Bioactivity and stability of endogenous fibrogenic factors in platelet-rich fibrin

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ABSTRACT

Platelet-rich fibrin (PRF®) is an autologous fibrin sealant (FS) enriched with a platelet concentrate (> 1,000,000 platelets/μL) produced by the automated Vivostat® system and used to enhance wound healing. The effects of PRF were compared with supernatant from thrombin-activated platelet concentrate, recombinant human platelet-derived growth factor (rhPDGF) isoforms, and a homologous FS in cultured normal human dermal fibroblasts. Also, the release of selected endogenous growth factors from PRF and their stability against proteolytic degradation were studied. The proliferative effect of PRF exceeded that of FS and rhPDGF-BB, although it was lower than thrombin-activated platelet concentrate possibly due to sustained growth factor release from platelets in PRF. Anti-PDGF antibody blocked the mitogenic effect of rhPDGF-BB but not that of PRF in growth-arrested fibroblasts. PRF promoted secretion of carboxy-terminal propeptide of type I collagen into conditioned medium while rhPDGF-AB had no significant effect on collagen biosynthesis. Limited proteolysis of PDGF-AB and no proteolysis of transforming growth factor-β1 (TGF-β1) in PRF were observed with trypsin treatment, whereas rhPDGF-AB and rhTGF-β1 in bovine serum albumin, matching the total protein concentration of PRF, were almost completely degraded after 24 hours at 37 °C. To conclude, PRF provides sustained release and protection against proteolytic degradation of endogenous fibrogenic factors important for wound healing.

Platelets are central in the initial hemostatic response to tissue injury. In addition to clotting factors, adhesive proteins, proteinases, and proteinase inhibitors, platelets also release a bolus of potent wound-healing promoting factors stored in the α-granules such as angiopoietins, connective tissue growth factor (CTGF), epidermal growth factor, insulin-like growth factor-I, platelet factor 4, platelet-derived growth factor (PDGF), transforming growth factor-α (TGF-α), TGF-β1, and vascular endothelial growth factor.[1] PDGF and TGF-β1 are the major platelet-derived mediators of fibrogenesis during wound repair.[2,3]

The effects of platelets and platelet-derived products on cutaneous wound healing in vivo are inconclusive. Thrombocytopenic mice showed a normal wound healing response and, remarkably, unaltered wound fluid levels of different growth factors in the immediate 24-hour postinjury period.[4] Topical supplementation with the supernatant of thrombin-activated human platelet concentrate elicited a pronounced connective tissue response while no positive effects were observed on wound contraction or epithelialization of full-thickness skin wounds in guinea pigs.[5] On the other hand, in diabetic rats a similar platelet product improved wound healing,[6] possibly due to PDGF deficiency in diabetic wounds.[7] In diabetic foot ulcer patients, Margolis et al.[8] concluded that platelet releasate was more effective (50% complete healing, n=6,252) than standard therapy (41%, n=20,347) within 32 weeks of the initiation of care in a retrospective study. In a randomized controlled trial, a platelet-rich plasma gel (AutoloGel™; Cytomedix, Rockville, MD) was claimed to promote the healing rate in 12 weeks (68.4%) of diabetic foot ulcers, although it was not statistically significant (p=0.126) compared with the saline gel group (42.9%) in the per-protocol population (n=40).[9] PDGF-BB (Regranex®; Ortho-McNeil Pharmaceutical, Raritan, NJ) is FDA-approved as a local adjunct in the treatment of diabetic foot ulcers.[10] No beneficial effects of different autologous platelet formulas in the treatment of venous leg ulcers have been documented.[11–13] This may be ascribed to excessive proteinase activity that attenuates growth factor bioactivity.[14]

BrdU	5-bromo-2′-deoxyuridine
BSA	Bovine serum albumin
CICP	C-terminal propeptide of type I collagen
ELISA	Enzyme-linked immunosorbent assay
FBM	Fibroblast basal medium
FCS	Fetal calf serum
FGM	Fibroblasts growth medium
FS	Fibrin sealant
NHDF	Normal human dermal fibroblast
PDGF	Platelet-derived growth factor
PRF	Platelet-rich fibrin
TGF-β1	Transforming growth factor-β1

The automated Vivostat® system (Vivolution A/S, Birkerød, Denmark) prepares an autologous fibrin biomatrix enriched with platelets—platelet-rich fibrin or PRF®—in about 30 minutes.[15] The fibrin(ogen) component may be important for the effectiveness of PRF®[16] and, for example, the presence of fibrin and fibrinogen may reduce the susceptibility of the endogenous polypeptidic growth factors to proteolytic degradation.[17] Furthermore, in vitro studies have shown increased migration and proliferation of fibroblasts with fibrinogen present.[18] Experimental studies in rats suggest that the formation of fibrin is necessary for collagen synthesis and biomechanical wound strength development.[19] The bioactivity of the PRF® product is unknown.

