Clin Oral Invest (2007) 11:369–376
DOI 10.1007/s00784-007-0123-2

ORIGINAL ARTICLE

# Levels of gingival tissue platelet activating factor after conventional and regenerative periodontal surgery

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Received: 7 July 2006 / Accepted: 26 April 2007 / Published online: 24 May 2007


**Abstract** The hypothesis, a relationship between gingival tissue platelet activating factor (PAF) levels and healing after periodontal surgery, was tested by measuring PAF levels in gingival tissues collected from sites that had undergone flap surgery and guided tissue regeneration (GTR) or flap surgery alone. Using a split-mouth design, 20 intrabony defects were randomly assigned to treatment with flap surgery and GTR (group 1) or with flap surgery alone (group 2). Gingival tissue samples were obtained at surgery (baseline) and at 6-month follow-up evaluation visit. One half of each sample was used for analysis of PAF levels by high-performance liquid chromatography, and the other half of the sample was used for histomorphometric analysis that included measurements of number and diameter of blood vessels. PAF levels and diameter of blood vessels were significantly decreased ($p<0.01$), and the number of blood vessels was significantly increased ($p<0.05$) in both groups after 6 months compared to the baseline values. Postoperative number of blood vessels were significantly higher in group 1 ($p<0.05$), whereas there was no significant difference in postoperative PAF levels between the two groups ($p>0.05$). Based on the reported results, it is suggested that a decrease in gingival PAF levels might be found after conventional and regenerative periodontal surgery.

**Keywords** Platelet activating factor · Gingival tissue · Periodontal surgery · Angiogenesis · High-performance liquid chromatography

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## Introduction

Periodontitis, an oral infectious disease, is characterized by clinical attachment loss, alveolar bone resorption, periodontal pocketing, and gingival inflammation [16, 52]. The goals of periodontal therapy are the elimination of the infection, arrest the disease progression, and regeneration of the periodontium [26, 41]. Periodontal regeneration comprises restoration of alveolar bone, cementum, and periodontal ligament lost because of periodontitis [26]. In guided tissue regeneration (GTR), placing a protective membrane between the exposed root surface and the mucoperiosteal flap allows repopulation of the root by cells of the periodontal ligament [25, 38, 40]. Periodontal regeneration involves a complex series of biological events [45]. The precise understanding of cellular components and biological events in periodontal wound healing could be useful to investigate more effective treatment modalities.

Platelet activating factor (PAF) is a potent phospholipid mediator synthesized by the thrombocytes, neutrophils, macrophages, eosinophils, and epithelial cells [3, 6, 14, 15, 28, 37, 51, 58]. This mediator is linked to many inflammatory and immune responses, including platelet stimulation, neutrophil and monocyte activation, increased vascular permeability, smooth muscle contraction, and bone resorption by osteoclasts [3, 4, 14, 15, 28, 60]. Elevated levels of PAF in gingival tissue [14, 37], gingival crevicular fluid [2, 14], and blood [3] in periodontal disease, and also higher concentrations of PAF in gingival tissue in periimplantitis [4] have been previously detected. A significant decrease in PAF levels of whole mixed saliva in subjects with chronic periodontitis after initial periodontal therapy has been demonstrated [43]. Data suggest that PAF levels in GCF have been decreased continuously by 24 weeks after flap surgery plus GTR and flap surgery alone [28], but the precise role of gingival tissue PAF levels during wound healing after periodontal surgery still remains unclear.

There are numerous studies on concentrations of inflammatory mediators in gingival tissue relevant to periodontal tissue destruction [18, 19, 24, 27, 32, 34, 54]. New blood vessel formation (angiogenesis), a highly orchestrated process, plays an important role in wound healing and inflammation [30, 59]. However, the role of PAF and angiogenesis in periodontal repair and regeneration has not yet been revealed. The aim of this study was to examine for the first time the levels of PAF and the number and diameter of blood vessels in gingival tissue samples collected from sites that have undergone either flap surgery and GTR or flap surgery alone.

