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Growth factor and proteinase profile of Vivostat® platelet-rich fibrin linked to tissue repair

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Vox Sanguinis

Background and Objectives Autologous platelet-rich fibrin (PRF®) is prepared by the automatic Vivostat® system. Conflicting results with Vivostat PRF in acute wound healing prompted us to examine its cellular and biomolecular composition. Specifically, platelets, selected growth factors and matrix metalloproteinase (MMP)-9 were quantified using novel analytical methods.

Materials and Methods Ten healthy non-thrombocytopenic volunteers donated blood for generation of intermediate fibrin-I and final PRF. Anticoagulated whole blood and serum procured in parallel served as baseline controls. Leucocyte, erythrocyte and platelet counts in whole blood and fibrin-I were determined by automated haematology analyser. Platelet concentration in PRF was quantified manually by stereologic analysis of Giemsa-stained tissue sections, and the total content of five growth factors and MMP-9 by enzyme-linked immunosorbent assays.

Results The number of leucocytes and erythrocytes was reduced ($P < 0{\cdot}001$), whereas platelets increased ($P < 0{\cdot}001$) in fibrin-I versus whole blood. PRF contained $982 \pm 206 \times 10^9$ platelets/l representing 3·9-fold ($P < 0{\cdot}001$) enrichment relative to whole blood. Growth factor abundance in Vivostat PRF and serum was in descending order: transforming growth factor-β1 [5·1-fold higher in PRF than serum, $P < 0{\cdot}001$] > platelet-derived growth factor (PDGF)-AB [2·5-fold, $P < 0{\cdot}01$] > PDGF-BB [1·6-fold, $P < 0{\cdot}05$] > vascular endothelial growth factor > basic fibroblast growth factor [75-fold, $P < 0{\cdot}001$]. MMP-9 was reduced 139-fold ($P < 0{\cdot}001$) compared with serum, reflecting leucocyte depletion in PRF.

Conclusion The gained knowledge on platelet enrichment and biomolecular constituents may guide clinicians in their optimal use of Vivostat PRF for tissue regenerative applications.

Key words: cellular therapy, cytokine, platelet concentrates, proteinase, wound healing.

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Introduction

Platelets are rich sources of endogenous growth factors (GFs) that are obligatory for wound healing [1–3]. Because it is assumed that superphysiological levels of GFs will stimulate hard and soft tissue repair, numerous protocols and instruments have been developed to concentrate autologous platelets from blood [4–6].

Autologous Vivostat® platelet-rich fibrin (PRF®) is prepared in two automated steps (Vivostat A/S, Allerød, Denmark). First, the machine makes an intermediate fibrin-I solution containing the enriched platelet fraction.

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Then, the final Vivostat PRF product composed of the platelet concentrate in polymerized fibrin-II is made *in situ* [7].

The bioactivity of Vivostat PRF has been documented in fibroblast cultures [7]. Intriguingly, the mitogenic effect was not blocked by the addition of neutralizing antibodies against the platelet-derived growth factor (PDGF)-AA, AB and BB isoforms [7]. Moreover, Vivostat PRF increased type I collagen synthesis, while PDGF-AB was ineffective [7]. These *in vitro* findings strongly suggest the presence of multiple trophic factors in Vivostat PRF.

Clinically, Vivostat PRF was found beneficial in the treatment for excised pilonidal sinus disease in a randomized clinical trial encompassing 100 patients [8]. A single application to the wound just before suturing was superior to an in-house platelet gel in terms of wound analgesia, postoperative complications and time to wound healing [8]. We have experience using Vivostat PRF from randomized clinical trials [9, 10]. In one trial, Vivostat PRF unexpectedly down-regulated type I collagen in a subcutaneous wound-healing model despite providing sustained and increased local levels of one GF, transforming growth factor (TGF)-β1 [10]. Obviously, the effects of exogenous GFs, delivered via Vivostat PRF, on basal wound-healing mechanisms are complex and sometimes unpredictable.

Published data on platelet and GF contents in Vivostat PRF are scarce possibly due to technical difficulties [7, 9–11]. Interestingly, Leitner *et al.* [12] applied an automated haematology analyser and observed increased number (4·0-fold) of platelets but decreased number of erythrocytes and leucocytes in Vivostat fibrin-I compared with whole blood from two healthy donors. Dohan Ehrenfest [13] used a high-speed dispersion tool to solubilize GFs in Choukroun's PRF membrane which is similar in consistency to Vivostat PRF. Choukroun's PRF membrane, as opposed to Vivostat PRF, contains significant numbers of leucocytes with preformed proteinases notably matrix metalloproteinase (MMP)-9.