We have compared the effects of PRF® with thrombin-activated platelet concentrate, recombinant human PDGF isoforms, and purified homologous fibrin sealant (FS; Tisseel®; Baxter Healthcare, Glendale, CA) on normal human dermal fibroblasts (NHDFs) with respect to proliferation and collagen synthesis. We also assessed the normal release rate of endogenous PDGF-AB and TGF-β1 from PRF® and the stability of these mediators in a proteolytic environment.

MATERIALS AND METHODS

Proteins and reagents

Recombinant human PDGF-AB (rhPDGF; 222-AB) was purchased from R&D Systems (Minneapolis, MN). rhPDGF-BB (P3201), TGF-β1 (T7039), trypsin (derived from porcine pancreas; 93614), bovine serum albumin (BSA; A-4503), and all other reagents and chemicals, unless otherwise stated, were purchased from Sigma-Aldrich (St. Louis, MO).

Preparation of Vivostat® PRF®, thrombin-activated platelet concentrate, and Tisseel®

Vivostat® PRF® was generated by an automatic process as described by the manufacturer (Vivolution A/S). In short, 120 mL whole blood was drawn by venipuncture from healthy male donors aged between 33 and 55 years, anti-coagulated with acid-citrate solution containing tranexamic acid (about 1 mg/mL in PRF®) and centrifuged. The resultant platelet-rich plasma (about 60 mL) was converted into an acid-soluble fibrin I polymer by the addition of batroxobin.[20] Batroxobin catalyzes the release of fibrinopeptide A from fibrinogen without activating factor XIII. The fibrin I polymer and the platelets were isolated by centrifugation and dissolved in 0.2 M acetate buffer (pH 4). The total protein concentration of this solution was 37.0 mg/mL as determined by the Bradford[21] assay (TP0100), the fibrinogen/fibrin concentration was 18.1 ± 1.4 mg/mL (mean ± SEM, $n=5$), and the platelet count was $1{,}270{,}000 \pm 143{,}000$ platelets/μL (Table 1). This corresponds to a mean 6.4-fold increase in platelet concentration in the fibrin I solution compared with the platelet concentration in whole blood. About 6 mL of Vivostat PRF® comprising cross-linked fibrin II polymer enriched with the platelets was obtained through neutralization of seven parts of the acidic fibrin I solution with one part of a 0.75 M carbonate/bicarbonate buffer (pH 10).[22] This polymerization process is thought to be mediated by activation of endogenous prothrombin to thrombin that activates endogenous factor XIII.

Thrombin-activated platelet concentrate was generated as described previously.[23] Briefly, 40 mL whole blood, drawn from a 36-year-old donor, was anti-coagulated with citrate–phosphate–dextrose and spun for 20 minutes at $135\times g$ at 4 °C without deceleration. The platelet count of the resultant platelet-rich plasma was determined using an automated hematology analyzer (SF-3000; Sysmex, Kobe, Japan). The platelets were pelleted at $750\times g$ for 10 minutes at 4 °C without deceleration and resuspended in a volume of phosphate-buffered saline (PBS) yielding a platelet count (989,000 platelets/μL) identical to that of PRF® produced in parallel from the same donor. The resuspended platelets were activated with human thrombin from the Tisseel® Duo Quick kit at 1 IU per 10^9 platelets. Supernatant was obtained after centrifugation at $950\times g$ for 5 minutes at 4 °C and used in the growth assay.

Table 1. Platelet counts in whole blood and PRF®, and fibrin concentrations in PRF® produced from five of the healthy male blood donors

	Blood donor				
	A	B	C	D	E
Age (years)	53	54	51	52	55
Platelet count (platelets/μL)					
Whole blood[a]	141,000	331,000	169,000	195,000	185,000
PRF®[b]	1,242,000	1,782,000	911,000	1,269,000	1,134,000
Fibrin(ogen) in PRF® (mg/mL)[b]	20.1	22.5	17.4	15.1	15.3
DNA synthesis[c]	12.2 ± 0.6[d]	25.9 ± 0.9[d]	ND	ND	ND

[a]Normal range: 150,000–400,000 platelets/μL.[33]
[b]Determined in fibrin I solution.
[c]Fold-increase over medium alone in the BrdU assay.[26]
[d]Mean ± SEM ($n=8$).
ND, not determined; PRF®, platelet rich fibrin.