## Materials and methods

### Study population

Ten systemically healthy patients (six men and four women) with the median value with quartiles of the age of 39.5 (36.8–48.0) years (range, 35–51 years) exhibiting radiographic evidence of bone loss were recruited for the study. The inclusion criteria consisted of patients with paired, similar vertical interproximal osseous defects (two-wall or three-wall) without furcation involvement in each of the two contralateral quadrants in the region including molars. The exclusion criteria were systemic diseases (i.e., diabetes mellitus, cancer, human immunodeficiency virus, bone metabolic diseases, or disorders that compromise wound healing), chronic high dose steroid therapy, radiation or immune-supressive therapy, pregnancy, lactation, allergy or sensitivity to any drug, and smoking. The subjects had no history of drug therapy for the last 6 months before recruitment to the study.

Initial periodontal therapy, which consisted of oral hygiene instruction, full-mouth scaling and root planing, and occlusal adjustment when indicated, was performed in all patients. Four to 6 weeks after completion of initial periodontal therapy, a periodontal re-evaluation was performed. Using a split-mouth design, 20 paired intrabony defects with a probing pocket depth of at least 6 mm and radiographic and intrasurgical osseous defect depth of at least 4 mm were randomly treated with either flap surgery and GTR (group 1) or flap surgery alone (group 2). Randomization was performed before surgery according to the flip of a coin.

The patients signed an informed consent form after receiving information about the study. The study protocol and consent forms were approved by the University Institutional Review Board.

### Clinical measurements and surgical procedure

Probing pocket depth (PPD) and clinical attachment level (CAL) measurements were made, and plaque index [50] and gingival index [31] scores were recorded immediately before surgery and at 6 months postoperatively by a Florida Probe (Florida Probe, Gainesville, FL). The measurements were recorded in six areas per tooth: mesiobuccal, distobuccal, midbuccal, mesiolingual, distolingual, and midlingual. All the clinical measurements were done by the same calibrated examiner who was blinded to the treatment modality.

All the operative procedures were performed on an outpatient basis under aseptic conditions by two experienced periodontal clinicians under local anesthesia. One of them performed the surgical procedure, and the other one assisted the procedure. After buccal and lingual intracrevicular incisions, full-thickness mucoperiosteal flaps were raised. All the granulation tissues were removed from the defects, and the roots were thoroughly scaled and planed by hand and ultrasonic instruments. The surgical sites were rinsed with sterile saline.

During surgery, depth of the intrabony component was determined as the distance from the alveolar bone crest to the bottom of the defect. This was the distance between the cemento-enamel junction (CEJ) and the bottom of the osseous defect minus the distance between the CEJ and the most coronal extension of the alveolar bone crest [48].

Atrisorb (Atrix Laboratories, Fort Collins, CO), an absorbable polylactide membrane for GTR, was prepared according to the manufacturer's instructions and placed over the defects in group 1. Application of Atrisorb was not performed in sites of group 2. Flaps were repositioned and secured with 4-0 silk suture utilizing an interrupted and vertical mattress suture technique. Primary closure was obtained in all cases. Sutures were removed 1 week after

surgery. Recall appointments for supragingival professional tooth cleaning and oral hygiene reinforcement were scheduled every other week during the first 2 months after surgery and once a month for the rest of the observation period.

