Platelet-rich Plasmas: Growth Factor Content and Roles in Wound Healing
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What is This?
Platelet-rich Plasmas: Growth Factor Content and Roles in Wound Healing

ABSTRACT
Platelet-rich plasmas (PRPs) are used in a variety of clinical applications, based on the premise that higher growth factor content should promote better healing. In this study, we have determined the effects of calcium and thrombin on the release of EGF, TGF-α, IGF-1, Ang-2 and IL-1β from PRPs, and assessed the mitogenic potential of PRP supernatants on osteoblast and endothelial cell division. ELISA assays indicate that (i) mean growth factor concentrations vary from traces (TGF-α) to 5.5 ng/mL (IGF-1), (ii) there are significant variations in growth factor concentrations between individuals, and (iii) calcium and thrombin regulate growth factor release, synthesis, and/or degradation in stereotyped patterns that are specific to each growth factor. PRP supernatants promote strong osteoblast and endothelial cell divisions, supporting the concept that PRPs may be beneficial in wound healing. Abbreviations: PRPs, platelet-rich plasmas; GFs, growth factors; EGF, epidermal growth factor; TGF-α, transforming growth factor-alpha; IGF-1, insulin-like growth factor-1; Ang-2, angiopoietin-2; IL-1β, interleukin-1 beta; HUVECs, human umbilical vein endothelial cells; hFOB 1.19, human fetal osteoblasts; and FBS, fetale bovine serum.

KEY WORDS: platelet-rich plasma, growth factors, endothelial cells, osteoblasts, wound healing.

INTRODUCTION
The use of platelet-rich plasmas (PRPs) to enhance bone regeneration and soft tissue maturation has increased dramatically in the fields of orthopedics, periodontics, maxillofacial surgery, urology, and plastic surgery over the last decade. However, controversies exist in the literature regarding the added benefit of this procedure. While some authors have reported significant increases in bone formation and maturation rates (Marx et al., 1998; Kim et al., 2002), others did not observe any improvement (Aghaloo et al., 2002; Fromu et al., 2002). Recent reviews have emphasized the need for additional research aiming to characterize PRPs in terms of growth factor (GF) content and their physiological roles in wound healing (Sanchez et al., 2003; Tozum and Demiralp, 2003; Freymiller and Aghaloo, 2004).

The wound-healing process is a complex mechanism characterized by four distinct, but overlapping, phases: hemostasis, inflammation, proliferation, and remodeling (Diegelmann and Evans, 2004). The proliferative phase includes blood vessel formation by endothelial cells and bone synthesis by osteoblasts. All these events are coordinated by cell-cell interactions and by soluble GFs released by various cell types. Thrombin represents a strong inducer of platelet activation leading to GF release (Furman et al., 1998). It is also known that particulate grafts, when combined with calcium- and thrombin-treated PRPs, possess better handling characteristics and higher GFs content (Tayapongsak et al., 1994; Marx et al., 1998; Fromu et al., 2002; Weberich et al., 2002). Typically, thrombin concentrations used in clinical applications vary between 100 and 200 units per mL (Marx et al., 1998), while platelet aggregation is maximum in the range of 0.5 to 4 units per mL (Maloney et al., 1998). To date, little is known about the calcium and thrombin concentrations needed to trigger optimal bone repair.

We have recently demonstrated that the release of PDGF-BB, TGF-β1, bFGF, and VEGF is significantly regulated by the amount of calcium and thrombin added to the PRPs, and that PRP supernatants are more mitogenic for endothelial cells than whole-blood supernatants, reinforcing the scientific evidence for a beneficial role of PRPs in tissue regeneration (Lacoste et al., 2003; Martineau et al., 2004). Other GFs—such as epidermal growth factor (EGF), transforming growth factor-alpha (TGF-α), insulin-like growth factor-1 (IGF-1), angiopoietin-2 (Ang-2), and interleukin-1beta (IL-1β)—are also known to play important roles in the wound-healing process (Karey and Sirbasku, 1989; Werner and Grose, 2003).