Tisseel® Duo Quick, a gift from Baxter A/S (Allerød, Denmark), comprises two 1-mL syringes. One syringe contains fibrinogen, fibronectin, bovine aprotonin, factor XIII, and plasminogen. The other syringe contains human thrombin (500 IU) and 40 μmol $CaCl_2$. Tisseel® was prepared according to the manufacturer's instructions. The protein concentration of Tisseel® is 50–65 mg/mL according to the manufacturer's product specification.

To standardize the dose in the different assays, clots of Vivostat® PRF® and Tisseel® of uniform volume (35 μL) were molded aseptically in a specially designed device made of polycarbonate with multiple cylindrical wells.

Representative Vivostat® PRF® clots were fixed in 6% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) at 4 °C overnight, osmicated, dehydrated, and then embedded in epoxy resin. Ultra-thin sections were cut and contrasted by uranyl acetate and lead citrate. The specimens were scrutinized in a Jeol 100 CX transmission electron microscope (Jeol, Tokyo, Japan).

Fibroblast culture studies

Primary adult NHDFs (CC-2511), derived from a 37-year-old Caucasian female, were purchased from Cambrex (Walkersville, MD) and expanded in fibroblast growth medium (FGM) with 10% fetal calf serum (FCS; Invitrogen, Carlsbad, CA) in 75 cm^2 culture flasks (Nunclon™; Nunc, Roskilde, Denmark) at 37 °C in humidified 5% CO_2/air. FGM is composed of fibroblast basal medium (FBM; Cambrex, CC-3131) containing gentamicin and amphotericin-B, and supplemented with growth factors (SingleQuots®; Cambrex, CC-4134). Cells subcultured four times in the presence of 0.05% trypsin–0.02% ethylenediaminetetraacetic acid (T3924) according to Cambrex's recommendation were used for the different assays.

In the growth assay, 2,000 fibroblasts per well were seeded in 100 μL of FBM+2% FCS and allowed to attach for 5 hours before 35 μL of control (FBM), 35 μL of PDGF-BB (57.1 ng/mL) in FBM, 35 μL of human albumin (50 mg/mL), 35 μL of the supernatant from thrombin-activated platelets, 35 μL of thrombin (1 IU/mL; Baxter) in PBS, one Tisseel® clot, or one PRF® clot was added together with 65 μL FBM+3% FCS to a final volume of 200 μL. One PRF® clot corresponds to 17.5% (vol/vol), which, in another study, yielded maximal mitogenic effect of activated platelet preparations in fibroblast cultures.[24] Fibroblasts were quantified over 96 hours of incubation at indicated time points indirectly by ATP bioluminescence.[25]

DNA synthesis was measured by 5-bromo-2′-deoxyuridine (BrdU) incorporation.[26] Fibroblasts were seeded in 96-well tissue culture plates (Nunclon™) at 10,000 cells per well and grown in FGM+10% FCS for 72 hours. Spent medium was then removed and quiescence was achieved by incubating the cells in 100 μL FBM+2% FCS for 24 hours. A polyclonal goat anti-PDGF neutralizing antibody (P6101) in 65 μL FBM+3% FCS, the normal goat IgG isotypic control (I5256) in 65 μL FBM+3% FCS, or 65 μL FBM+3% FCS alone was added together with 35 μL of FBM alone (control), one PRF® clot, or 35 μL of FBM with PDGF-BB (57.1 ng/mL), yielding a final volume of 200 μL per well. Cells were incubated for 24 hours with BrdU present at 10 μmol/L for the final 2 hours except for the background control that received no BrdU. BrdU incorporation was measured colorimetrically using an enzyme-linked immunosorbent assay (ELISA) kit (11 647 229; Roche Diagnostics, Roche Applied Science, Mannheim, Germany).

Collagen de novo biosynthesis was measured by the release of type I C-terminal collagen propeptide (CICP) into the culture medium from fibroblasts according to conditions described elsewhere.[27] Fibroblasts (50,000/well) were seeded in 24-well plates in FGM+10% FCS and grown for 72 hours to confluence. Fibroblasts were washed with 1× FBM and quiescence was induced by incubating the cells in FBM+2% FCS for 24 hours. The cells were then washed with 1× FBM and treated for 24 hours at 37 °C with control medium comprising 710 μL FBM supplemented with 2 mg/mL BSA (FBM+0.2% BSA), six 35 μL-PRF® clots, and 500 μL FBM+0.2% BSA, or with 710 μL of FBM+0.2% BSA supplemented with PDGF-AB (10 ng/mL) per well. PRF® background controls comprised six PRF® clots in 500 μL FBM+0.2% BSA incubated simultaneously in cell-free wells. Conditioned medium was spun and stored at −80 °C. Collagen synthesis assay was performed using the Metra CICP ELISA kit (Catalog no.: 8003) as per the manufacturer's (Quidel Corporation, San Diego, CA) instructions.