## Tissue preparation and PAF analysis

Gingival tissue samples containing both epithelium and connective tissue of groups 1 and 2 were collected at periodontal surgery (baseline) and at 6 months after surgery. Standardized biopsies of the gingival papilla (3 mm height; 2 mm width) were taken from the area of the intrabony defect at the buccal aspect of the gingiva before raising the flap [10].The baseline and postoperative biopsies were removed from the same site in the same way. The wound created by harvesting for postoperative biopsies was left for secondary healing. One half of the tissue biopsy was placed into a sterile polypropylene tube containing 1 ml phosphate buffered solution (PBS) and kept at −70°C until PAF analysis. Before grinding, the tissue was blotted, weighed in a microbalance, and then placed into a sufficient volume of PBS (4°C, pH 7.0) containing a protease inhibitor (5 μg/ml aprotinin [Trasylol IV solution, Bayer, Leverkusen, Germany] and 1 mM ethylenediaminetetraacetic acid), to a dilution of 10 mg tissue/ml PBS plus protease inhibitor solution. The samples were homogenized four times at 8,500 rpm for 30 s with 10-s intervals by a homogenizer (Ultra Turrax T25, IKA Labortechnik, Staufen, Germany). The homogenate was processed two times with freeze–thaw procedures and then sonicated three times by an ultrasonicator (MSE Soniprep 150, Sanyo Gallenkamp PLC, Leicestershire, UK) at 4–5 μ for 30 s with 10-s intervals [9]. The homogenate was centrifuged (Refrigerated Centrifuge, 3K30, Sigma, Osterode, Germany) at 15,000 rpm for 16 min, and the supernatant was collected for PAF analysis. These supernatant preparation processes were performed on ice medium at ~0–4°C.

The derivatization of PAF to coumarin carbamoyl derivatives was performed as previously described [28, 49]. The fluorescent compound, 7-diethylamino-coumarin-3-carbonylazide (DEACZ; Molecular Probes, Eugene, OR), was used for the derivatization of PAF. By the derivatization, synthetic PAFs (1-0-hexadecyl-2-0-acetyl-*sn*-glycero-3-phosphocholine [PAF $C_{16}$]; D-α-phosphatidylcholine, β-acetyl-γ-*O*-hexadecyl; 1,2-didecanoyl-*sn*-glycero-3-phosphocholine; 1,2-Di-*O*-hexadecyl-*sn*-glycero-phosphocoline; Sigma-Aldrich Chemical, St. Louis, MO) were converted into their corresponding carbamoyl derivatives. Briefly, 100 μl of a 1-mg/ml solution of DEACZ in anhydrous toluene (derivatization solution) was added on the standard samples containing synthetic PAFs in the organic phase (mobile phase) and then heated at 80°C. After 3 h of derivatization, the reaction vials were cooled, and the florescent derivatives were directly analyzed by reverse-phase high-performance liquid chromatography (HPLC) and then quantified by fluorimetric detection [28]. The procedure described above was applied to the gingival tissue samples after an extraction process with 100 μl volume of the mobile phase as the extractant used.

The separation of coumarin carbamoyl derivatives of PAF was performed on a reverse-phase C-8 Nova-Pack column by a model HPLC, Perkin Elmer series 3, (Optimize Technologies, Oregon City, OR) with an isocratic run of a triplet solvent mixture (methanol/chloroform/water, 75:20:5; v/v) containing 250 mg/l tetramethylammonium chloride (Fluka Chemie GmbH, Buchs, Switzerland). The fluorescence detection was at an excitation wavelength of 400 nm and an emission wavelength of 480 nm. A typical chromatogram of coumarin carbamoyl derivatives, obtained under these experimental conditions, is shown in Fig. 1.

For the quantification, different amounts of synthetic PAFs (0.01–1 μg) were dissolved in 1 ml of the mobile

