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Growth factors and cytokines in autologous platelet concentrate and their correlation to periodontal regeneration outcomes

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***Abstract*:**

Aim: To determine the concentration of naturally available biologic mediators in autologous platelet concentrates and their correlation with periodontal regeneration outcomes.

Material and methods: In 25 patients with two intra-bony defects each, an autologous platelet concentrate (APC) was prepared by a laboratory thrombocyte apheresis technique pre-operatively. Both defects were treated using a bioresorbable guided tissue regeneration-membrane in combination with tricalciumphosphate (TCP). In the test defect, APC was additionally applied. In the APC, platelets were counted and the levels of growth factors and cytokines were determined by ELISA. Correlations between the platelet counts or the growth factor/cytokine levels and the potential clinical and radiographic regeneration outcomes due to APC were calculated after 3, 6, and 12 months.

Results: The APC contained 2.2×10^6 platelets/μl, which was 7.9 times more than in the venous blood. Transforming growth factor-β1 (TGF-β1), insulin-like growth factor-I (IGF-I), platelet-derived growth factor-AB (PDGF-AB), PDGF-BB, vascular endothelial growth factor (VEGF), and epidermal growth factor (EGF) were found in the APC, whereas interleukin-1β (IL-1β), IL-6, tumor necrosis factor α (TNFα), IL-4, and IL-10 were not detectable. The regression analysis showed a weak correlation between the platelet counts or the growth factor levels and the clinical and radiographic regeneration outcomes ($r^2 \leqslant 0.4$).

Conclusion: Autologous platelet concentrate contains relatively high concentrations of PDGF-AB, PDGF-BB, TGF-β1, and IGF-I, but their potential influence on periodontal regeneration remains unclear.

Keywords: apheresis; clinical trials; cytokines; growth factors; periodontal therapy; platelet concentrate; platelets; regeneration

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In the recent years, increased efforts have been focused on understanding the mechanisms of and factors required for restoring periodontal tissues in order to increase the predictability of regenerative therapy. The basic cellular mechanisms of periodontal regeneration are proliferation and migration of periodontal ligament (PDL) cells, the differentiation of cementoblasts and osteoblasts and the synthesis of extracellular matrix (ECM) (Bartold et al. 2006, Polimeni et al. 2006). These events are controlled by biological mediators like growth factors, morphogenetic proteins, ECM proteins, and others, which are produced by monocytes, platelets, and resident tissue cells like PDL cells, osteoblasts, cementoblasts, and endothelial cells. Furthermore, the process of periodontal regeneration also involves inflammatory aspects (Slots et al. 1999). Several studies have shown that periodontal regeneration may be enhanced by the therapeutic application of specific growth factors (Cochran & Wozney 1999,

Mumford et al. 2001, Camelo et al. 2003, Marcopoulou et al. 2003, Nevins et al. 2003, Sarment et al. 2006). However, important questions, like selection or a combination of certain growth factors, their right concentrations, or the selection of an appropriate carrier system, still need to be resolved. Furthermore, so far, there are no recombinant human growth factors readily available for clinical use.

As a natural source, platelets contain a number of different growth factors including platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), and insulin-like Growth Factor (IGF) (Gawaz 2001). Although a positive effect of platelets on osteoblasts has been observed in vitro (Gruber et al. 2002, Weibrich et al. 2002b), others could not detect any influence of platelet concentrates on bone regeneration in animal studies (Aghaloo et al. 2002). Applying an autologous platelet concentrate (APC) in combination with autologous bone grafts for the treatment of mandibular continuity defects in 44 patients, Marx et al. (1998) found histologically and radiographically an accelerated new bone formation and bone density gain after 6 months compared with control defects without using APC. Furthermore, some case studies reported promising results using APC in alveolar ridge and sinus augmentation procedures (Kassolis et al. 2000, Robiony et al. 2002, Maiorana et al. 2003, Kassolis & Reynolds 2005). Kim et al. (2002) suggested the additional use of APC to accelerate the osseointegration of titanium implants, whereas Weibrich et al. (2004) could not show any additional benefit.