Release and proteolysis of endogenous growth factors in PRF®

PRF® clots were weighed and mixed with the corresponding volume of PBS in 2-mL microcentrifuge polyethylene tubes on an ice-water bath, and finely dispersed 5×30 seconds at 30,000 r.p.m. using a T10 Ultra-Turrax® (IKA-Werke, Staufen, Germany) equipped with a 5-mm dispersing tool (S10N-5G). Dispersed PRF®, PDGF-AB (20 ng/mL, final concentration) in 17 mg/mL BSA dissolved in PBS, or TGF-β1 (50 ng/mL) in 17 mg/mL BSA dissolved in PBS was incubated in a total volume of 60 μL in Nunc-Immuno™ Minisorp polyethylene tubes (466982; Nunc). The BSA concentration in the PDGF-AB and TGF-β1 groups matched the total protein concentration of PRF®.[22] To mimic the degradation of growth factors by serine proteinases in chronic wounds,[14] trypsin in PBS (2 μg/mL) was added to a final concentration of 1 μg/mL to dispersed PRF® clots and recombinant growth factors as suggested by Krishnaswami et al.[28] After incubation for 0 (baseline), 10, 90, and 1,440 minutes at 37 °C, the reactions were stopped by the addition of 10 μL of a 7× concentrated proteinase inhibitor cocktail (Complete® EDTA-free, 11836170; Roche Diagnostics) to each tube. The samples were first incubated at 57 °C for 10 minutes, then overnight at 4 °C, and finally centrifuged at 16,000×*g* for 1 hour.[29] Supernatants were assayed for PDGF-AB (DuoSet®; DY222) and TGF-β1 (DuoSet®; DY240) concentrations using ELISA kits according to the manufacturer's (R&D Systems) instructions including activation of latent TGF-β1 with 1 N hydrochloric acid.

Statistical analyses

The differences between the means between test groups were evaluated for statistical significance using one-way analysis of variance (ANOVA). Where a two-sided

statistically significant difference ($p < 0.05$) was identified, the statistical significance of differences between individual test groups were assessed using the post hoc Dunnett-T3 test. Statistical analyses were performed using SPSS software (version 10.0; Chicago, IL). Data are expressed as mean ± SEM (standard error of the mean).

RESULTS

Ultrastructural studies of PRF®

Transmission electron microscopy examination of PRF® revealed that granules are less dense and partially or completely empty in some thrombocytes in PRF® compared with thrombocytes from peripheral blood (Figure 1).[30]

Effect of PRF® on fibroblast proliferation

In the fibroblast growth assay (Figure 2), PRF® promoted fibroblast growth over an extended time period, exceeding the effect of a single application with rhPDGF-BB at 70 hours ($p < 0.001$) and 96 hours ($p < 0.001$). Treatment with the homologous FS resulted in a small (30%) but statistically significant increase in the number of fibroblasts while treatment with human albumin had no significant ($p=0.641$) effect on fibroblast numbers compared with the control at the end of the 96-hour incubation period (Figure 2A). In another experiment, two other comparators were included. All the treatments increased fibroblast density from 48 hours onward compared with medium alone (FBM+2% FCS). Again, PRF® stimulated fibroblast growth more than rhPDGF-BB at 72 hours ($p < 0.001$) and 96 hours ($p < 0.001$). No significant differences at any time point were found between PRF® and the optimized culture medium (FGM+10% FCS) containing multiple growth factors.[26] Supernatant from thrombin-activated platelet concentrate increased the number of fibroblasts

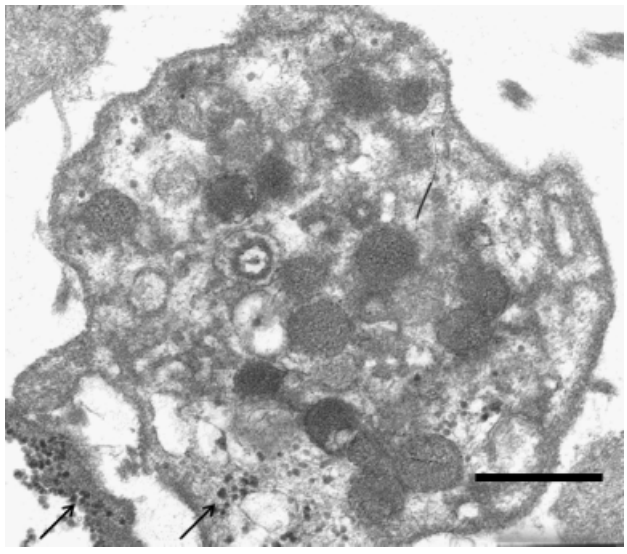

Figure 1. Ultrastructure of representative thrombocyte in PRF®. Glycogen particles are arrowed. Scale bar: 0.4 μm. PRF®, platelet-rich fibrin.