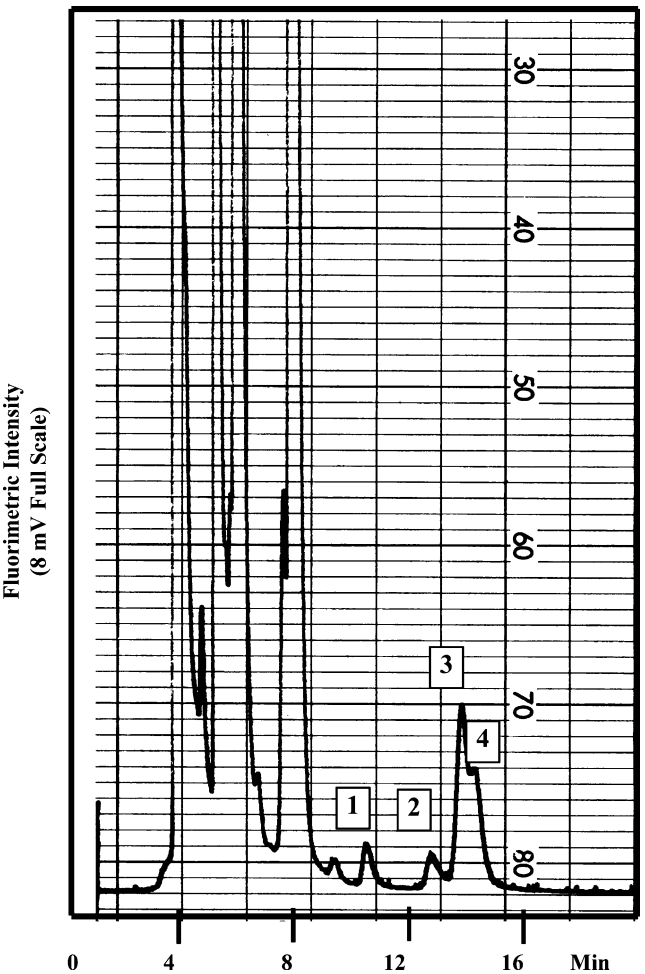


**Fig. 1** Reversed phase HPLC of the gingival tissue. Peaks: (*1*) 1-0-hexadecyl-2-0-acetyl-*sn*-glycero-3-phosphocholine (PAF $C_{16}$). (*2*) D-α-phosphatidylcholine, β-acetyl-γ-*O*-hexadecyl. (*3*) 1,2-didecanoyl-*sn*-glycero-3-phosphocholine. (4) 1,2-Di-*O*-hexadecyl-*sn*-glycero-3-phosphocoline

phase and treated as described above. Calibration curves were obtained from the peak heights of the each coumarin carbamoyl derivative of the PAF chromatogram, and the concentration of PAF was determined using these calibration curves. The laboratory personnel working with PAF analysis were blinded to the treatment modality.

### Histomorphometric assessment

The other half of each gingival tissue sample was fixed in 10% neutral-buffered formalin and embedded into paraffin. Serial sections of samples were obtained in the bucco-lingual direction at 4 μm thickness, which were then stained with hematoxylin and eosin (H and E). In the H-and-E-stained sections, histomorphometric analysis was performed by a light microscope (BH2 Research Microscope, Olympus, Tokyo, Japan), and the microscopic images were digitized by a video camera (Objective 3.3×, F10 CCD Camera, Panasonic, Osaka, Japan) and transferred to a monitor. In the gingival connective tissue, the number of cross-sectioned blood vessels as an indicator of angiogenesis was counted in three individual fields of $60\times60$-$\mu m^2$ area per section at an objective magnification of 40× [9]. The diameter of two selected (the largest and the smallest) cross-sectioned blood vessels per section as an indicator of vasodilatation was measured at a randomly selected magnification area (objective magnification of 100×) [9]. The mean of measurements was used for data analysis.

### Statistical analysis

The statistical analysis was performed using a commercially available software program (SPSS version 12.0, SPSS, Chicago, IL). For the statistical analysis of PPD and CAL, only the recordings representing the deepest clinical site of each defect were used [41]. The Wilcoxon signed ranks nonparametric test was used for the intragroup and intergroup comparisons of the data.

Data are shown as medians with 25–75% quartiles. The significance levels was $\alpha=0.05$.

## Results

### Clinical findings

Healing of the defects treated with both surgical techniques was uneventful. Neither allergic reactions nor suppuration or abscesses were observed in any surgical site. In addition, no cases of membrane exposure were detected. Intragroup comparisons showed that the PPD decreased and CAL improved significantly at 6 months postoperatively, compared to the preoperative data ($p<0.01$). The changes in PPD were 4.0 (4.0–5.3) mm in group 1 and 5.0 (4.0–5.3) mm in group 2. The preoperative CAL was found to be improved by an average of 4.0 (3.0–5.0) mm in group 1 and 5.0 (4.0–5.0) mm in group 2. No statistically significant difference in any of the clinical parameters was observed between the groups ($p>0.05$) as shown in Table 1.