Basic data and exhaustive studies on thrombocyte growth factor levels in a platelet concentrate are lacking. So far, only a limited number of controlled studies on the biological effects of APC have been published (Tayapongsak et al. 1994, Marx et al. 1998, Aghaloo et al. 2002, Weibrich et al. 2002b, Jensen et al. 2005). Furthermore, to date, only very few studies have reported on the application of a platelet concentrate in regenerative periodontal therapy (Camargo et al. 2002, Lekovic et al. 2002, Okuda et al. 2005). And, to the best of our knowledge, there are no studies in the literature analysing the isolated influence of APC on periodontal regeneration outcomes. Although some data exist on growth factor levels in APC prepared by different methods (Aghaloo et al. 2002, Weibrich et al. 2002a, c, d, 2003a, b), no information is available correlating these levels with the corresponding hard and soft tissue healing outcomes.

APCs were reported to also contain inflammatory cytokines, e.g. interleukin-1β (IL-1β), tumor necrosis factor (TNF), and IL-6 (Wadhwa et al. 2000, Edvardsen et al. 2001, Addas-Carvalho et al. 2004). Possibly, these cytokines may influence the regeneration process, either as pro-inflammatory or anti-inflammatory mediators. Pro-inflammatory cytokines like IL-1β, IL-6, or TNFα are closely associated with disease onset and progression by inducing and enhancing bone resorption (Okada & Murakami 1998). In contrast, antiinflammatory cytokines like IL-4 and IL-10 are known to suppress the synthesis and secretion of proinflammatory cytokines (Alexander & Damoulis 1994, Gorska et al. 2003). Although proinflammatory cytokines were detected in platelet concentrates (Wadhwa et al. 2000, Edvardsen et al. 2001, Addas-Carvalho et al. 2004), anti-inflammatory cytokines have not yet been examined.

This paper describes the laboratory analysis accompanying a randomized clinical prospective split-mouth study on the influence of APC on periodontal regeneration in intra-bony defects (Christgau et al. 2006). The aim of the present study was to determine the concentrations of growth factors and inflammatory cytokines detectable in APCs and to correlate the analytical results with the platelet counts as well as with the potential clinical and radiographic regeneration effects due to the APC application found in the clinical split-mouth study.

Materials and Methods

Study design

The clinical study design and the procedure of patient selection has been reported in detail elsewhere (Christgau et al. 2006). In brief, each of the 25 systemically healthy patients showed one pair of contra-laterally located deep intra-bony, inter-proximal periodontal defects. Applying the split-mouth design, the effect of additional application of APC on the regeneration outcome following guided tissue regeneration (GTR) therapy was analysed.

Preparation of the APC

The thrombocytes were collected using an apheresis technique at the Division of Transfusion Medicine at the Institute of Laboratory Medicine and Clinical Chemistry, University Clinic of Regensburg. All platelet-apheresis procedures were performed with the Spectra cell separator (Cobe BCT, Lakewood, CO, USA) using the software version 5.1 and the platelets standard program with a leucocyte reduction system (LRS). The cell separator was set in accordance with the manufacturer's recommendations and controlled by the German regional authorities according to the German law of Medicine Products. All patients fulfilled the German national criteria for blood donation (Wissenschaftlicher Beirat der Bundesärztekammer & Paul Ehrlich Institut 2000). They were eligible for platelet-apheresis, if the platelet count before apheresis was higher than $150 \times 10^3/\mu$l and if a suitable venous access was available. All procedures were performed using dual-needle sets. The aphereses were processed at a flow rate of 50–80 ml/min. The ratio of acid-citrate-dextrose (ACD) to blood was held constant throughout the procedures at a 1:9 ratio without any additional systemic anticoagulation, e.g. heparin. According to the recommendation of the German Society of Blood Transfusion and Immunohematology (DGTI) (Deutsche Gesellschaft für Transfusionsmedizin und Immunhämatologie 1998), each platelet collection was stopped when either the fourfold amount of whole blood volume was processed or the time for thrombocyte-apheresis reached a maximum of 5 h. The apheresis was continued until adequate numbers of at least 10^{10} thrombocytes were collected. The volume of platelet concentrates was adjusted to 50 ml.

Under sterile conditions, the platelet concentrate was carefully transferred to sterile Falcon tubes (Falcon Blue Max 50 ml, Becton Dickinson Labware, Franklin Lakes, NJ, USA) using a sampling site coupler (Baxter-Fenwal, Dearfield, IL, USA) and pink canules (diameter 1.2 mm) at a pulling rate of about 10 ml/min. Short-term storing was obtained in a platelet incubator (Helmer Inc., Noblesville, NJ, USA) at 22°C. Finally, 2.5 ml of the platelet concentrate was used for clinical application (Christgau et al. 2006), and about 30 ml of the platelet concentrate was prepared for laboratory analyses as described below.

