Differential Growth Factor Retention by Platelet-Rich Plasma Composites

Rick C. Tsay, DMD, MD,* Jennifer Vo, MS,† Andrea Burke, BS,‡ Sidney B. Eisig, DDS,§ Helen H. Lu, PhD,¶ and Regina Landesberg, DMD, PhD‖

Purpose: This study evaluates the temporal sequence and growth factor release from platelet-rich plasma (PRP) combined with different bone substitutes (BS), to identify an optimal substrate for extended growth factor retention.

Materials and Methods: PRP was clotted with bovine thrombin or thrombin receptor activator peptide-6 (TRAP). In addition, PRP was clotted using Allogro (Ceramed, Lakewood, CO), BioGlass (Mo-Sci, Rolla, MN), or BioOss (Osteohealth, Shirley, NY). The effects of media exchange and BS on platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β) release were quantified via enzyme-linked immunosorbent assay.

Results: At day 1, the thrombin group released 36% more PDGF than the TRAP group and 80% more than the BS groups. At 7 days, PDGF release was the greatest for the TRAP group. PDGF release was minimal for all groups at day 14, with BS groups retaining 60% more PDGF than thrombin clots. Similarly, the thrombin group released the greatest amount of TGF-β (81.4% of the total), whereas TRAP and BS groups released significantly less TGF-β at day 1. Compared with thrombin, TRAP retained 39.2% more TGF-β, whereas BS groups retained even greater levels (Allogro, 54.3%; BioOss, 45.8%; BioGlass, 67.0%). No significant difference in TGF-β release was observed among the substitutes after day 1. The BS groups continued to retain TGF-β after 14 days, whereas all TGF-β in the thrombin clots was depleted.

Conclusions: PRP preparation with thrombin results in a large, immediate release of growth factors that could be lost into the interstitium in vivo. TRAP-BS may prove more efficacious than thrombin in sustaining growth factor levels critical for the cascade of events leading to bone formation.

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Platelet-rich plasma (PRP) is derived from plasma enriched for platelets and may be efficacious in enhancing wound healing and increasing the rate of bone graft healing in oral and maxillofacial surgery.1,2 Platelets are known to contain a number of growth factors, such as platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), insulin-like growth factors (IGFs), epidermal growth factor (EGF), and epithelial cell growth factor (ECGF).3,4 In the early stages of wound healing following bone fractures or surgical interventions, platelets are activated by the coagulation cascade, particularly thrombin and subendothelial collagen. Activated platelets subsequently release the content of their granules into the wound site. Current methods of PRP preparation use bovine thrombin for clotting, which has been associated with the formation of antibodies to clotting factors V and XI and thrombin, resulting in life-threatening coagulopathies.5 Thrombin, a serine protease, acts through the activation of specific thrombin receptors to elicit

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*Chief Resident, Division of Oral and Maxillofacial Surgery, School of Dental and Oral Surgery, Columbia University, New York, NY.
†Graduate Student, Department of Biomedical Engineering, School of Engineering and Applied Sciences, Columbia University, New York, NY.
‡Dental Student, Harvard School of Dental Medicine, Boston, MA.
§Professor and Chair, Hospital Dentistry/Oral and Maxillofacial Surgery, School of Dental and Oral Surgery, Columbia University, New York-Presbyterian Hospital, New York, NY.
¶Assistant Professor, Department of Biomedical Engineering, Fu Foundation School of Engineering, Columbia University, New York, NY.
‖Assistant Professor, Division of Oral and Maxillofacial Surgery, School of Dental and Oral Surgery, Columbia University, New York, NY.

Address correspondence and reprint requests to Dr Landesberg: Columbia University, School of Dental and Oral Surgery, Division of Oral and Maxillofacial Surgery, 650 W 168th St, Box 20, New York, NY 10032; e-mail: rl351@columbia.edu

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a variety of cellular responses. The thrombin receptors from human platelets have been sequenced and cloned, and they belong to the 7 transmembrane–spanning domain receptor family coupled to G proteins (Fig 1). Thrombin binds to and cleaves its receptor between amino acid residues Arg41 and Ser41 to generate a new amino terminus. The newly generated amino-terminal segment of 14–amino acid peptide SFLLRNPDNKYEPF functions as a “tethered ligand” and activates the receptor. Thrombin receptor activator peptide-6 SFLLRN (TRAP) is a synthetic peptide corresponding to the amino peptide sequence and mimics thrombin in eliciting thrombin-signaled cell responses in platelets.