### Changes in PAF levels

Gingival tissue PAF levels for groups 1 and 2 are shown in Table 2. Intragroup analysis showed significant decreases in PAF levels for both groups at 6 months postoperatively compared to the baseline values ($p<0.01$). Intergroup analysis demonstrated no significant differences in PAF levels between the study groups at baseline and after 6 months ($p>0.05$).

### Histomorphometric findings

The number of blood vessels was significantly increased ($p<0.05$), whereas the diameter of the blood vessels was significantly decreased ($p<0.01$) in both treatment groups at 6 months postoperatively compared to the baseline values

**Table 1** Clinical findings of the study groups

| | Group 1[a] | | | Group 2[a] | | |
|---|---|---|---|---|---|---|
| | Preoperative | Postoperative | Significance ($p$ value)[b] | Preoperative | Postoperative | Significance ($p$ value)[b] |
| PPD | 7.0 (6.8–8.3) | 3.0 (2.0–3.0) | <0.01 | 7.5 (7.0–9.0) | 3.0 (2.0–3.0) | <0.01 |
| CAL | 7.5 (7.0–8.5) | 4.0 (3.0–4.0) | <0.01 | 8.0 (7.8–9.0) | 4.0 (3.0–4.0) | <0.01 |
| INTRA | 5.0 (4.8–6.0) | – | – | 5.0 (4.0–5.3) | – | – |
| PI | 0.7 (0.6–0.7) | 0.7 (0.6–0.7) | >0.05 | 0.7 (0.6–0.7) | 0.6 (0.6–0.7) | >0.05 |
| GI | 1.5 (1.4–1.5) | 0.4 (0.2–0.4) | <0.01 | 1.4 (1.3–1.5) | 0.3 (0.2–0.4) | <0.01 |

Data are expressed as the medians with 25–75% quartiles.

*PPD* Probing pocket depth (mm), *CAL* clinical attachment loss (mm), *INTRA* depth of intrabony component (mm), *PI* plaque index, *GI* gingival index

[a] No significant difference between the values of the groups ($p>0.05$)

[b] Wilcoxon signed ranks test

**Table 2** Gingival tissue PAF levels and histomorphometric findings of the study groups

| | Group 1 | | | Group 2 | | |
|---|---|---|---|---|---|---|
| | Baseline | Postoperative | Significance ($p$ value)[c] | Baseline | Postoperative | Significance ($p$ value)[c] |
| PAF | 87.5 (78.8–96.3) | 45.0 (38.8–50.0) | <0.01 | 85.0 (68.8–110.0) | 42.5 (33.8–46.3) | <0.01 |
| NBVP | 1.3 (0.9–2.0) | 3.3 (3.0–3.9)[a] | <0.01 | 1.5 (1.0–1.8) | 2.3 (1.9–2.7) | <0.05 |
| DBVP | 15.9 (15.2–16.5)[b] | 12.1 (10.8–12.9) | <0.01 | 18.9 (18.0–19.7) | 13.2 (11.9–13.8) | <0.01 |

Data are expressed as the medians with 25–75% quartiles.
*PAF* Platelet activating factor (ng/ml), *NBVP* number of blood vessels, *DBVP* diameter of blood vessels (μm)
[a] Significantly different from group 2 ($p<0.05$)
[b] Significantly different from group 2 ($p<0.01$)
[c] Wilcoxon signed ranks test

(Table 2, Fig. 2a–d). Intergroup analysis demonstrated that the postoperative number of blood vessels was significantly higher in group 1 compared to those in group 2 ($p<0.05$).