Clinical study

For clinical use, the platelet concentrate was reactivated in the following way: 2.5 ml of the platelet concentrate was transferred from the Falcon tube to a 5-ml syringe. In a 6:1 ratio, 0.5 ml of a sterile 10% calciumchloride ($CaCl_2$) solution was added in order to compensate the anticoagulating citrate effect. A preliminary experiment showed that this 6:1 ratio corresponded to the minimal amount of $CaCl_2$ inducing the clotting of the platelet concentrate.

All surgeries were performed by one surgeon (M. C.) according to the principles of GTR. After thorough defect debridement and root-conditioning with 24% ethylenediamine tetraacetic acid (EDTA)-Gel (PrefGel, Straumann, Freiburg, Germany), in the test defects reactivated APC was applied onto the root surfaces. Then, the defects were filled with β-tricalcium phosphate (TCP) granules (Ceros, Mathys, Bettlach, Switzerland), which were also soaked with APC for at least 30 min. before application. Finally, the defects were covered with a bioresorbable GTR membrane (Resolut XT, W.L. Gore, Flagstaff, AZ, USA). Control defects were treated in the same way, but without application of APC. In control sites, TCP granules were soaked with patient's blood instead of APC.

For the correlation analysis, in the present study, the clinical regeneration outcome was assessed by using the vertical relative attachment gain (V-rAG) (Christgau et al. 2006) after 3, 6, and 12 months. Hard tissue changes in the defect area after 3, 6, and 12 months were assessed by the changes in bone density (corrected mean grey level) and the area of bone density changes using quantitative digital subtraction radiography (Christgau et al. 1998, 2006). For extrapolation of the additional benefit of APC, the differences in the clinical and radiographic parameters between test and control sites were calculated for further analysis.

Analysis of the APC

Platelet counts

Platelet and leucocyte numbers in the venous blood samples as well as in the platelet concentrates were determined using a routine automatic counting system (ADVIA 120, Bayer, Leverkusen, Germany).

Preparation of APC samples for further analysis

About 30 ml of the original platelet concentrate was used for the laboratory analysis. The samples were aliquoted under sterile conditions using a hood (HS 12/2, Heraeus Instruments, Hanau, Germany) and stored at $-86^{\circ}C$ ($-86^{\circ}C$ Freezer, Model 923, Forma Scientific Inc., Marjetta, OH, USA).

Determination of growth factor and cytokine levels

For each patient, the samples were analysed for the following growth factors and cytokines using commercially available ELISA kits (Quantikine, R&D Systems, Minneapolis, MN, USA): PDGF-AB (Cat. No. DHD00B), PDGF-BB (Cat. No. DBB00), TGF-β1 (Cat. No. DB100), IGF-I (Cat. No. DG100), vascular endothelial growth factor (VEGF: Cat. No. DVE00), epidermal growth factor (EGF: Cat. No. DEG00), IL-1β (Cat. No. DLB50), IL-6 (Cat. No. D6050), TNFα (Cat. No. DTA00C), IL-4 (Cat. No. D4050), and IL-10 (Cat. No. D1000B). Immediately before analysis, the aliquots were thawed at room temperature and cytolysis was performed by ultrasonics (Sonorex Super RK512H, Bandelin electronic, Berlin, Germany) at 3×30 s.

In a preliminary experiment, the different ELISA kits were validated in measuring their respective growth factors and cytokines in the platelet concentrates and the appropriate dilutions were determined. In contrast to the manufacturer's recommendations, the following dilutions had to be used: PDGF-AB, $\times 100$ (instead of $\times 50$); TGF-ß1, $\times 600$ (instead of $\times 30$); and VEGF, $\times 2$ (instead of $\times 1$).

The measurements of the growth factor and cytokine levels were performed according to the manufacturer's instructions. The standard and sample solutions were pipetted on a 96-well plate. After the corresponding incubation time, the 96-well plate was washed automatically (Columbus Microplate Strip Washer, Tecan Group Ltd., Maennedorf, Switzerland) with a prepared washing buffer. Afterwards, a solution of corresponding polyclonal antibodies (conjugated to horseradish peroxidase) was added and incubated. After another washing procedure, the substrate solution was added to start the enzyme-catalysed reaction, which was stopped by the addition of a stop solution. The concentrations of the growth factors and cytokines were photometrically determined using the Tecan Sunrise photometer (Tecan Group Ltd.). Standard dilutions of isolated growth factor and cytokine solutions were used to create calibration curves. For each patient, all measurements were performed in duplicate. Only data with a variation coefficient of less than 15% were used for further evaluation. Otherwise, the analysis was repeated until the variation coefficient between the duplicate measurements was $<15\%$. The actual growth factor and cytokine concentrations were calculated by the optical densities using the calibration curves and the Table Curve 2D software, version 4.0 (Systat Software Inc., Point Richmond, CA, USA).