Overall, there is a need to establish the cellular and biomolecular composition of Vivostat PRF to aid clinicians in their selection of proper indications for this topical therapy. Thus, the specific aims of this study were to (1) measure the total number of platelets but also leucocytes and erythrocytes in the intermediate fibrin-I solution and compare these values with baseline, that is, whole blood from the same healthy donors; (2) enumerate platelets and (3) quantify the total content of the GFs basic fibroblast growth factor (bFGF), PDGF-AB, PDGF-BB, TGF-β1 and vascular endothelial growth factor (VEGF), and the proteinase MMP-9 in Vivostat PRF. For these purposes, novel methods were applied for the quantification of platelets, GFs and MMP-9 in Vivostat PRF. Serum obtained from the same individuals was used as reference. Serum contains representative factors released locally at the initiation of the wound-healing cascade.

Materials and methods

Participants

Five male and five female healthy volunteers aged 28–55 years (39 ± 10 years) and with body mass index of 22·7 ± 2·5 kg/m^2 were enrolled. The participants were non-thrombocytopenic (>150 × 10^9 platelets/l) and non-smoking and had not ingested substances with known specific effects on platelets, for example acetylsalicylic acid within 4 days. Blood was drawn 8:00–12:00.

Procurement and further treatment of whole blood, serum, Vivostat fibrin-I and Vivostat PRF

The study design is depicted in Fig. 1. A 2-ml whole venous blood sample was collected in ethylenediaminetetraacetic acid (EDTA)-containing tubes (Vacuette® 454217; Greiner Bio-One, Frickenhausen, Germany) for determination of blood counts using a haematology-automated analyser (Sysmex XE-2100; Sysmex Corporation, Kobe, Japan). Two more 5-ml blood samples were drawn into serum separator tubes (Vacuette 454067) that were gently inverted eight times. One blood sample was allowed to clot for 30 min at room temperature for later bFGF, PDGF-AB, PDGF-BB, VEGF and MMP-9 determination. The other was incubated for 18 h at 4°C for complete release and determination of TGF-β1. The tubes were centrifuged at 1800 g for 10 min, and supernatants aliquoted into two separate polypropylene cryogenic vials (Greiner Bio-One) kept at −80°C until analysed.

From 120 ml of whole blood, 5·2 ± 0·3 (n = 10) ml intermediate fibrin-I solution (pH 4) was produced in the Vivostat machine (Fig. 1). Aliquots (0·25 ml) were mixed with 0·75 ml 0·2 M acetate buffer (pH 4) with 24 mM calcium chloride by gentle inversion 6 times in 1·5-ml polypropylene microcentrifuge tubes (MCT-150; Axygen, Union City, CA, USA). Leucocyte, erythrocyte and platelet concentrations were determined in two fibrin-I aliquots from each participant within 4 h using the Sysmex XE-2100 instrument.

From the generated Vivostat PRF (Fig. 1), one aliquot was sprayed into 10 mm × 10 mm × 5 mm Cryomold® (Tissue-Tek®; Miles, Elkhart, IN, USA) that was fixed in 4% phosphate-buffered paraformaldehyde (pH 7·4) overnight at 4°C and embedded in paraffin for platelet