FIGURE 1. Schematic of the function of thrombin receptor activator peptide-6 [TRAP]. Thrombin binds to and cleaves its receptor between amino acid residues Arg41 and Ser41 to generate a new amino terminus. The newly generated amino-terminal segment of 14–amino acid peptide SFLLRNPDNKYEPF functions as a “tethered ligand” and activates the receptor. Thrombin receptor activator peptide-6 SFLLRN (TRAP) is a synthetic peptide corresponding to the amino peptide sequence and mimics thrombin in eliciting thrombin-signaled cell responses in platelets.


FIGURE 2. Clot diameter and distribution. Differences in clot diameter were observed between the thrombin, thrombin receptor activator peptide-6 [TRAP], and platelet-rich plasma (PRP) composites AlloGlo (AG), BioOss (BO), and BioGlass (BG). Larger and more evenly distributed clots were observed for the PRP composite groups. Tsay et al. Differential GF Retention by PRP Composites. J Oral Maxillofac Surg 2005.

be under the control of a number of growth factors. Initially, chemotaxis of osteoblast precursors to the site of bone regeneration is mediated by structural proteins such as collagen and/or osteocalcin, as well as growth factors such as PDGF and TGFβ. This is followed by proliferation of osteoblasts. PDGF, TGFβ, fibroblast growth factor (FGF), and IGF-I and -II have all been shown to stimulate osteoblast growth. The differentiation of osteoblasts into mature bone cells is also controlled by growth factors, most significantly by IGF-1 and the bone morphogenetic proteins (BMPs).

The aforementioned growth factors within the PRP are believed to mediate normal bone healing and regeneration. The efficiency of these factors in enhancing bone regeneration is likely dependent on the dosage, spatial distribution, and temporal sequencing of the available growth factors. Current methods of PRP preparation have reported platelet enrichments of 300% to 700%, whereas assays for growth factors in PRP showed a 7-fold increase in TGFβ and a 30-fold increase of PDGF using enzyme-linked immunosorbent assay (ELISA). Whether these enhanced levels of growth factors in PRP are locally available to the osteoblast at the critical time has not been investigated.

Growth factors activated at the appropriate temporal sequence and spatial distribution have a profound effect on bone regeneration. PDGF has been shown to stimulate mitogenesis and proliferation of mesenchymal-derived cells such as osteoblasts in bone healing. TGFβ is a mitogenic and chemotactic factor that induces proliferation and

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differentiation of mesenchymal cells into osteoblasts. In vitro studies showed that the combination of cytokines and growth factors increased osteoblast proliferation and differentiation. The spatial and temporal localization of the growth factors is critical in bone cellular growth and differentiation. Although PRP has proved to be effective in enhancing bone graft healing, the temporal sequence and levels of growth factors released from the PRP composite have not been well studied.

Bone regeneration requires an osteogenic cell source, growth factors, and nutrient supplies. PRP alone does not have osteoconductive or osteoinductive effects on bone regeneration and is usually used in conjunction with bone grafts or bone substitute materials. BioOss is a bone substitute derived from bovine bone after the removal of all organic materials. The morphologic structure of BioOss resembles that of human cancellous bone. The porous nature of BioOss provides a scaffold for the formation of the new bone. AlloGro is demineralized freeze-dried bone allograft (DFDBA). DFDBA has been used extensively in bone grafting, as it is known to have osteoinductive characteristics that will enhance bone cell growth. 45S5 bioactive glass (BioGlass) is a melt-derived bioactive glass ceramic that has been reported to be osteoconductive and osteointegrative. In vitro studies have shown that BioGlass has the ability to stimulate the growth and estrogenic differentiation of human osteoblast-like cells.

The dominant mechanism governing growth factor release from the composites of PRP and bone substrate is diffusion. This process is driven by the local growth factor concentration gradient present at the graft site. In vitro models of growth factor release must take into account several processes, which are unique in vivo. Specifically, the temporal concentration and spatial distribution of growth factors within the graft site are expected to vary as a function of fluid infiltration during the initial repair response, as well as the subsequent uptake of available growth factors for cellular function during the bone regeneration stage. Reported in vitro growth factor release studies may adapt either the static or dynamic mode of incubation. In the static mode, no media exchange is performed and concentration values will eventually reach steady state. In the dynamic mode, fresh solution is added periodically to the system to emulate the location changes in growth factor concentration and utilization. For the current study, the effects of the frequency of media exchange on the kinetics of growth factor release are examined under both static and dynamic incubation conditions.