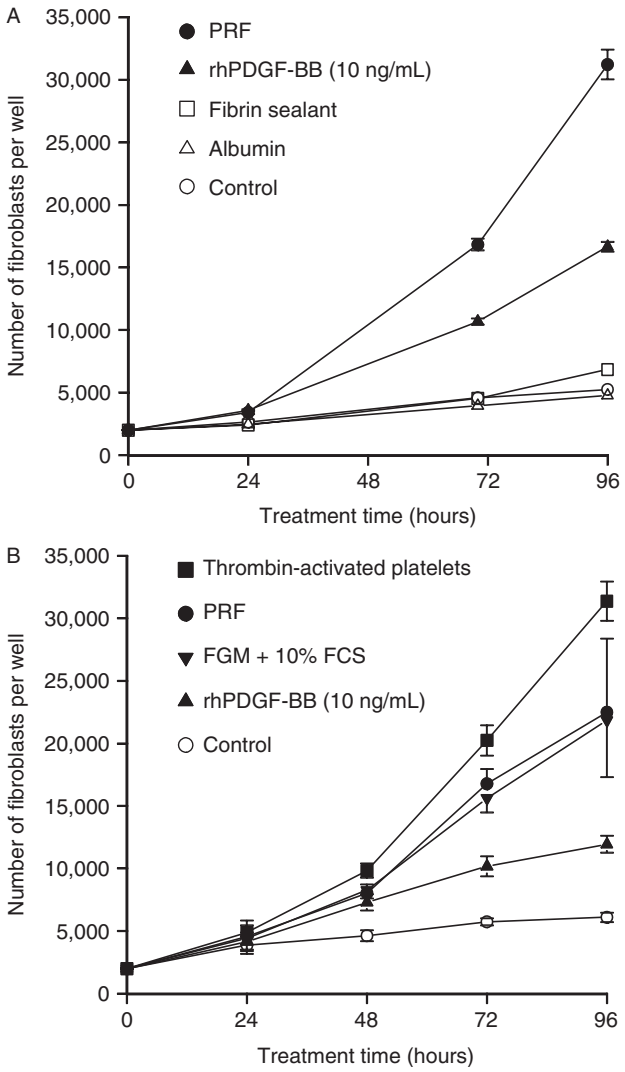

Figure 2. Effect of treatments on fibroblast growth assessed by the ViaLight® Plus (Cambrex) assay in 96-well plates. At the indicated time points, wells were emptied, and 100 μL of FBM and 50 μL of lysis reagent were added. After 10 minutes of incubation at ambient temperature, 100 μL supernatant was transferred to white luminescence plate and the emitted light measured in microplate luminometer (LUMIstar; BMG Labtech, Offenburg, Germany). (A) Each point in the graph represents the mean of 8 replicates ± SEM. For reasons of clarity SEM are omitted for the control, albumin and fibrin sealant groups. 24 hours: PRF® vs. control, $p=0.009$; PDGF-BB vs. control, $p < 0.001$; 70 hours: PRF® vs. control, $p < 0.001$; PDGF-BB vs. control, $p < 0.001$; albumin vs. control, $p < 0.041$. 96 hours: PRF® vs. control, $p < 0.001$; PDGF-BB vs. control, $p < 0.001$; Fibrin sealant vs. control, $p=0.003$. PRF® was produced from male donor B presented in Table 1. (B) Each point in the graph represents the mean of 12 replicates ± SEM. All four treatments significantly ($p < 0.001$) increased the number of fibroblasts compared to control at 48, 72, and 96 hours. PRF® and the supernatant of thrombin-activated platelet concentrate were made in parallel from the same male blood donor. PRF®, platelet-rich fibrin; PDGF, platelet-derived growth factor; SEM, standard error of mean.

