the other two factors. The differences between Ca- and CaT-activated samples were not substantial.

On the contrary, measurements performed after 2 days show that the interfaces in Ca-activated PRGF samples retained significantly more of these growth factors than did the interfaces in the nonactivated samples (panels B in Figs. 4–6). In the case of PDGF-AB, a difference was observed between Ca- and CaT-activated samples [Figure 4(B)]: the concentration of PDGF-AB in the media above the CaT samples was lower than that above the Ca-activated ones although the difference was not statistically significant (p < 0.05). For this growth factor, 35% of the total detected amount was found in Ca-activated samples after 2 days and 8% in the nonactivated ones. In addition, for VEGF the retention in activated samples was considerably higher than in nonactivated samples (41% of total amount detected for Ca activated after 2 days vs. ~15% in nonactivated samples). For the VEGF and TGF-β1, the differences due to thrombin were not significant. PpP samples showed insignificant amounts of these growth factors and will not be discussed further.

**Total amount of growth factors.** The total amount of the different growth factors found for each interface can be analyzed by summing the different amounts of the growth factors obtained during the entire assay (days 0, 2, 4, and 9), and the amounts released from the interface by summing the amounts of GFs released after incubation with the media (Supporting Information Table S1).

For HGF, both the total amount and the amount released were independent of the platelet concentration or activation [Figure 7(A,B)]. The total amount in CaT samples was slightly lower than in the Ca or NA samples. The amount of HGF detected after the removal of the supernatant in the CaT samples [Figure 7(B)] was slightly higher than in the Ca or NA samples, but otherwise also independent of platelet concentration or activation. We note that the differences in the amount of HGF due to thrombin are not statistically significant.

The total amounts of the three PDGFs strongly depended on the platelet concentration, as expected. Therefore, higher amounts of these growth factors were present in the PRGF-derived samples than in the PpP-derived ones [Figure 7(C,E,G)]. Nonactivated PRGF samples showed substantially higher amounts of TGF-β1 (1460 pg) than Ca-activated samples (722 pg), and slightly higher amounts of PDGF-AB (154 pg in nonactivated samples vs. 148 pg in Ca-activated samples). For VEGF, the opposite behavior was observed: the total amount was observed in Ca samples (15 pg) was higher than the amount in nonactivated ones (13 pg). No significant effect of thrombin on the total amounts of these three factors detected in this study was observed.

In contrast, the amounts of the PDGFs retained and released by the interfaces [Figure 7(D,F,H)] were much higher in the Ca-activated samples than in the nonactivated ones: 72 pg versus 24 pg for PDGF-AB, 168 pg versus 93 pg for TGF-β1, and 7 pg versus 3 pg for VEGF.

Thrombin had an interesting effect on the release of PDGF-AB: the amount of this growth factor that is released in CaT-activated samples is almost the same as in the nonactivated ones [Figure 7(D)]: 27 pg and 24 pg, compared with 72 pg in the Ca-activated samples. This observation is all the more significant considering that, as we said above, the thrombin effect on the total amount of PDGF-AB measured...
was not so high [Figure 7(C)]. Therefore, a significant amount of this growth factor remains associated with the fibrin matrix.

The amounts of the other two growth factors released in CaT-activated samples were lower, but not significantly so, than in the Ca-activated ones. In summary, activation with thrombin does not appear to affect the retention of the growth factors by the interfaces but does decrease their release, most significantly in the case of PDGF-AB.

**DISCUSSION**

The aim of this study was to examine the fibrin network formation and growth factor release from implant surfaces treated with PRGF-Endoret and to compare them with the ones obtained with PpP. Besides platelet content, the interfaces varied also in the method of activation (no activation, activation with Ca or with Ca and thrombin).

Activation of platelets plays an essential role in the clot formation.1-5 It is not surprising, therefore, that we found a
fibrin network in the activated samples [Figure 2(A,D)] and no such network from the surfaces in the nonactivated samples [Figure 2(E,F)]. Platelet concentration and the kind of activator used (Ca or CaT) had effects in terms of fiber thickness and morphology. Fibrils were thicker in the Ca-activated PRGF preparation than in all the other preparations. The decrease in fiber thickness on addition of thrombin (PRGF-ACa vs. PRGF-ACaT) is consistent with previous observations of Weisel et al. 42 and Ryan et al., 41 who showed that fiber thickness in fibrin gels prepared in the absence of platelets as well as in clots correlated inversely with the thrombin concentration. Interestingly, the addition of external thrombin to the PpP preparations did not compensate for the lack of endogenous thrombin in terms of fibril thickness. In other words, thrombin concentration is not the only factor that controls fibrin gel morphology. Weisel showed that platelet factor 4, for example, leads to an increase in the fiber thickness. 42 It would be interesting to further examine in future studies the mechanical properties of these interfaces.