## Discussion

In the present study, gingival tissue PAF levels, angiogenesis, and vasodilatation were evaluated in gingival connective tissue at baseline and at 6 months postoperatively. The clinical findings of this study showed that the treatment of intrabony periodontal defects with flap surgery plus GTR and flap surgery alone might lead to a significant PPD reduction and CAL gain. No significant differences in any of the clinical parameters were observed between the groups. Based on the available evidence from the literature, many studies have observed greater reduction in the amount of PPD and gains in CAL [1, 12] and radiographic bone fill and defect depth reduction [1] in the flap surgery plus GTR groups, while others have found no statistically significant differences in PPD reduction and CAL gains [28, 33] and radiographic changes in bone height [33] between the treatments with flap surgery plus GTR and flap surgery alone, which is in agreement with our findings. It is well documented that gain in CAL after any type of conventional and regenerative periodontal treatment is dependent on the initial PPD; that is, the deeper the initial PPD, the greater the PPD reduction and CAL gain [12, 42]. In addition, depth of the intrabony component is the determining factor for the maximally possible attachment gain [39]. In this way, the deeper depth of the intrabony component may be related to the greater CAL gains [12]. Between the two treatment groups, there were no differences in terms of initial PPD and intrabony defect depth.

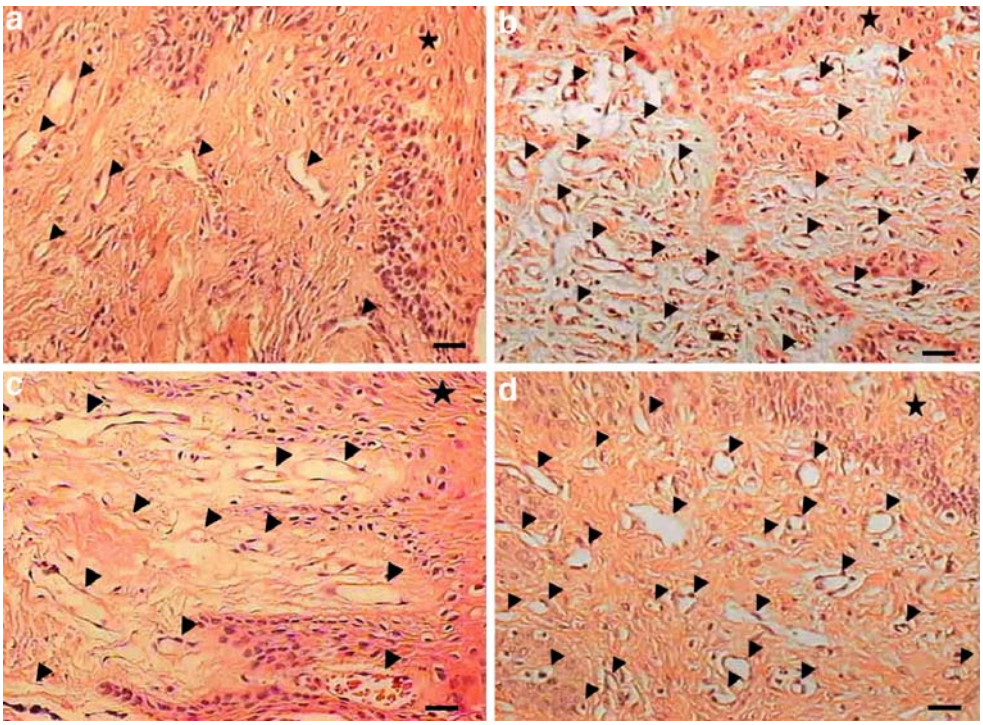


**Fig. 2** **a**–**d** Micrographs of gingival tissue stained with H and E (scale bar=50 μm). *Asterisks* indicate epithelium, and *arrowheads* indicate blood vessels. **a** Baseline view of group 1. **b** More prominent increase in the number of blood vessels at 6 months postoperatively view of group 1. **c** Baseline view of group 2. **d** Increase in the number of blood vessels at 6 months postoperatively view of group 2

The results obtained in the present study might have been influenced by the defect characteristics and center and/or operator effect, which may depend on differences in the enrolled patients, technical ability, clinical organization, and experience of the clinicians or a combination of these factors [46]. When interpreting the clinical findings of this study, it has to be pointed out that the CAL gain detected in the flap surgery group is somewhat higher than those reported in the literature [8, 48]. Postoperative infection, an important factor in the healing process, was not detected in any treatment group. Therefore, greater CAL gain might also be related to this finding.