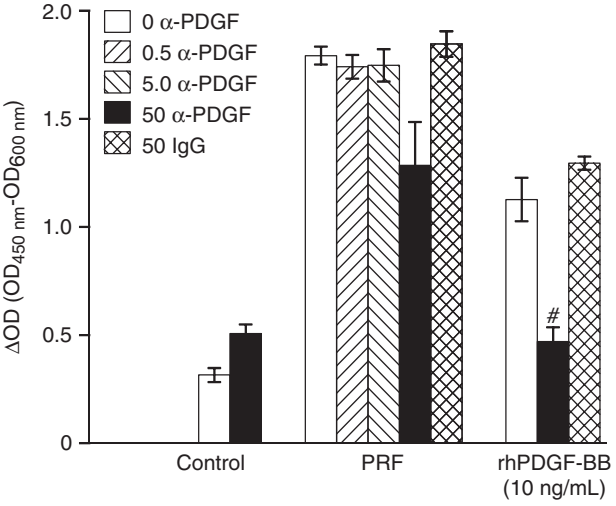

Figure 3. Effect of neutralizing anti-PDGF (α-PDGF) antibody at the indicated concentrations in μg/mL on DNA synthesis measured by BrdU incorporation. Mean ± SEM (n=6). #p=0.013 vs. no addition of antibody (0 α-PDGF) and $p < 0.001$ vs. the isotypic IgG control (IgG). A 33-year-old healthy male donated blood for PRF®. PRF®, platelet-rich fibrin; PDGF, platelet-derived growth factor; SEM, standard error of mean.

significantly more compared with PRF® and the optimized culture medium at 48, 72, and 96 hours (Figure 2B). Corresponding thrombin concentration did not increase the fibroblast count compared with control (data not shown).

To elucidate the origin of mitogenic substances, PRF® clots and the positive PDGF-BB control were incubated with a neutralizing antibody against the PDGF-AA, PDGF-AB, and PDGF-BB isoforms (Figure 3). Although both PRF® ($p < 0.001$) and PDGF-BB (p=0.006) significantly stimulated DNA synthesis in growth-arrested fibroblasts compared with the control medium alone, only the mitogenic activity of recombinant PDGF-BB was blocked completely by the neutralizing antibody. The stimulatory effect of PRF® on DNA synthesis was not significantly antagonized by the antibody compared with no addition of antibody (p=0.560) or compared with the isotypic IgG control (p=0.465).

The mitogenic effect of PRF® produced from a mildly thrombocytopenic donor was significantly ($p < 0.001$) reduced compared with a donor with a normal platelet blood count (Table 1).

Effect of PRF® on collagen biosynthesis by dermal fibroblasts

Collagen synthesis was assessed by the levels of the C-terminal of type I procollagen in the medium using an ELISA assay. We found that the assay was linear over 24 hours and then reached steady-state levels under serum-free culture conditions (Figure 4A). Supplemental serum (2%) suppressed procollagen processing possibly due to the inhibitory effect of α2-macroglobulin on C-proteinase activity.[31] In another study, reduced type I collagen mRNA levels were found in adult dermal fibroblasts cultured at