The purposes of this study were therefore to assess: (i) the concentrations of EGF, TGF-α, IGF-1, Ang-2, and IL-1β released from PRPs after treatment with different concentrations of calcium and thrombin; and (ii) the effects of supernatants from calcium- and thrombin-treated PRPs on endothelial cell and osteoblast proliferation in vitro.
MATERIALS & METHODS

Blood Harvesting and Preparation of Platelet-rich Plasmas

300 mL of whole blood were collected from each of the five donors who had provided informed consent. The study was approved by the Ethics Committee of Université Laval, Québec, Canada (#2001-076.1). PRPs were prepared with the Platelet Concentrate Collection System (PCCS) from Implant Innovations Inc. (3L, West Palm Beach, FL, USA), as previously reported (Martineau et al., 2004). Complete blood counts from whole-blood samples and PRPs were evaluated in a Gen-S flow cytometer counter (Beckman-Coulter, Fullerton, CA, USA).

Treatment of PRPs with Calcium and Thrombin

Thrombin (Thrombostat®, Parke-Davis, Scarborough, ON, Canada) and calcium were added to PRP and whole-blood samples at the following final concentrations: (condition A) 142.8 U/mL of thrombin and 14.3 mg/mL of CaCl₂·2H₂O; (conditions B and C) five- and 25-fold dilutions of condition A, respectively. Condition A refers to the concentrations used in clinical applications, while condition C is similar to the concentrations reported to induce maximal in vitro platelet aggregation (Maloney et al., 1998; Marx et al., 1998). Calcium- and thrombin-treated PRP aliquots were incubated for 0.5 hr, 1 hr, 6 hrs, 24 hrs, 3 days, and 6 days at 37°C in the presence of 5% CO₂ (Auto-Flow incubator, NuAire Inc., Plymouth, MN, USA). Samples were centrifuged for 10 min at 4000 g to collect PRP supernatants. Non-activated PRP samples and PRPs were evaluated in a Gen-S flow cytometer counter (Beckman-Coulter, Fullerton, CA, USA).

Determination of Growth Factor Concentrations

GF concentrations in PRP and whole-blood supernatants were determined by sandwich-ELISA. EGF concentrations were measured with the use of an ELISA Development kit according to the manufacturer's instructions (Peprotech Inc., Rocky Hill, NJ, USA). TGF-α, IGF-1, Ang-2, and IL-1β concentrations were determined by DuoSet ELISA Development kits (R&D Systems, Inc., Minneapolis, MN, USA). Triplicates were performed for all assays.

Cell Culture and Reagents

Human umbilical vein endothelial cells (HUVECs, Clonetics/Biowhittaker, Walkersville, MD, USA) were expanded in a complete medium consisting of M199 (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Wisent, Saint-Laurent, QC, Canada), 15 U/mL of heparin, 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 15 μg/mL of endothelial cell growth supplement (ECGS) (Sigma Co., Oakville, ON, Canada). Cells were incubated at 37°C in the presence of 5% CO₂. HUVECs from 2nd and 3rd passages only were used in the experiments.

Transformed human osteoblasts (hFOB 1.19, American Type Culture Collection, Rockville, MD, USA) were expanded in a complete medium consisting of DMEM/F12 (Sigma Co., Oakville, ON, Canada) supplemented with 10% FBS, 100 U/mL of penicillin, 100 μg/mL of streptomycin (HUVEC), or DMEM/F12 supplemented with 1% FBS, 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 0.3 mg/mL of G-418 (Invitrogen, Burlington, ON, Canada). The cells were incubated at 34°C in the presence of 5% CO₂.

Proliferation Assays

HUVECs and hFOB 1.19 were seeded at densities of 2500 and 1800 cells per well, respectively, in 0.25% gelatine-coated 96-well plates in their corresponding complete medium for 24 hrs. Cells were then incubated in the following minimal medium: M199 supplemented with 1% FBS, 100 U/mL of penicillin, and 100 μg/mL of streptomycin (HUVEC), or DMEM/F12 supplemented with 1% FBS, 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 0.3 mg/mL of G-418 (hFOB 1.19). Cells were incubated for 5 days in minimal medium containing 10% (v/v) of PRP supernatants, including a change of medium at day 3. Cell culture experiments were performed in triplicate.