Correlation analysis

Correlations between the number of platelets in the platelet concentrate or the concentrations of the growth factors (PDGF-AB, PDGF-BB, TGF-ß1, IGF-I, VEGF, and EGF) and inflammatory cytokines (IL-1β, TNFα, IL-6, IL-4, IL-10) on the one hand, and the differences of the clinical (V-rAG) and radiographic (amount and area of bone density changes) healing outcomes between test and control sites on the other were calculated using simple regression analysis (Software Sigma Plot 2002 for Windows, Version 8.0, Systat Software Inc.; second-order graph). The regression coefficient r^2 was determined.

Results

In the following, only the median values are reported.

Analysis of the APC

The analytical results of the APC are reported in Tables 1 and 2.

Number of platelets

The venous blood contained 276×10^3 platelets/μl, whereas in the APC 2163×10^3 platelets/μl were found. This complies with an enrichment factor of 7.9 (Table 1). The APC was contaminated with a maximum of 2×10^3 leucocytes/μl.

Growth factor levels

The following growth factors could be detected in the APC: PDGF-AB, PDGF-BB, TGF-β1, IGF-I, VEGF, and EGF. Their concentrations are reported in Table 2.

Cytokine levels

In the present study, none of the investigated cytokines revealed a concentration above the detection limits (i.e. IL-1β: 3.9×10^{-3} ng/ml; IL-6: 3.12×10^{-3} ng/ml, TNFα: 15.6×10^{-3} ng/ml, IL-4: 31.2×10^{-3} ng/ml, IL-10: 7.8×10^{-3} ng/ml) of the ELISA tests used.

Regeneration outcomes

The clinical and radiographic healing results of this study have been reported in detail elsewhere (Christgau et al. 2006). In this paper, they are mentioned only as far as necessary for the understanding of the correlation analyses.

As shown in Table 3, the test and control defects showed a V-rAG of more than 70% after 3, 6, and 12 months. Clinically, no significant differences could be found between test and control sites.

The evaluation of hard tissue changes by digital subtraction radiography after 3, 6, and 12 months revealed a statistically significant amount and area of bone density gain in test and control sites compared with the baseline situation (Table 3). Only after 6 months, the test sites showed a significantly higher increase in bone density compared with the control sites.

The differences between the test and control sites for the investigated clinical and radiographic parameters are reported in Table 4. Although there seems to be a slight tendency for better healing results in favour of the test procedure after 3 and 6 months (positive median values) and in favour of the control procedure after 12 months (negative median values), the data show a great variability and do not reveal a clear superiority of one of the procedures.

Table 1. Median value (with 25/75-percentiles) and mean value (with standard deviation, SD) of the platelet counts in venous blood (blood), autologous platelet concentrate (APC), and its enrichment factor ($n = 25$)

	Blood (10^3 platelets/μl)	APC (10^3 platelets/μl)	Enrichment factor
Median	276	2163	7.9
25/75%-ile	225/320	1527/2671	5.8/9.5
Mean ($\pm$ SD)	273 ($\pm$ 56)	2134 ($\pm$ 782)	7.8 ($\pm$ 2.5)

Table 2. Median value (with 25/75-percentiles) and mean value (with standard deviation, SD) of the concentration of platelet-derived growth factor-AB (PDGF-AB), platelet-derived growth factor-BB (PDGF-BB), transforming growth factor-β1 (TGF-β1), insulin-like growth factor-I (IGF-I), vascular endothelial growth factor (VEGF), and epidermal growth factor (EGF) in the autologous platelet concentrate (APC) ($n = 25$)

	PDGF-AB (ng/ml)	PDGF-BB (ng/ml)	TGF-β1 (ng/ml)	IGF-I (ng/ml)	VEGF (ng/ml)	EGF (ng/ml)
Median	36.3	14.4	301.7	76.7	0.4	1.7
25/75%-ile	26.2/52.6	10.9/20.7	217.5/430.3	62.6/93.8	0.1/0.6	1.2/2.4
Mean ($\pm$ SD)	39.0 ($\pm$ 18.1)	15.8 ($\pm$ 7.9)	331.6 ($\pm$ 155)	78.1 ($\pm$ 21.5)	0.4 ($\pm$ 0.4)	1.8 ($\pm$ 1.0)

Table 3. Clinical and radiographic results: median value (with 25/75-percentiles) and mean value (with standard deviation, SD) of the vertical relative attachment gain (V-rAG), the bone density gain (corrected mean grey-level Δ (ER-CR)), and the relative area of radiographic bone density changes in the defect (rel. area) in test and control sites after 3, 6, and 12 months ($n = 25$).