To identify the optimal substrate for growth factor retention, this study evaluates the temporal sequence and factor release levels from PRP composites with different bone substitutes. Differences in growth factor retention by these substitutes are expected. In addition, the effects of the media exchange mode are examined. It is hypothesized that the dynamic mode of media exchange increases the bioavailability of the PRP-derived growth factors, thereby altering the kinetics of their release.

Materials and Methods

PREPARATION OF PLATELET-RICH PLASMA

PRP was prepared through a modification of the method of Landesberg et al. Sixty milliliters of venous blood from healthy adult volunteers was mixed with ACD Solution B in 9.0 mL Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ). The ACD solution contained 13.2 g/L trisodium citrate, 4.8 g/L citric acid, and 14.7 g/L dextrose. The samples were centrifuged at 2000 g for 15 minutes (ACE Surgical Supply Company, Inc, Brockton, MA). The plasma and buffy coat layers were removed, placed into 10-mL tubes, and spun at 2000 g for an additional 10 minutes. The upper half of the preparation was designated platelet-poor plasma (PPP) and subsequently discarded. The lower half of the plasma and the pellet were resuspended and pooled to be the PRP.

PREPARATION OF PLATELET-RICH PLASMA–BONE SUBSTITUTE COMPOSITES

PRP composites were prepared by mixing PRP and TRAP with bone substitutes commonly used in the clinical setting. Twenty-four-well plates (Corning Inc, Corning, NY) were coated with 1% bovine serum albumin (Sigma, St Louis, MO) and incubated for 1.5 hours at 37°C. Sterilized BioOss (25 mg, 0.5 to 1.0 mm; Osteohealth, Shirley, NY), AlloGro (25 mg; Ceramed, Lakewood, CO), and 45S5 Bioactive Glass (25 mg, 300 μm; MOSCI, MN) were uniformly dispersed within the wells. Fresh PRP (0.5 mL) aliquots were then dispensed into the precoated 24-well plates, and 30 μL of 10% calcium chloride solution was added to each well. Experiments were performed in triplicate with the addition of bovine thrombin (75 units) or TRAP (100 μmol/L; H2N-Ser-Phe-Leu-Leu Arg-Asn-NH2). TRAP was used to clot PRP for all bone substitute groups examined (Fig 2).

GROWTH FACTOR RELEASE—EFFECTS OF BONE SUBSTITUTE AND INCUBATION MODE

The temporal release of growth factors was examined as a function of bone substitute and mode of
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Results

The greatest volume of supernatant was collected for all groups at day 1. At all time points, the largest volume was measured in the thrombin group. At day 1, the thrombin group released the greatest amount of PDGF-AB (P < .05, 52,526 pg), approximately 36% more than the TRAP group (20,642 pg). PDGF-AB release from the bone substitute groups, BioGlass (8,757 pg), BioOss (6,847 pg), and AlloGro (5,519 pg), was approximately 80% less than that from the thrombin group (Fig 3). At 7 days, the TRAP group had the greatest PDGF-AB release (11,624 pg) compared with thrombin (5,068 pg), whereas BioGlass (5,304 pg), BioOss (6,646 pg), and AlloGro (3,775 pg) had similar levels measured. At 14 days, all groups released minimal amounts of growth factor. All bone substitute groups retained on average 60% more PDGF-AB than the thrombin group after 14 days.

The amount of TGFβ released by the PRP composite is shown (Fig 5). Similar to the case with PDGF-AB, the thrombin group released the greatest amount of TGFβ at day 1 post clotting. The total amount of TGFβ contained in the original PRP volume was measured to be 13,982 ± 2,673.81 pg. In the thrombin group (TH), over 81.4% of the growth factor was already released from the clot. In contrast, the TRAP-alone and the PRP composite groups released significantly lower levels of TGFβ (P < .05). Compared with the thrombin group, clotting of PRP with TRAP retained 39.2% more of the growth factor, whereas the bone substrate groups retained significantly greater amounts of TGFβ (AlloGro, 54.3%; BioOss, 45.8%; BioGlass, 67.0%). Within the bone substitute groups, BioGlass had the greatest TGFβ retention compared with the 2 other groups tested (P < .05). No significant differences in growth factor release were observed among the bone substrates at the remaining time points. After 14 days, all of the TGFβ in thrombin clots was released, whereas the bone substrates re-

Sample incubation. Specifically, the PRP composites were allowed to clot, and all samples were incubated in a humidified environment at 37°C for up to 14 days. The medium was exchanged for all groups at 1 day after incubation. The volume of fluid released from the clot was measured, and an equal volume of fresh Dulbecco’s modified Eagle’s medium (DMEM; Mediatech, Herdon, VA) without serum was added back to each well. Subsequently, in the dynamic incubation mode (group 1), the fluid released from the clot was collected and an equal volume of fresh DMEM was added back into the well at 3, 7, and 14 days. In the static mode (group 2), the medium was exchanged only at the designated time points (7 and 14 days). All collected supernatant samples were stored at -70°C before analyses.