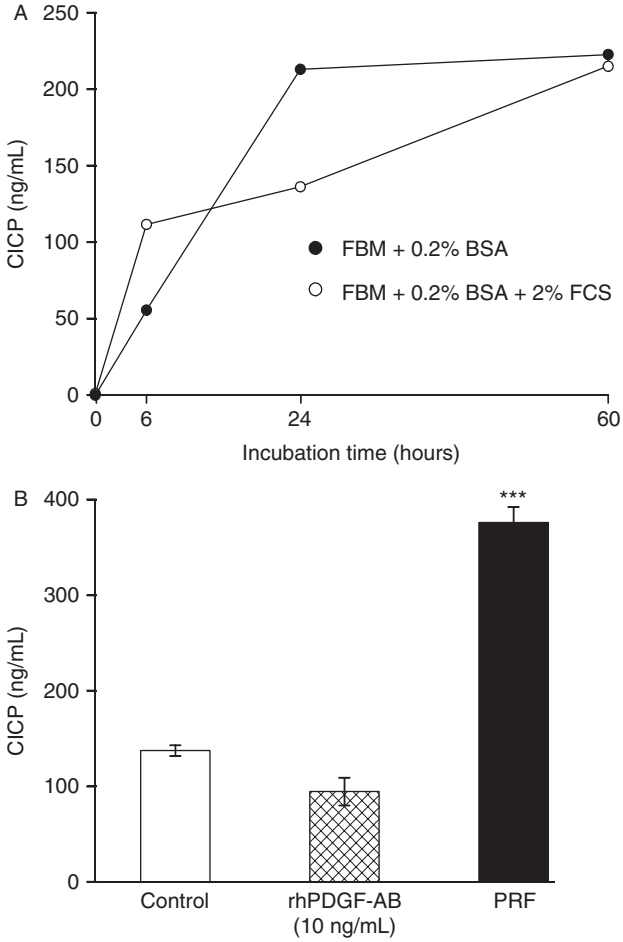

Figure 4. Type I collagen synthesis in confluent and non-proliferating normal human dermal fibroblasts. (A) Cells were cultured in FBM+0.2% BSA or in FBM+0.2% BSA+2% FCS. At the time points indicated two wells for each condition were emptied, media briefly spun and stored at −80 °C until analyzed in duplicates for C-terminal propeptide of type I collagen (CICP). (B) Effect of PRF® and PDGF-AB treatment for 24 hours compared with control-treated fibroblasts grown in FBM+0.2% BSA alone. The CICP level in medium from incubated PRF® clots without fibroblasts was 1.7 ng/mL. Mean ± SEM (n=6). ***$p < 0.001$ vs. control. A 33-year-old healthy male donated blood for the PRF®. BSA, bovine serum albumin; FBM, fibroblast basal medium; PRF®, platelet-rich fibrin; PDGF, platelet-derived growth factor; SEM, standard error of mean.

low density in 10% FCS compared with no FCS present.[32] PRF® increased the release of type I collagen pro-peptides into the medium almost two-fold compared with control-treated fibroblasts, whereas rhPDGF-AB, the main PDGF isoform in human platelets, had no significant (p=0.097) effect on type I collagen biosynthesis (Figure 4B).

Release and stability of endogenous PDGF-AB and TGF-β1 in PRF®

To determine liberation from and stability of endogenous growth factors PDGF-AB (Figure 5A) and TGF-β1

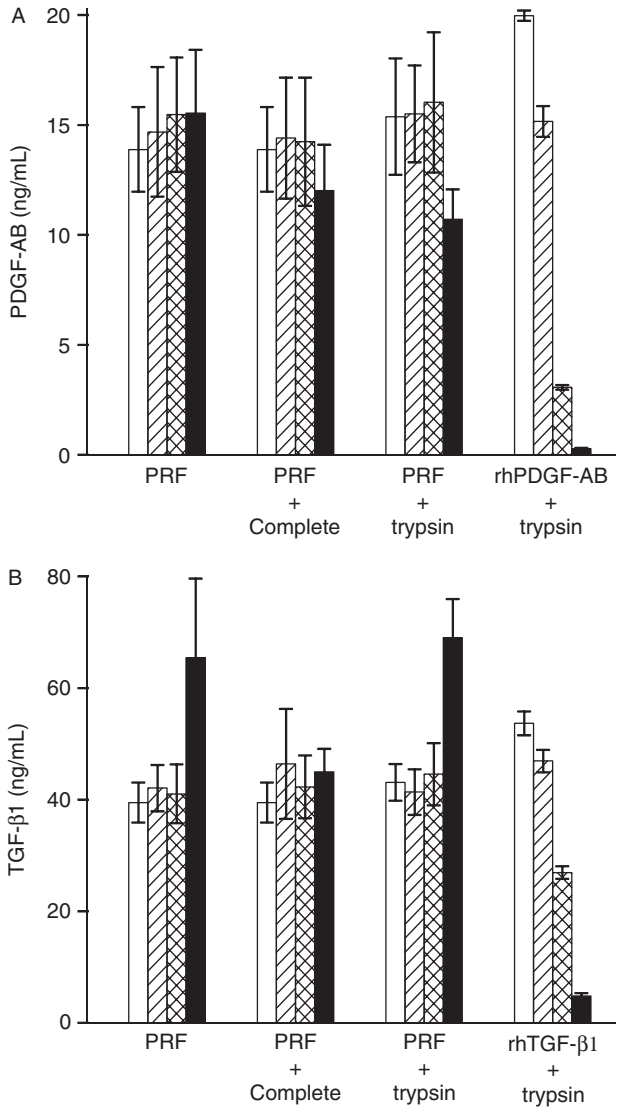

Figure 5. Release of and the effect of trypsin treatment on PDGF-AB (A) and TGF-β1 (B) levels, measured by ELISA, in medium before incubation (open bars) and after 10 minutes (hatched bars), 90 minutes (crosshatched bars), and 1,440 minutes (closed bars) of incubation of PRF® or of recombinant PDGF-AB (20 ng/mL) or TGF-β1 (50 ng/mL) at 37 °C in PBS, PBS+proteinase inhibitor cocktail (Complete) or in PBS+trypsin (1 μg/mL). PRF® was derived from the three male blood donors C, D and E presented in Table 1. Mean ± SEM (n=3). ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; PRF®, platelet-rich fibrin; TGF, transforming growth factor; SEM, standard error of mean.