	Test sites			Control sites		
	V-rAG (%)	Δ (ER-CR)	rel. area (%)	V-rAG (%)	Δ (ER-CR)	rel. area (%)
Changes after 3 months						
Median	83.3	6.0†	84.5†	75.0	6.0†	78.3†
25/75%-ile	64.6/100.0	0.5/22.0	57.8/96.2	53.5/88.9	1.0/13.5	63.7/89.3
Mean ($\pm$ SD)	82.1 ($\pm$ 25.1)	10.4 ($\pm$ 13.1)	80.6 ($\pm$ 37.6)	72.4 ($\pm$ 30.3)	6.9 ($\pm$ 7.2)	81.6 ($\pm$ 27.3)
Changes after 6 months						
Median	71.4	8.5*†	80.8†	70.0	7.0*†	73.8†
25/75%-ile	61.2/88.7	0.8/20.5	55.8/102.3	50.0/87.5	2.0/10.0	61.7/87.1
Mean ($\pm$ SD)	73.3 ($\pm$ 19.4)	11.8 ($\pm$ 12.9)	81.4 ($\pm$ 32.9)	70.9 ($\pm$ 28.4)	6.5 ($\pm$ 5.7)	72.3 ($\pm$ 26.2)
Changes after 12 months						
Median	71.4	8.0†	77.9†	71.4	10.0†	80.7†
25/75%-ile	62.5/81.6	$-$ 1.0/20.3	67.9/100.0	55.6/85.4	5.0/16.0	67.9/100.0
mean ($\pm$ SD)	70.1 ($\pm$ 15.8)	9.7 ($\pm$ 12.3)	80.6 ($\pm$ 19.9)	70.6 ($\pm$ 21.8)	11.1 ($\pm$ 8.0)	79.5 ($\pm$ 23.8)

Test sites EDTA+TCP+APC+GTR.
Control sites EDTA+TCP+GTR.
Wilcoxon' signed-rank test ($\alpha = 0.05$).
*Statistically significant differences between test and control defects.
†Statistically significant change compared with baseline.

Correlation analysis

Scatter plots of platelet counts of each patient *versus* their corresponding growth factor levels showed a weak correlation ($r^2 \leqslant 0.4$). The same was true for scatter plots of the platelet counts *versus* the differences of the clinical and radiographic parameters between the test and control sites ($r^2 \leqslant 0.3$). Furthermore, there was a weak correlation between the individual APC growth factor levels of each patient and differences of the clinical and radiographic healing outcomes between the test and control sites ($r^2 \leqslant 0.4$) (Table 5). Figure 1 shows an example of a scatter plot illustrating the weak correlation between the platelet count and the difference in V-rAG between the test and control sites. The cytokine levels found in the present study were excluded from the correlation analyses because they were too low and below the detection limits of the ELISA kits used.

Discussion

Discussion of the methods

In the present study, the APC was produced by a closed apheresis system applying a continuous cell separation procedure under optimally standardized conditions. In this way, the platelets were extracted, while almost all other blood components were reinfused in contrast to other commercially available chairside systems. Thus, the procedure used in the present study was less invasive for the patient and enabled a minimal contamination with leucocytes and erythrocytes. Compared with chairside systems, higher platelet counts could be achieved (Weibrich et al. 2003b).

The APC aliquots for analysis were frozen at -86°C. According to previous studies, the freezing process had no influence on the growth factor levels in platelet concentrates (Sekido et al. 1987). Others used freezing as a method of platelet lysis to facilitate the release of growth factors (Ito et al. 1993). Repeated freeze–thaw cycles were avoided in order to prevent negative influences on the expression of cytokines as reported by Kvarnstrom et al. (2004).

As suggested by other studies (Loppnow et al. 1998, Gruber et al. 2002), cytolysis of the platelets was achieved by ultrasonics. In contrast, Weibrich et al. (2002a, c, d, 2003a) and Landesberg et al. (2000) centrifuged their samples for cytolysis. Okuda et al. (2003) avoided any pre-treatment of the APC before analysis.