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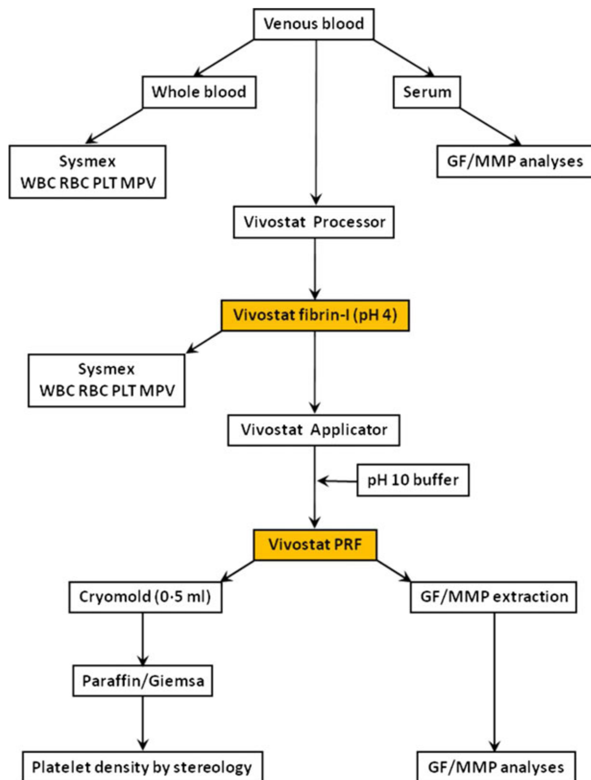

Fig. 1 Sampling schedule and analysis steps of whole blood, serum, Vivostat intermediate fibrin-I and PRF. In the Processor unit, the Vivostat acid-soluble fibrin-I polymer (pH 4) is isolated together with platelet concentrate and other cells. Vivostat PRF is produced by neutralization of seven parts of the platelet-enriched acidic fibrin-I (pH 4) with 1 part of 0·75 M carbonate/bicarbonate buffer (pH 10) [7, 10] in the Applicator unit. GF, growth factor; MMP, matrix metalloproteinase; MPV, mean platelet volume; PLT, platelet; RBC, red blood cell; WBC, white blood cell.

quantification. Additional two Vivostat PRF aliquots (each ~0·25 g) per participant were used for tissue extraction.

Choukroun's PRF membrane

Five millilitres of venous blood was drawn into plastic tubes coated with micronized silica particles (Vacuette 454204) from two healthy male volunteers (29 and 59 years) and one female (54 years) according to the standard protocol [13]. The tubes were immediately centrifuged at 400 g for 12 min and supernatant removed. The fibrin clot was isolated from the erythrocyte fraction in the bottom and pressed gently between non-woven swabs producing the Choukroun's PRF membrane (~0·20 g). Serum was isolated in parallel from 5 ml blood drawn into identical tubes that were left for 30 min before being centrifuged at 1800 g for 10 min. PRF tissue extract and serum was analysed by gelatin zymography.

Platelet enumeration in Vivostat PRF by stereology

The paraffin blocks were cut exhaustively into 70-μm-thick sections. Five systematically and randomly sampled sections were mounted on gelatin-coated glass slides, deparaffinized, stained with Giemsa for 45 min, dehydrated and cover-slipped. Platelets were counted by light microscopy using 100 × oil immersion objective with a numerical aperture of 1·4 (BH2; Olympus, Hamburg, Germany) [14]. The area of the counting frame, 31·3 μm^2, multiplied by the disector height, 10 μm, gave the dimensions of the optical disector (Fig. 2). The total number of platelets in the specimen, N (platelets), was estimated from: $N(\text{platelets}) = \frac{\sum Q^-}{\sum V(\text{dis})} * V(\text{ref})$, where ΣQ^- is the total number of counted platelets, ΣV (dis) is the volume of all disectors, and V(ref) is the volume of the Cryomold. The density was finally calculated from: $N_V = N$ (platelets)/V (ref).

PRF tissue extraction

All procedures were carried out at 0–4°C. PRF samples were dispersed within 4 h in T-PER® (ThermoScientific, Rockford, IL, USA) reagent (3 ml/g PRF) using T10 Ultra-Turrax® (IKA-Werke, Staufen, Germany) [7]. The homogenate was centrifuged at 16 000 g for 10 min and supernatant aspirated. The pellet (<10% of the weight of Vivostat PRF) was dispersed in the same volume T-PER. This homogenate was centrifuged, and three parts of this supernatant was mixed with four parts of the first PRF extract. Samples were kept in the cryogenic vials (Greiner Bio-One) at −80°C for later analysis. This method was validated using TGF-β1 as prototypic GF. About 90% of the endogenous TGF-β1 content was present in the first

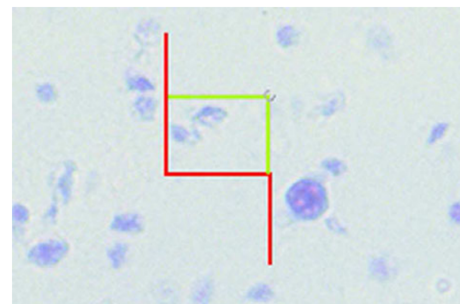

Fig. 2 Representative paraffin section (70 μm) stained with Giemsa for stereologic quantification of platelets in Vivostat PRF. The red line indicates the exclusion line, while the green line the inclusion line. In this example, the two platelets inside the counting frame (31·3 μm^2) were counted, while the one platelet inside the counting frame hitting the red exclusion line was not counted.