The effect of PRP supernatants on the proliferation rates of HUVECs and hFOB 1.19 was determined by [³H]-thymidine incorporation as previously described (specific activity, 86.4 Ci/mmol; Perkin-Elmer, Boston, MA, USA) (Martineau et al., 2004). Thymidine incorporation was measured by an LS-6500 Multi-Purpose Scintillation Counter (Beckman Coulter, Mississauga, ON, Canada). We normalized the proliferation rates by dividing the DPM counts of a specific experiment by the DPM counts obtained in minimal medium.

Data Analysis

Student’s t test analyses were performed with InStat 3.0 software (Graphpad Softwares Inc., San Diego, CA, USA).

RESULTS

Whole-blood samples were collected from five healthy volunteers, and PRPs were prepared according to the PCCS system. This technique allowed us to concentrate platelets 5.5-fold on average with a platelet recovery rate of 77.7%. During this process, most of the red blood cells were

<table>
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<tr>
<th>Table 1. Platelet, Red Blood Cell, and Leukocyte Concentrations, Yields, and Recoveries from Five Donors</th>
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<tr>
<td>Whole Blood, Mean (SD)*</td>
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<tr>
<td>Platelets</td>
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<td>Red blood cells</td>
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<td>Detailed leukocytes’ relative values</td>
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<td>Neutrophils</td>
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* SD, standard deviation.
Table 2. Growth Factor Concentrations in Whole Blood and PRP Supernatants

<table>
<thead>
<tr>
<th></th>
<th>Non-activated Samples (t = 0 hrs), pg/mL</th>
<th>Activated Samples, Immediate Releaseb (pg/mL)</th>
<th>Activated Samples, Maximal Release (pg/mL)</th>
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<tbody>
<tr>
<td></td>
<td>Whole blood, Mean (SD)</td>
<td>PRP, Mean (SD)</td>
<td>PRP/Whole-blood Ratio</td>
</tr>
<tr>
<td>EGF</td>
<td>12 (40)</td>
<td>57 (77)</td>
<td>4.6 x</td>
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<tr>
<td>IGF-1</td>
<td>3000 (2175)</td>
<td>5550 (2075)</td>
<td>1.9 x</td>
</tr>
<tr>
<td>Ang-2</td>
<td>130 (260)</td>
<td>425 (405)</td>
<td>3.2 x</td>
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<tr>
<td>IL-1β</td>
<td>NDd</td>
<td>ND</td>
<td>-</td>
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</table>

a GF concentrations in non-activated whole blood and PRP supernatants (t = 0 hrs). GF concentrations corresponding to immediate release are defined as concentrations detected in whole blood and PRP supernatants, collected 0.5 hr after activation with high calcium and thrombin concentrations (condition A). GF concentrations corresponding to maximal release are defined as the highest mean GF concentrations detected in supernatants after PRP activation. Conditions (A, B, or C) and activation times leading to maximal extracellular GF concentrations are provided. Data represent mean GF concentrations and standard deviations from five donors.
b t = 0.5 hr, Condition A.
c SD: standard deviation.
d ND: not detected.

Table 2. Growth Factor Concentrations in Whole Blood and PRP Supernatants

- Eliminated, and a 2-fold increase in total leukocytes was observed, with a recovery rate of 26.8% (Table 1). A two- to four-fold increase in GF content was generally noted in PRP supernatants, as compared with whole-blood supernatants (Table 2).

Kinetics of Growth Factor Release into PRP Supernatants

Low levels of EGF were detected in supernatants from non-activated PRPs (57 ± 77 pg/mL; Table 2). Immediate increases (4.7- to 11-fold; p < 0.0005) in EGF extracellular concentrations were observed after the addition of all concentrations of calcium and thrombin (Fig. 1A). Highest EGF levels in PRP supernatants were detected at t = 6 hrs with condition A (595 ± 195 pg/mL), followed by a slight decrease over the six-day incubation period.