According to previous studies (Landesberg et al. 2000, Gruber et al. 2002, Weibrich et al. 2002c, d, 2003a, Okuda et al. 2003), the growth factor and cytokine levels were determined by using commercially available ELISA kits. In a preliminary assessment, the appropriate solutions had to be determined. The optical densities caused by the growth factor and cytokine concentrations in the APCs had to be located in the linear part of the standard curves. Protein detection by ELISA does not necessarily reflect only the levels of biologically active cytokines. It often also detects degraded proteins, aggregated or precursor forms, and the results can be affected by the presence of soluble receptors, antagonists, binding proteins, and other non-specific inhibitory molecules (Thorpe et al. 1992).

In the past, several studies reported the potential influence of some growth factors in periodontal regeneration. A phase I/II-study (Howell et al. 1997) showed a significant increase in bone defect fill after application of a PDGF-BB/IGF-I combination (150 μg/ml) during open flap surgery. Other authors examined the clinical and histological outcomes after application of recombinant PDGF-BB in combination with allogenic bone (Nevins et al. 2003). They reported a reduction in probing pocket depth and a gain in clinical attachment. They found histological

Table 4. Differences of the clinical and radiographic results between test and control sites

	Δ (test–control)		
	V-rAG (%)	Δ (ER-CR)	Rel. area (%)
Changes after 3 months			
Median	8.3	2.0	0.3
25/75%-ile	− 13.4/33.8	− 5.0/13.0	− 22.7/18.3
Mean (± SD)	9.71 (± 32.5)	3.6 (± 14.8)	− 1.5 (± 50.5)
Changes after 6 months			
Median	8.3	4.5	7.6
25/75%-ile	− 23.8/33.4	− 3.8/13.0	− 9.2/39.7
Mean (± SD)	6.9 (± 34.6)	5.6 (± 12.8)	10.2 (± 38.8)
Changes after 12 months			
Median	− 5.5	− 3.0	− 4.5
25/75%-ile	− 20.0/21.5	− 10.8/1.8	− 16.3/1.6
Mean (± SD)	2.0 (± 28,7)	− 3.1 (± 12.8)	− 3.2 (± 25.6)

V-rAG, vertical relative attachment gain.

Table 5. Correlation coefficient r^2 of the correlations between platelet count in the APC (platelet count), growth factor levels (PDGF-AB, PDGF-BB, TGF-β1, IGF-I, VEGF, EGF) and the differences between test and control site of the following clinical and radiographic healing parameters: vertical relative attachment gain (V-rAG), bone density gain (corrected mean grey level, Δ(ER-CR)), relative area of the radiographic bone density changes in the defect (rel. area) after 3, 6, and 12 months

r^2	Platelet count	PDGF-AB	PDGF-BB	TGF-β1	IGF-I	VEGF	EGF
Platelet count	–	0,268	0,409	0.036	0.025	0.227	0.239
V-rAG (months)							
0–3	0.081	0.088	0.027	0.019	0.190	0.041	0.049
0–6	0.113	0.108	0.117	0.191	0.091	0.127	0.073
0–12	0.214	0.042	0.037	0.085	0.326	0.066	0.006
Δ (ER-CR) (months)							
0–3	0.086	0.086	0.074	0.094	0.055	0.097	0.134
0–6	0.002	0.040	0.076	0.061	0.061	0.003	0.105
0–12	0.107	0.077	0.120	0.132	0.037	0.086	0.091
Rel. area (months)							
0–3	0.189	0.312	0.284	0.258	0.010	0.218	0.372
0–6	0.169	0.081	0.096	0.052	0.165	0.007	0.113
0–12	0.286	0.039	0.188	0.194	0.010	0.136	0.096

PDGF-BB, platelet-derived growth factor-BB; TGF-β1, transforming growth factor-β1; IGF-I, insulin-like growth factor-I; VEGF, vascular endothelial growth factor; EGF, epidermal growth factor; APC, autologous platelet concentrate.