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Vivostat PRF extract. Furthermore, 30 ng of active rhTGF-β1 (R&D Systems, Minneapolis, MN, USA) added to Vivostat PRF aliquots (~0·20 g) was completely recovered. Finally, addition of an EDTA-free proteinase inhibitor cocktail to T-PER had no effect on the measured TGF-β1 levels.

Enzyme-linked immunosorbent assay (ELISA) and total protein analyses

bFGF (HSFB00D), PDGF-AB (DHD00B), PDGF-BB (DBB00), TGF-β1 (DB100B), VEGF (DVE00) and MMP-9 (DY911) were determined in duplicate using enzyme-linked immunosorbent assay kits from R&D Systems. Total TGF-β1 levels were determined after treatment of the samples with 1 N hydrogen chloride. Detection limits for the GFs were 0·03, 1·7, 15, 4·6 and 9·0 pg/ml according to the manufacturer. The total protein was determined using the Bradford method with bovine serum albumin standard. GF and MMP-9 concentrations were expressed in pg/ml or pg/mg total protein content of Vivostat PRF extract or serum.

Statistical analyses

The mean of the two Vivostat fibrin-I and PRF samples from each participant was compared with whole blood and serum with paired *t*-test. In addition, Pearson correlation coefficients (*r*) between the platelet and GF measurements of the two Vivostat fibrin-I and PRF samples from each participant were calculated using R version 3.0 (The R Foundation for Statistical Computing, Vienna, Austria). $P < 0{\cdot}05$ was considered statistically significant. Numerical data are given as mean ± standard deviation (SD) unless stated otherwise.

Results

Whole blood and Vivostat fibrin-I analyses

The mean whole blood and the intermediate soluble fibrin-I counts from the ten donors are presented in Table 1. The number of leucocytes and erythrocytes was significantly reduced in fibrin-I compared with whole blood counts. In contrast, the impedance platelet count in fibrin-I was increased ($P < 0{\cdot}001$) 4·4-fold compared with the whole blood [15]. Fibrin-I platelet counts correlated strongly ($r = 0{\cdot}997$, $P < 0{\cdot}001$). Platelet clumping was absent in all blood samples but occurred in five of the 10 fibrin-I samples. Importantly, this did not have a significant ($P = 0{\cdot}71$) impact on platelet counts in fibrin-I. The mean platelet volume in the Vivostat fibrin-I acidic solution was increased by 9·3% ($P < 0{\cdot}05$) compared with whole blood (Table 1).

Table 1 Sysmex measurements of anticoagulated whole blood and Vivostat fibrin-I

	Whole blood (n = 10)	Fibrin-I (n = 10)
Leucocytes (10^9/l)	5·56 ± 1·02	0·27 ± 0·30***
Erythrocytes (10^{12}/l)	5·06 ± 0·38	0·09 ± 0·06***
Platelets (10^9/l)	253 ± 47	1128 ± 407***,[a]
Mean platelet volume (fl)	10·7 ± 0·7	11·7 ± 1·2*

[a]Fibrin-I samples (n = 5) with platelet clumping: 1179 ± 468 × 10^9 platelets/l.
Fibrin-I versus whole blood: *$P < 0{\cdot}05$, ***$P < 0{\cdot}001$.

Vivostat and Choukroun's PRF analyses

The platelet concentration in Vivostat PRF was 982 ± 206 × 10^9/l in the ten individuals as quantified by stereological analysis of Giemsa-stained tissue sections. This concentration is 3·9 times higher ($P < 0{\cdot}001$) than in whole blood.