Quantification of Platelet-Derived Growth Factor and Transforming Growth Factor-β

Supernatant collected from all time points for both groups 1 and 2 were assayed for PDGF-AB and TGFβ content using diagnostic kits from R & D Systems (Minneapolis, MN). Both assays are based on a sandwich enzyme immunoassay technique. The PDGF-AB assay uses a precoated microtiter plate with a monoclonal antibody to PDGF-AA. Preparation and dilution of samples and standards were performed as directed by the manufacturer. Both the standards and the samples were incubated for 3 hours at room temperature. The plate was washed with buffer, and a conjugated antibody to PDGF-BB was added to the wells and incubated at room temperature for 1 hour. The plate was then washed, and substrate was added for 20 minutes at room temperature. The reaction was stopped, and absorbance was determined at 450 nm using a spectrophotometer (SpectraFluor Plus; Tecan, Maennedorf, Switzerland). A standard curve was generated, and the PDGF-AB levels (pg/mL) of each sample were determined. The total amount of growth factors was calculated based on the amount of supernatant obtained after clot retraction.

Statistical Analyses

All quantitative results were expressed as mean ± SD. Multiway analysis of variance (ANOVA) was performed and the Tukey-Kramer test was used to compare between the means. Significance was determined at P < .05.
tained approximately 44% more of this growth factor compared with the thrombin group.

The effects of media exchange on PDGF and TGFβ release are shown (Figs 6, 7, respectively). Figures 3 and 4 compare specifically the release profile of PDGF under dynamic (group 1) versus static (group 2) modes of media exchange as shown in Figure 6. In terms of PDGF release, a significant difference in release was observed only for the BioGlass substrate as a function of media exchange (P < .05). Group 1 released greater amounts of PDGF compared with group 2 (P < .05, n = 3). No significant difference in release was observed for thrombin, TRAP, and all other bone substitutes tested as a function of frequency of media exchange. In contrast, TGFβ release from the substrates was affected by media exchange in the PRP composite formed with AlloGro (P < .05). Group 1 released a significantly greater amount of the factor compared with group 2, where the medium was exchanged less frequently. Media exchange was found to have no significant effect on the retention of either PDGF-AB or TGFβ by the BioOss group.

Discussion

In this study, we evaluated the release of growth factors PDGF-AB and TGFβ from different preparations of PRP and monitored the effect of the frequency of media exchange on the release profiles. Current methods of preparing PRP use commercially available thrombin derived from bovine plasma, which has been associated with the development of antibodies to clotting factors V and XI and thrombin, resulting in the risk of life-threatening coagulopathies. We have therefore developed an alternative method for PRP clotting using TRAP, a synthetic peptide that mimics thrombin in eliciting thrombin-signal cell responses in platelets. Previous studies in our laboratory have shown that TRAP results in significantly less clot retraction than thrombin while providing excellent working time in the preparation of PRP. Because TRAP is a synthesized peptide, it is devoid of contaminated coagulation factors present in bovine thrombin, negating the risk of serious coagulopathies. We therefore chose to use TRAP to activate clotting of PRP in the present study. The results from this study show that TRAP, when used alone, retained more PDGF-AB than thrombin. Moreover, when TRAP was combined with BioOss, BioGlass, or AlloGro, approximately 60% more growth factor was retained compared with thrombin. These results suggest that PRP with TRAP and TRAP plus bone substitutes are potentially superior to PRP prepared with thrombin.