In the periodontal literature, PAF has been analyzed in gingival tissues [4, 14, 37] as well as in gingival crevicular fluid [2, 14, 15], saliva [17, 35, 43, 44], and blood [3]. A significant elevation of gingival tissue PAF levels has been found in periodontitis and gingivitis groups compared to periodontally healthy controls [14]. In addition, data indicating significantly higher levels of PAF in inflamed gingiva from periodontitis patients than in healthy gingiva have suggested that the higher levels of PAF in the inflamed gingival tissue might have been due to an increase in PAF production [37]. This present study focused on the changes in gingival tissue PAF levels during periodontal repair and regeneration, and the results clearly showed a significant decrease in gingival tissue PAF levels after flap surgery plus GTR and flap surgery alone. Despite the presence of studies [2, 3, 14, 17, 37] analyzing the role of PAF in the pathogenesis of periodontal disease, there are a few experimental studies related to the gingival tissue PAF levels in periodontal therapy [7, 56, 57]. The application of omega-3 fatty acid [57] and the combination of selective cyclooxygenase-2 inhibitor and omega-3 fatty acid administration [56] have been demonstrated to reduce gingival PAF levels in the endotoxin-induced periodontitis in rats.

In light of the presented reports, PAF is considered to be an important mediator of alveolar bone and connective tissue destruction in periodontitis [14, 15, 56, 57]. Based on this information, our study suggests that the decrease in gingival PAF levels after periodontal surgical therapies might be due to the repair and regeneration of the gingival connective tissue. In addition, a significant increase in angiogenesis was found as well as a decrease in gingival PAF levels after these therapies. A series of morphological changes, which occurred in the wound healing process after flap surgery, reflects the metabolic condition of the tissue [36]. The role of angiogenesis in promoting either the progression or the healing of periodontal lesions is not clear, as angiogenesis is a prominent feature of both inflammation and healing [5]. In a recent experimental study, new blood vessels in the repaired periosteal tissue have been shown to regress after mucoperiosteal flap surgery on postoperative day 21 because of a decrease in bone resorption and addition [36]. This is consistent with the reports related to the angiogenesis, which have suggested a close relationship between bone metabolism and the morphology and function of the microcirculation [20–22, 36].

Stimulated angiogenesis facilitates an increased delivery of immune and hematopoietic precursor cells to the affected region and causes endothelial cells to display multiple key regulatory signals on their cell surfaces that trigger the activation, transmigration, differentiation, and/or function of such cells [11, 13, 23, 29, 53]. A significant increase in angiogenesis seems to be associated with uncompleted healing activity at 6 months postoperatively in both treatment groups of our study. Likewise, healing activity may be more prominent in the GTR treatment group because of the significantly greater increase in angiogenesis. An alternative interpretation for this finding is that the increased number of blood vessels may be caused by a foreign body reaction because it is known that polylactide membrane, a solid polymer of polylactic acid, causes a foreign-body reaction [47, 55]. Vessel enlargement is an important histopathological feature of the inflamed tissues. The decrease in gingival PAF levels and vasodilatation could be due to resolution of inflammation, and the increase in angiogenesis could be associated with uncompleted healing activity, as angiogenesis is accepted to be an important process in healing.

Based on the reported results, it is suggested that a decrease in gingival PAF levels might be found after conventional and regenerative periodontal surgery. Additional studies should be undertaken about the role of PAF and angiogenesis on wound healing mechanisms after periodontal regenerative therapies to further strengthen the findings of the present study.

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