The concentration of bFGF in Vivostat PRF was increased 75 times ($P < 0{\cdot}001$) compared with that in serum of the same individuals. PDGF-AB, the major PDGF isoform in human serum, was increased 2·5-fold ($P < 0{\cdot}01$), while the PDGF-BB concentration was 1·6-fold or 60% higher ($P < 0{\cdot}05$) in Vivostat PRF compared with serum. The total level of TGF-β1 was 5·1-fold higher ($P < 0{\cdot}001$) in Vivostat PRF compared with serum. Of the total TGF-β1 content in Vivostat PRF, 1-2% was endogenously active. Active TGF-β1 was undetectable in serum. The level of the angiogenic factor VEGF did not differ significantly ($P = 0{\cdot}30$) between Vivostat PRF and serum. Correlation coefficients between the two Vivostat PRF samples from the ten individuals were 0·988 for bFGF, 0·990 for PDGF-AB, 0·944 for PDGF-BB, 0·977 for TGF-β1 and 0·971 for VEGF; all $P < 0{\cdot}001$. The data for TGF-β1 are shown in Fig. 3. MMP-9 levels were lowered 139 times ($P < 0{\cdot}001$) in Vivostat PRF compared with serum (Table 2). The difference between Vivostat PRF and serum levels of the five GFs and MMP-9 was more pronounced when normalized to the total soluble protein content (Fig. 4). Statistical analyses using nonparametric Wilcoxon test yielded the same conclusions for all comparisons between Vivostat PRF and serum.

Electrophoretic analysis indicated that the distribution and amount of major proteins were similar in serum and Vivostat PRF extract (Fig. 5).

MMP-9 was also measured by gelatin zymography, which monitors functional activity of the different MMP-9 molecular species. This analysis confirmed the ELISA results of considerably reduced MMP-9 activity in Vivostat PRF compared with serum (Fig. 6a). Conversely,

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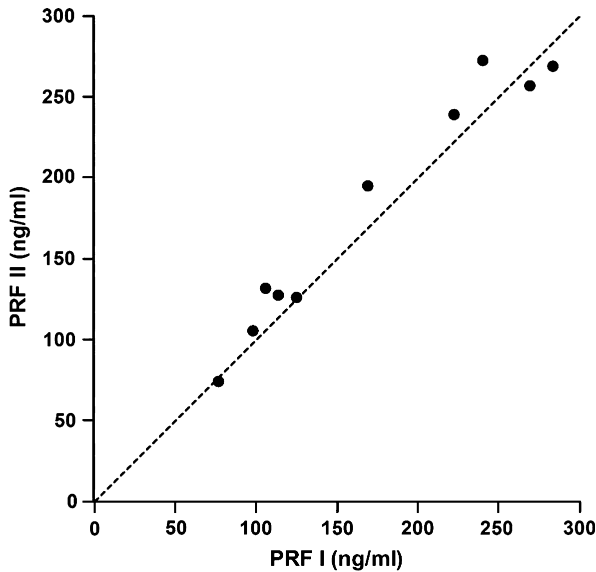

Fig. 3 TGF-β1 concentration of the two Vivostat PRF samples (PRF I and PRF II) from the ten donors. The dotted line indicates the ideal outcome ($r = 1.000$).

Table 2 Growth factor and proteinase (MMP-9) concentrations (pg/ml) in serum and in tissue extracts of Vivostat PRF

	Serum		
Analyte	Reference[a]	Present study ($n = 10$)	Vivostat PRF ($n = 10$)
bFGF	0·776	0·81 ± 0·46	61·0 ± 30·5***
PDGF-AB	20 126	18 600 ± 2400	47 300 ± 21 200**
PDGF-BB	3478	3610 ± 1180	5770 ± 3130*
TGF-β1	39 592	34 500 ± 4700	175 000 ± 75 800***
VEGF	220	339 ± 123	406 ± 141
MMP-9	436 000	702 000 ± 88 300	5050 ± 560***

[a]bFGF determined by R&D Systems in 30, PDGF-AB in 31, PDGF-BB in 65, TGF-β1 in 23, VEGF in 37 and MMP-9 in 37 apparently healthy volunteers.
Vivostat PRF versus serum: *$P < 0.05$, ** $P < 0.01$, ***$P < 0.001$.

Choukroun's PRF membrane contained substantially more MMP-9 than serum from the same three donors (Fig. 6b).

Discussion

This is the first study that has quantified the number of platelets in the final Vivostat PRF product, and its total content of growth factors (GFs) and one proteinase (MMP-9) involved in wound healing.