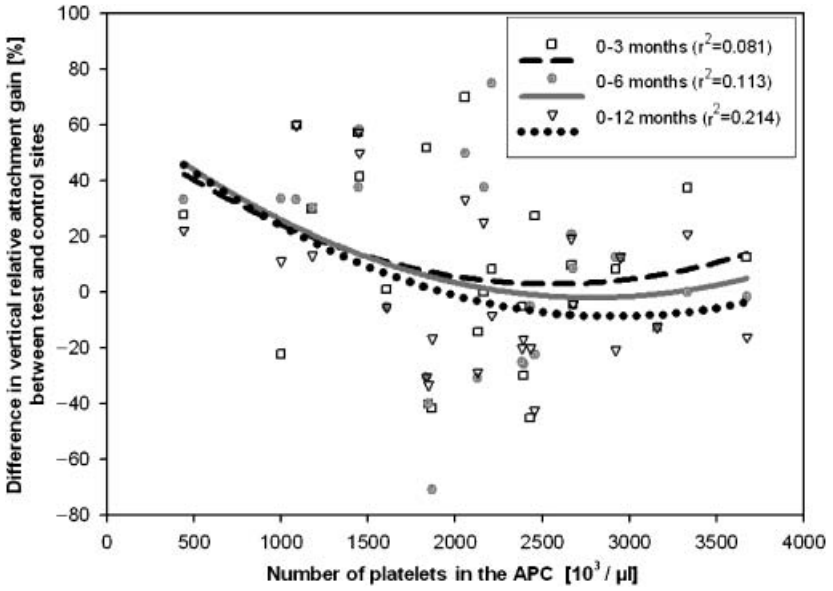

Fig. 1. Correlation (with quadratic regression) between the number of platelets in the autologous platelet concentrate (APC) and the difference in vertical relative attachment gain between the test and control sites.

evidence for regeneration with newly formed cementum, PDL, and alveolar bone in most of the defects. For both factors (PDGF-BB and IGF-I), similar results were found in animal studies (Lynch et al. 1989, 1991, Rutherford et al. 1992, Giannobile et al. 1994, 1996). TGF-β has been tested only in the animal model. Wikesjö et al. (1998) found superior histological healing outcomes in class II furcation defects of beagle dogs following GTR therapy, when 20 μg of recombinant TGF-β was additionally applied. VEGF is expected to promote angiogenesis during osteogenesis. This might also have a positive influence on the periodontal regeneration (Deckers et al. 2000). Only a few studies have dealt with the role of EGF in periodontal tissues. Immunohistochemical examinations in cats have shown the presence of EGF in periodontal tissue after application of orthodontic forces (Guajardo et al. 2000). The authors concluded that EGF may be important in the rebuilding processes of periodontal tissues.

Recently, some authors have reported the existence of inflammatory cytokines (IL-1β, IL-6, IL-8, TNFα) in APC (Muylle et al. 1993, Wadhwa et al. 1996, 1997, 2000, 2002, Edvardsen et al. 2001, Addas-Carvalho et al. 2004). For example, IL-1β, IL-6, and TNFα are known to have proinflammatory effects (Okada & Murakami 1998). In contrast, IL-4 and IL-10 are anti-inflammatory cytokines (Gorska et al. 2003).

Discussion of the results

In the present study, the platelet counts obtained in the venous blood were similar to those reported by other studies (Marx et al. 1998, Weibrich et al. 2002c, d, 2003a, Okuda et al. 2003,) and within the normal range of 150×10^3–300×10^3 platelets/μl (Gawaz 2001). In the APC, 2163×10^3 platelets/μl could be detected. This is far more than in platelet concentrates produced by other laboratory procedures: 1408×10^3 platelets/μl (Weibrich et al. 2002d), and 270×10^3 platelets/μl (Appel et al. 2002). The variability of platelet counts in the present platelet concentrates (441×10^3–3672×10^3 platelets/μl) was in accordance with other studies (Weibrich et al. 2002a, c, d, 2003a, Okuda et al. 2003).

In the APCs, the PDGF-AB levels were by far lower (36.6 ng/ml) than in previous studies. Weibrich et al. (2002d) found a PDGF-AB concentration of 109.6 ng/ml in a platelet concentrate produced by discontinous cell separation. In another study, Weibrich et al. (2002c) found 314.1 ng PDGF-AB per ml with the Curasan PRP Kit and 251.8 ng/ml with the PCCS system. In the present study, the level of PDGF-BB (14.4 ng/ml) was similar to other studies (Weibrich et al. 2002a). In contrast, Weibrich et al. (2002d) found lower PDGF-BB levels of 7.8 ng/ml. The level of IGF-I in the present study (76.7 ng/ml) was similar to the findings of other authors, examining platelet concentrates produced by discontinous cell separation (Weibrich et al. 2002a, d), or by chairside systems (Weibrich et al. 2002c). In the present study, the APC contained 301.7 ng of TGF-β1/ml. The TGF-β1 levels of previous studies showed a wide scattering. Weibrich et al. (2002a, d) found 221 and 162.3 ng/ml in platelet concentrates produced by discontinous cell separation. In contrast, in platelet concentrates produced by chairside systems, different concentrations had been detected (Curasan PRP-Kit: 79.7 ng/ml; PCCS system: 467.1 ng/ml) (Weibrich et al. 2002c). The EGF levels of the present study (1.7 ng/ml) were far smaller than in the study of Ito et al. (1993), who found 34 ng EGF/ml. Concerning VEGF levels in the APC, no data exist in the literature that could be compared with the present study.