In contrast to EGF, IGF-1 concentrations in supernatants from non-activated PRPs were significantly higher compared with all other GFs and did not increase after the addition of calcium and thrombin (5.5 ± 2.1 ng/mL; Table 2, Fig. 1B). A decrease in IGF-1 level was observed over the six-day incubation period in all conditions tested.

Moderate Ang-2 concentrations were detected in supernatants from non-activated PRPs (425 ± 400 pg/mL; Table 2). Highest Ang-2 concentrations were detected in PRP supernatants within the first hour following activation with low concentrations of calcium and thrombin (mean, 1760 ± 450 pg/mL; Fig. 1C). This peak was followed by marked Ang-2 degradation over time.

IL-1β concentrations in supernatants from non-activated PRPs were below the detection threshold in all volunteers (< 2 pg/mL; Fig. 1D). IL-1β levels in PRP supernatants reached the detection level only after 6 hrs of incubation with conditions B and C. From that point, an increase in extracellular IL-1β was observed (up to 430 pg/mL), which was especially evident when PRPs were treated with condition B (greater than 200-fold increase at day 6, as compared with t = 0 hrs; p < 0.05). IL-1β levels detected at later times in PRP supernatants (24 hrs to 6 days) were greater than the total IL-1β concentration measured after PRP lysis (mean, 1.4 pg/mL), suggesting a de novo synthesis.

Mean TGF-α concentrations were either not detectable or very close to the detection threshold over the entire incubation period (< 4 pg/mL), suggesting that this GF is present at trace levels, and that TGF-α release does not seem to be significantly regulated by calcium and thrombin (data not shown).

Endothelial and Osteoblast Cell Proliferation

Significant increases in endothelial cell division (up to 24-fold; p < 0.0001) were observed with the addition of PRP supernatants, as compared with minimal medium alone (Fig. 2A). Supernatants from calcium- and thrombin-treated PRPs were significantly more mitogenic for endothelial cells than were supernatants from non-activated PRPs (p < 0.05). This was especially evident with condition A-treated PRP supernatants. Time before supernatant collection also had an important effect on endothelial cell proliferation: Highest HUVEC division was observed with PRP supernatants collected within the first 24 hrs of treatment, followed by a decrease over the six-day incubation period.

As noted with HUVECs, osteoblast proliferation was significantly increased upon the addition of PRP supernatants (up to 22-fold as compared with minimal medium alone; p < 0.0005; Fig. 2B). While a four- to eight-fold increase in HUVEC division was noted with activated PRP supernatants (as compared with non-activated PRP supernatants), PRP activation had limited effect on osteoblast proliferation. However, condition B was more mitogenic for osteoblasts than was condition A (p < 0.005).

DISCUSSION

PRPs from five healthy donors were prepared according to the PCCS system, with platelet yields and recoveries similar to others already published (Marx et al., 1998; Appel et al., 2002; Weibrich et al., 2002). This method also allowed us to concentrate leukocytes 2.1-fold on average, while substantial reduction of the concentration of red blood cells was observed (Table 1).

As previously reported for TGF-β, PDGF, VEGF, and bFGF (Weibrich et al., 2002; Lacoste et al., 2003; Martineau et al., 2004), important variations in GF concentrations were detected between individuals having very similar platelet counts (Tables 1 and 2). This was especially evident with the cytokine IL-1β, in which a 100-fold variation was observed in
PRP supernatants from five different donors (data not shown). The inter-individual variation could explain, in part, some of the controversial results obtained with PRPs in different bone regeneration studies (Aghaloo et al., 2002; Froum et al., 2002).