We found that Vivostat PRF was enriched by platelets by a factor of 3·9 compared with whole blood. This value is comparable to that of the intermediate Vivostat fibrin-I (4·4×) when taking into account the dilution by the pH 10 buffer (0·875×) at the *in situ* formation of Vivostat PRF

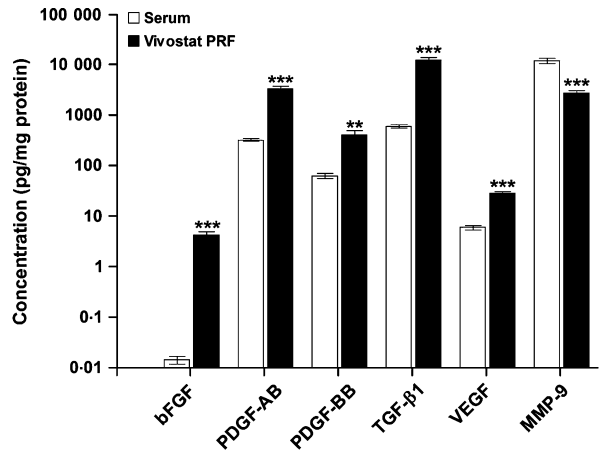

Fig. 4 Growth factor and proteinase (MMP-9) concentrations in sera and Vivostat PRF extracts from the ten healthy donors normalized to the total soluble protein content of the serum (58·9 ± 9·5 mg/ml) and PRF extract (1·9 ± 0·2 mg/ml) samples. Error bars, standard error of the mean. ** $P < 0.01$, ***$P < 0.001$.

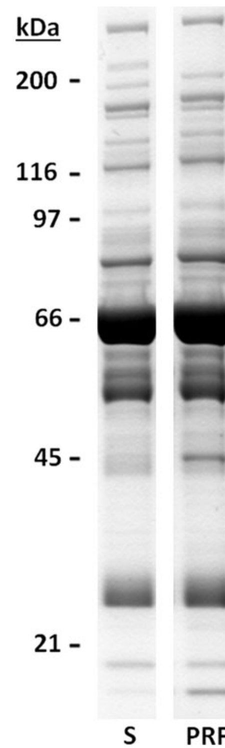

Fig. 5 Serum (S) and Vivostat PRF extract (PRF) at 5 μg total proteins per lane were electrophoresed on NuPAGE® 4–12% Bis-Tris gel under non-reduced conditions at constant 200 V (Life Technologies, Carlsbad, CA, USA). The gel was stained with Colloidal Blue (Life Technologies).

[12]. It should be emphasized that the morphology of the counted platelets was not assessed. However, previous ultrastructural and bioactivity studies attest to the fact that the platelets in Vivostat PRF are minimally activated or degranulated [7, 12].

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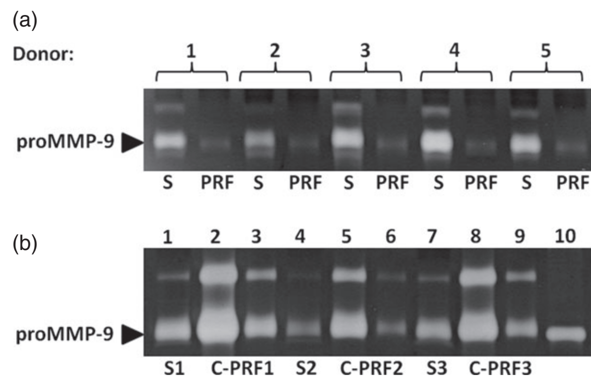

Fig. 6 MMP-9 activity in serum, Vivostat PRF and Choukrouns PRF membrane by zymography using 10% gels containing 1 mg/ml gelatin, reagents and instructions from Life Technologies [31]. In principle, gelatin zymography detects all molecular forms of MMP-9 that are separated electrophoretically according to molecular weights. The substrate (gelatin) incorporated in the gel is degraded by active as well as latent MMP-9 in the incubation step that follows (40 h at 37°C). After staining gelatin with Colloidal Blue, the gelatinolytic action of MMP-9 is seen as clearings. (a) Sera (S) and Vivostat PRF extract (PRF) from five (1–5) donors were loaded with 7·5 μg total proteins per lane. (b) Sera (S) and Choukrouns PRF membrane extract containing ~20 mg/ml of proteins (C-PRF) from three donors (1–3). Lanes 1, 2, 4, 5, 7 and 8: 7·5 μg total proteins loaded; 3, 6 and 9: 0·75 μg total proteins loaded; 10: 50 pg rhMMP-9.