The release of PDGF-AB and TGFβ from PRP and PRP composites was determined. There were no significant differences in PDGF-AB release among any of the bone substitutes at all time points. These results suggest that the retention of PDGF-AB by the TRAP–bone substitute composites is relatively nonspecific to the tested substitutes. In contrast, differences in TGFβ release were observed among the various bone substitutes as a function of time. Growth factor retention is dependent on both the substitute used and the specific growth factor examined. Unlike the results for PDGF-AB, there was a large release of TGFβ (40% to 50%) at day 1 in all bone substitute groups. This suggests that the bone substitutes have a much greater potential for the retention of PDGF-AB. Al-
through IGF-1 was not tested in this study, it is known to be a component of PRP and is critical to osteoblast differentiation in the later stages of bone regeneration, making it extremely important to delay its release. IGF-1 is considerably smaller than PDGF-AB and TGFβ (PDGF, 30 kDa; TGFβ, 24 kDa; IGF-1, 7.6 kDa) and may have a different release profile than the growth factors tested. The release of IGF-1 in PRP preparations and novel delivery modalities will be included in our future investigations.

We examined the effects of media exchange on growth factor release from PRP and the PRP composites. The rationale was to identify an optimal and realistic in vitro model that will mimic the local bone grafting environment in which the growth factors will be delivered. PRP-derived growth factors at the grafting site will most likely be released from the platelets and used during bone healing. However, continuous interstitial fluid exchange or infiltration of vasculature will alter the local concentration of the growth factors. The frequency of media exchange was used to mimic this continuous process. In all experiments, PRP clot medium was changed at day 1. Group 1 (dynamic mode) had additional media changes at 3 days, whereas in group 2 (static mode) no additional changes were performed between 1 and 7 days. In the static mode, it is anticipated that the growth factor release level would reach steady state and then remain at the same levels. While it was observed that mean value release of PDGF-AB was consistently greater in the group where the medium was more frequently exchanged (dynamic mode), the release profile reached a plateau in both cases after 7 days. If growth factor release was based solely on equilibration, it would be expected that the accumulation factor levels from day 1 to day 7 in the dynamic group would be equal to the total amount of growth factor released for the static group within the same period. Interestingly, we found that PDGF release from the BioGlass sample was lower in group 2 than in group 1. In contrast, TGFβ was significantly less in group 2 of the AlloGro sample, leading us to conclude that AlloGro and BioOss enhance the retention of PDGF-AB, whereas BioOss and BioGlass promote retention of TGFβ.

In this study, we evaluated growth factor retention by a select group of bone substitutes. In terms of substrate chemistry, AlloGro is based on demineralized bone matrix, which is composed of organics, whereas BioOss is deproteinated bone with a calcium phosphate matrix. Bioactive glass develops a surface calcium phosphate layer, which has been shown to promote bone bonding. Calcium phosphate ceramic-based materials have been combined with a variety of growth factors, including TGFβ, to successfully promote bone healing in vitro and in vivo. The specificity or the chem-
The medium was exchanged less frequently. Media exchange was significantly a greater amount of the factor compared with group 2, where BioGlass have enhanced retention of TGF\( \beta \) during the interme-
diate phase, and IGF-1 and BMPs during the final early phases, followed by TGF\( \beta \) during the final phase of bone differentiation and maturation.\(^{25}\) The results in a large immediate release of growth factor into the supernatant, which could be lost into the intervention method. The bone regeneration potential of the biomaterial substrate tested here is unclear at this time. Future studies will examine the effects of substitute chemistry on the temporal retention of PRP-derived growth factors relevant in bone regeneration.

At the present, there exist several schools of thought regarding the clinical efficacy of PRP in enhancing bone regeneration in oral and maxillofacial surgery.\(^ {30, 31}\) Based on the known sequence of events that takes place during bone regeneration, it is desirable to have PDGF and TGF\( \beta \) present in the early phases, followed by TGF\( \beta \) during the intermediate phase, and IGF-1 and BMPs during the final phase of bone differentiation and maturation.\(^ {25}\) The observed difference in growth factor retention and temporal availability reported in the present study suggests that the bioavailabilities of the associated growth factors are a function of the PRP preparation method. The bone regeneration potential of PRP may be controlled by matching growth factor release profiles with the known cascade for bone healing. Consistent clinical outcomes using PRP-based technology will require significant research efforts into the bioavailability of relevant growth factors.

In summary, preparation of PRP with thrombin results in a large immediate release of growth factor into the supernatant, which could be lost into the interstitium in vivo. Materials other than thrombin such as TRAP and bone substitutes may prove more efficacious in sustaining growth factor levels critical for the cascade of events leading to bone formation. Growth factor retention was a function of both the substrate used and the specific growth factor examined. These findings will aid in the engineering of novel delivery systems to enhance bone regeneration.

References

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