Kinetics experiments were performed over a six-day incubation period, based on the fact that the angiogenic and osteogenic processes are triggered during the first week following an injury (Diegelmann and Evans, 2004). Of particular interest was the major increase in IL-1β observed in PRP supernatants treated with condition B (up to 200-fold increase at day 6 as compared with non-activated PRPs). These concentrations (mean, 425 pg/mL) were greater than total IL-1β levels measured after PRP lysis (mean, 1.4 pg/mL; data not shown), suggesting a de novo synthesis. While it is recognized that platelets do not contain DNA, micro-array hybridization studies have suggested that platelets contain as many as 1500 different mRNAs, including the one encoding for IL-1β (Bugert et al., 2003). However, PRPs consist of a mixture of erythrocytes, platelets, and leukocytes, and, in a recent study, a significant increase in IL-1β was observed from PRPs only when the concentration of leukocytes was above 10^8 cells, suggesting that IL-1β synthesis from PRPs was mainly related to leukocytes (Hartwig et al., 2002). Table 1 shows that 30% of total monocytes were recovered following PRP preparation. It has also recently been shown that the expression of IL-1β by monocytes can be regulated by thrombin, in a time-dependent manner (Naldini et al., 2002). Additional experiments are needed to clarify the relative contributions of platelets and leukocytes to IL-1β synthesis.

The addition of calcium and thrombin to PRPs had different effects on extracellular GF concentration (Fig. 1). EGF and Ang-2 levels increased immediately after treatment, IGF-1 decreased slowly, and a delayed increase in IL-1β concentration was observed over time. These patterns were observed for all donors, suggesting that calcium and thrombin regulate GF release, synthesis, and/or degradation in stereotyped patterns that are specific to each growth factor. The amounts of calcium and thrombin added to PRPs also had

Figure 1. GF and cytokine concentrations in PRP supernatants over a six-day incubation period at 37°C. (A) Mean EGF concentration (pg/mL). (B) Mean IGF-1 concentration (pg/mL). (C) Mean Ang-2 concentration (pg/mL). (D) Mean IL-1β concentration (pg/mL). Final thrombin and CaCl₂·2H₂O concentrations added in PRPs were: (for condition A) 142.8 U/mL and 14.3 mg/mL; (for condition B) 28.56 U/mL and 2.86 mg/mL; and (for condition C) 5.71 U/mL and 0.57 mg/mL, respectively. Bars on histogram indicate mean ± SEM (standard error of mean). Numbers above the column represent the ratio (fold) obtained when the GF/cytokine concentration at each time was divided by the GF/cytokine concentration at t = 0 hrs. All assays were performed in triplicate for each donor, and each column represents the mean of five donors.
different effects on extracellular GF content: Highest EGF levels were observed with condition A, IL-1β concentrations were maximal with condition B, and the greatest amounts of Ang-2 were detected with condition C. These results suggest that different mechanisms may regulate extracellular GF concentration. Platelets are known to have multiple vesicles, including alpha-granules, dense bodies, and lysosomes (Harrison and Cramer, 1993; Heijnen et al., 1999). EGF is known to be present in platelets (Li et al., 2001), more studies are needed to characterize the localization of Ang-2. In contrast, no immediate increase in extracellular IGF-1 or IL-1β was detected after the addition of calcium and thrombin, even if these GFs/cytokines had been identified in platelets (Karey and Sirbasku, 1989; Kobayashi et al., 2002), reinforcing the existence of different mechanisms for GF release, synthesis, or degradation.

Supernatants from activated PRPs appeared to be more mitogenic for HUVECs than did non-activated PRP supernatants. This was especially evident with condition A-treated PRP supernatants (Fig. 2A). It has previously been reported that thrombin alone can stimulate endothelial cell division (Lafay et al., 1998; Borrelli et al., 2001), but these and previous results suggest that HUVEC division is predominantly related to GF release from PRPs and not from thrombin per se (Lacoste et al., 2003). Interestingly, osteoblast proliferation almost reached its maximum with non-activated PRP supernatants (mean, 14-fold increase, as compared with minimal medium alone), suggesting that endogenous extracellular GFs from non-activated PRPs were present in sufficient amounts. PRP activation had little additional benefit on osteoblast division, which is in contrast to endothelial cell behavior, and confirms that the proliferation of these 2 cell types is regulated by different growth factors. Since angiogenesis precedes osteogenesis in the wound-healing process, and since osteoblasts are peri-vascular cells, it is plausible that stimulation of blood vessel formation with high calcium and thrombin concentrations may help to up-regulate osteogenic activity. Additional experiments are needed to determine which calcium and thrombin concentrations are optimal for bone formation in vivo.

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