The effect of the different preparation conditions on the ability of the interface to retain and then release the growth factors was examined by first measuring the growth factor concentration in the supernatant above the interfaces, and then by measuring the amount of growth factors released by the interface over time. In the case of the activated samples, the interface is a clot attached to the TiO2 surface. In the case of the nonactivated preparations, the interface is a layer of adsorbed proteins and, in the PRGF preparations, platelets. Surprisingly, both kinds of interfaces were able to retain the plasmatic growth factor HGF, whose concentration in the supernatant was independent of platelet concentration and activation. This retention suggests that HGF may adsorb to the surface of the implant directly or through a previously adsorbed protein. The adsorption of HGF to the surface is not altered by fibrinogen-to-fibrin conversion, because the kinetics of HGF release from the interfaces is independent of activation.

In contrast, Ca- and CaT-activated interfaces were better at retaining the PDGFs PDGF-AB, TGF-β1, and VEGF, judging from their higher concentrations in the supernatants above the nonactivated, PpP-derived interfaces. Thrombin had little effect on the ability of the activated interfaces to retain PDGFs. However, using thrombin as an activator impaired the ability of the activated interfaces to release PDGF-AB into the media: the concentration of this growth factor above the CaT-activated PRGF interfaces was comparable to that above the nonactivated ones, considerably lower than above the Ca-activated ones. This inhibitory effect of thrombin at high concentrations has been previously reported for PDGF released by endothelial cells. 43

The amount of growth factors detected over the interfaces can be furthermore compared with the values found in the literature. The concentration of HGF normal blood is ~316 pg/mL 44,45 whereas the total amount measured in our assays was ~504 pg/mL [averaged over all samples, Figure 7(A)]. In the case of PDGF, we found ~3.5 ng/mL in activated PRGF and ~5.5 ng/mL in the nonactivated PRGF preparations [Figure 7(C)]. In blood of healthy patients, the concentration of this factor is reported to vary between 0.2 and 18 ng/mL. 46,47 The concentration of TGFβ-1 in blood range between 6 and 29 ng/mL 44,47 compared with 2.3 ng/mL in the Ca-activated PRGF and 5.5 ng/mL in the nonactivated PRGF [Figure 7(E)]. The VEGF concentration found in Ca-activated PRGF was ~350 pg/mL, and in the NA-PRGF ~450 pg/mL, compared with between 9 and 227 pg/mL in normal blood. 44,47 These comparisons show that growth factor concentrations vary significantly from donor to donor, which would be an additional argument for the use of autologous preparations.

Arguably the most important finding of our study is related to the time frame over which the growth factors are released. As already mentioned above, the concentration of HGF in the media above the interfaces remained significant over a period of 9 days. The concentration of the platelet-derived factors, in contrast, remains significant only over 2 days. After 4 days, the concentration of the three PDGFs drops to the level observed in PpP preparations—that is, in the absence of platelets. However, within that time period, their concentration in the media above the PRGF-derived Ca-activated interfaces is considerably higher than that in the PpP-derived interfaces. The latter is indicative of the concentration of these growth factors in the normal blood plasma; these growth factors are stored inside the platelets and are only released topically at the site of their activation.

The presence of these factors at the wound sites has pivotal effects for healing and tissue regeneration. HGF, which is found in plasma, has several biological activities that include mitogenesis, mitogenesis, morphogenesis, and growth inhibition. HGF is a potent mitogen for different epithelial and endothelial cells 49 and also for hepatocytes 50 and plays an important role in epithelial-mesenchymal interactions during organogenesis and organ regeneration. 51 PDGF is implicated in several physiological processes such as organogenesis, 52 cardiovascular system development, 53 angiogenesis, 54,55 cell proliferation, and migration. 56,57,68 In the case of wound healing, it stimulates chemotaxis and proliferation of cells to increase granulation tissue formation. 59–61 TGFβ-1 is modulates the immune response, 62,64 induces differentiation of osteoblast precursor cells, 51 and plays a central role in wound healing. 44,65 VEGF is a chemo-attractant for macrophages and granulocytes 66–70 that are implicated in the inflammatory phase of the wound-healing process, and it also plays a major role in angiogenesis. 71–74

In the context of implant integration, the healing that follows the surgery generally proceeds along the same lines that the normal wound healing. 75,76 but it occurs in the presence of an implant that is foreign to the surrounding biological tissue. 76 Thus, to obtain optimal osseointegration (referring to the direct contact between new formed bone and the implant), the inflammatory reaction against the implant should be controlled. The use of a PRGF-Endoret-derived fibrin scaffold, which is enriched in HGF among
other factors may result essential in reducing the inflammatory process.77

Finally, although implant surface activation with PRGF-Endoret is an important step forward in terms of managing implant-body interactions, combining it with specific pharmacological agents should open new ways to treat risk patients suffering of, for example metabolic bone diseases, diabetes, or osteoporosis. For these purposes, the modulation of fibrin matrix formation, platelet activation and delivery kinetics investigated in this work is of paramount importance.

CONCLUSIONS

This study shows that the type of platelet activation plays a role in the fibrin network formation. Furthermore, platelet activation permits to delay their growth factor secretion independently of the type of activator used. Thus, for future clinical applications, plasma preparations should be activated to permit growth factors release and thereby facilitate implant surface osseointegration.

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