The cytokine levels in the present study were below the detection limit of the ELISA kits used. Wadhwa et al. (1996) detected IL-1β and IL-6 in much higher concentrations in platelet concentrates of different preparation methods than in the present study. Furthermore, Muylle et al. (1993) found increased levels of IL-6, IL-1β, and TNF α in platelet concentrates, but also higher leucocyte counts ($>3 \times 10^3$/μl) than in the present study ($<2 \times 10^3$/μl). In contrast, Edvardsen et al. (2001) detected several bioactive substances like histamine or plasminogen activator inhibitor, but no IL-6. In the present study, the investigated pro- and anti-inflammatory cytokines seem to have no influence on the regeneration outcome. The low cytokine levels in the present study may possibly reflect the high purity of the APC with only slight leucocyte contamination.

The correlation analyses examined the potential dependencies between the growth factor levels and the platelet counts in the APC and the potential clinical and radiographic healing effects of the additional APC application. In line with previous studies on bone regeneration (Weibrich et al. 2002d, Jensen et al. 2005), only a weak correlation could be shown between the platelet counts and the corresponding growth factor levels. Weibrich et al. (2002d) found similar weak correlations between

platelet counts and the levels of PDGF-AB, PDGF-BB, TGF-β1, TGF-β2, and IGF-I. These authors explained their findings as being due to the high individual variability of cellular production and storage of these cytokines. Weibrich et al. (2002d) suggested that the growth factor level of an individual is influenced by several partly still unknown biologic factors. In contrast, in another study (Weibrich et al. 2002c), significant correlations between the platelet counts and PDGF-AB as well as TGF-β1 levels were detected, depending on the APC preparation system. The authors regarded the higher leucocyte number found in that study to be the source of the increased PDGF-AB level. Furthermore, they could not find a correlation between the IGF-I level and the platelet counts.

In the present study, only very weak correlations were found between the potential clinical and radiographic healing effects of APC (shown by the differences between the test and control sites) and the platelet counts or growth factor levels. As this was the first study investigating the potential influence of APC on the periodontal healing outcome, the present results cannot be compared with previous findings (Marx et al. 1998, Anitua 1999, Dugrillon et al. 2002). The weak correlation between the growth factor levels and the healing outcomes may be explained by the following: first, there may have already been sufficient amounts of the investigated growth factors naturally occuring in the periodontal wound. Thus, the therapeutical application might not have an additional effect on the periodontal wound healing. Second, the half-life time of the growth factors might be too short to have any significant influence on the long-term changes of the investigated periodontal healing parameters.

Conclusions

In the present study, APC was applied in deep intra-bony periodontal defects in addition to GTR therapy. The laboratory analysis revealed large amounts of the growth factors PDGF-AB, PDGF-BB, TGF-β1, and IGF-I in theAPC. However, only very weak correlations could be found between the growth factor levels and the potential additional periodontal healing outcomes due to APC. The levels of the inflammatory mediators investigated were below the detection limits. Thus, within the limits of this study, the potential influence of an APC and its growth factors on the periodontal regeneration process remains unclear and needs further investigation.

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Clinical Relevance

Scientific rationale: In the past, several studies have demonstrated positive effects of recombinant growth factors and negative effects of inflammatory cytokines on the regeneration of periodontal tissues. So far, recombinant growth factors are not yet available for clinical use, but platelet concentrates as a natural source of growth factors seem to have beneficial effects on bone healing and may also promote periodontal regeneration.

Principal findings: Our analysis revealed relatively high concentrations of PDGF-AB, PDGF-BB, TGF-β1, and IGF-I in APC, while inflammatory mediators were not detectable. However, we could not find a relevant correlation between the platelet counts or the growth factor/cytokine levels and the periodontal healing effects due to APC.

Practical implications: The potential influence of APC on periodontal regeneration is questionable.