(Figure 5B) over time, dispersed PRF® clots were incubated without or with trypsin over 24 hours. The baseline level of PDGF-AB was 13.9 ± 1.9 ng/mL (n=3) and of TGF-β1 was 39.5 ± 3.6 ng/mL (n=3). After 24 hours of incubation of PRF® clots at 37 °C, the levels had risen to 15.5 ± 2.9 and 65.4 ± 14.2 ng/mL, respectively. Intriguingly, the levels of both PDGF-AB and TGF-β1 were lower when PRF® was coincubated with a proteinase inhibitor cocktail (Complete EDTA-free) that blocks serine and cysteine proteinases. Control levels of recombinant PDGF-AB and TGF-β1 incubated in parallel in PBS with or without Complete did not change appreciably over the 24-hour period (data not shown).

With the addition of trypsin, the recombinant growth factors were degraded almost to completion in 24 hours at 37 °C. In sharp contrast, endogenous PDGF-AB levels decreased only slightly relative to start levels and TGF-β1 levels were still elevated by about 60% at 24 hours with PRF® incubated with trypsin (Figure 5).

DISCUSSION

The platelet concentration in Vivostat PRF is above 1,000,000 platelets/μL, which is the working definition of an autologous platelet-rich product with healing potential.[33] In the present in vitro investigations, PRF® also enhanced the proliferation of human dermal fibroblasts and their synthesis of type I collagen.

We could demonstrate increased levels of PDGF-AB and TGF-β1 from PRF® with prolonged incubation. Corroborating these findings, Leitner et al.[15] reported that the PDGF-AB levels increased continuously for at least 72 hours from PRF® clots incubated in PBS. In contrast, thrombin completely activates platelets within 10 minutes[34] and may explain the increased fibroblast proliferation with supernatant from the thrombin-activated platelet concentrate. The lower growth factor levels with a proteinase inhibitor present indicate that the platelets in PRF® were activated by endogenous proteinases. Buchta et al.[22] determined the thrombin concentration of Vivostat® fibrin clot as 1.5 IU/mL and plasminogen as 41 μg/mL. Increased release of growth factors with time from PRF® was possibly mediated by either of the proteinase-activated receptors (PAR) 1 and 4 on the platelets.[35] In addition, platelet activation is associated with liberation of plasminogen activator inhibitor-I[2] and thrombospondin-1,[28] which may provide protection of the released endogenous growth factors.

Interestingly, we were unable to block the mitogenic effect of PRF® with an anti-PDGF neutralizing antibody. This was also indicated by Celotti et al.[36] using batroxobin-treated platelet-rich plasma gel in an osteoblastic cell line. Platelet-rich plasma also increased proliferation of periodontal ligament fibroblasts significantly more than the combination of recombinant PDGF-AB and TGF-β1 at the corresponding endogenous concentrations.[3] PRF® is a complex mixture of growth-promoting mediators and there are many candidates. For example, plasmin-induced DNA synthesis in a human fibroblast cell line via PAR-1 activation was recently reported.[37]

The homologous FS devoid of growth factors showed lower proliferative activity compared with PRF®. This indicates that the bioactivity of PRF® was primarily attributed to the platelets in our two-dimensional in vitro assay, although there are some structural differences in the fibrin in PRF® and Tisseel®.[22] In another study, Michel et al.[38] observed that by lowering thrombin from 250 to 50 U/mL and the final calcium concentration from 20 to 2 mmol/L this resulted in a three-fold increase in fibroblast

proliferation. Moreover, this composition of the FS increased the synthesis of type I and type III collagens compared with medium alone.[38]

PRF® strongly stimulated the synthesis of type I collagen in the dermal fibroblasts as observed previously for a platelet lysate formula.[24] This was unlikely attributed to endogenous PDGF-AB, the major PDGF isoform of platelets. Lepistö et al.[39] even reported decreased mRNA levels for the pro α1 (I) chain in the presence of PDGF-AB at 10 ng/mL and above in cultured normal human dermal fibroblasts. TGF-β1 enhances collagen synthesis possibly through CTGF,[40] another platelet-derived protein. The fibrin(ogen) content of PRF® may also contribute to the increased collagen synthesis.[38]

The use of autologous PRF® in wound management is largely dependent on the effect and stability of the PRF®-derived growth factors. We have shown that at least two growth factors are protected from proteolytic degradation in PRF® by multiple mechanisms. This may be advantageous in the treatment of chronic wounds characterized by high proteinase activity.[14] These findings together with the observed fibrogenic properties indicate that PRF® may be a useful adjuvant in the management of problematic wounds.

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