Furthermore, total levels of bFGF, PDGF-AB, PDGF-BB and TGF-β1 were significantly increased in Vivostat PRF compared with serum. It is difficult to comment on the content of GFs in Vivostat PRF in relation to other platelet concentrate products [16–18] although a survey of nine different commercial systems showed that Vivostat PRF had the third highest PDGF-AB content [5]. We would rather advocate parallel measurements of serum that would account for analytical variations for benchmarking purposes. The variability of GF levels in Vivostat PRF was comparable to other platelet concentrates [16–18] and was seemingly largely due to inherent differences among the donors.

The concentration of TGF-β1 was 5·1 times higher in Vivostat PRF than in serum and was the most abundant GF. This could be explained by the relative abundance of TGF-β1 in platelets compared with other cells [19, 20]. The measured TGF-β1 includes intracellular and fibrin-bound TGF-β1 in latent as well as in active forms. We could document that TGF-β1 present in any of these forms in Vivostat PRF were recovered and detected by the developed analytical methods. TGF-β1 is secreted as a latent complex [19, 21]. Notably, 1-2% of the total TGF-β1 content in Vivostat PRF was endogenously active. One caveat is that this active TGF-β1 was possibly derived from latent TGF-β1 activated during the preparation of the Vivostat PRF extract [19].

Our findings may have pharmacokinetic relevance. In a randomized clinical trial, local TGF-β1 levels were still increased twofold 10 days after a single application of Vivostat PRF to the wound site compared with topical application of albumin [10]. This suggests that Vivostat PRF provides increased levels of TGF-β1 and possibly other GFs over an extended treatment period. Experiments in mice have clearly shown that sustained release of GFs by non-activated platelets is superior to instant delivery of the GF bolus from thrombin-activated platelets [22]. In patients, Vivostat PRF treatment for excised chronic pilonidal sinus disease also accelerated wound closure compared with autologous thrombin-activated platelets [8].

In another randomized clinical trial, topical Vivostat PRF attenuated type I collagen synthesis and deposition by the wound fibroblasts [10]. One possible explanation for this intriguing finding is that the TGF-β1 activity was antagonized by bFGF derived from Vivostat PRF leading to the antifibrotic effect [23]. The bFGF concentration in Vivostat PRF was also increased the most relative baseline sera perhaps due to its high affinity to fibrin(ogen) [24] that is increased about sixfold in Vivostat PRF [7, 10]. Actually, de la Puente *et al.* [25] showed that bFGF combined with fibrin decreased collagen formation by dermal fibroblasts compared with fibrin alone. This example illustrates the complexity in the regulation of wound healing.

The proteinase MMP-9 is another important wound-healing factor. The level of MMP-9 is critical for wound repair, and deficiency in as well as excessive MMP-9 is detrimental [26, 27]. Furthermore, delayed healing of diabetic wounds was due to increased MMP-9 activity [28]. MMP-9 was markedly decreased in Vivostat PRF. In contrast, the leucocyte-rich Choukroun's PRF membrane contained massive amounts of MMP-9. These results agree with a recent study concluding that platelets alone are devoid of MMP-9, but leucocyte contamination proportionally increases the MMP-9 content [29]. The clinical consequences of MMP-9 supplementation are unknown but will probably depend on the type and stage of the wound.

Admittedly, we quantified only six specific proteins, albeit crucial for wound healing, of the more than 1100 known platelet proteins [30].

In summary, we have characterized Vivostat PRF derived from healthy individuals in terms of platelet numbers in addition to some GFs and one proteinase involved in wound healing. This initial knowledge is important for the proper use of Vivostat PRF although the results should be reproduced in the actual patient categories treated with the product.

Acknowledgements

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performed the growth factor/MMP analyses and wrote the manuscript; Karina Rasmussen was responsible for the haematological analyses by the Sysmex instrument; Dr. Pakkenberg supervised the stereologic analyses and interpretations; and Dr. Jørgensen was in charge of blood sampling and generation of Vivostat products.

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