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Periodontitis is associated with platelet activation

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Abstract

There is an epidemiological association between periodontitis and cardiovascular disease (CVD). In periodontitis, low grade systemic inflammation and bacteremia occur regularly. Such events may contribute to platelet activation and subsequent pro-coagulant state. This study aimed to investigate platelet activation in periodontitis patients.

The study is composed of two parts. In the first part, plasma levels of soluble(s) P-selectin and sCD40 ligand were measured as general markers of platelet activation in periodontitis patients ($n = 85$) and in healthy controls ($n = 35$). In the second part, surface-exposed P-selectin and the ligand-binding conformation of the glycoprotein IIb–IIIa complex (binding of PAC-1 antibody) were determined on individual platelets in whole blood of periodontitis patients ($n = 18$) and controls ($n = 16$). Patients had significantly elevated plasma levels of sP-selectin ($P < 0.001$) and increased binding of PAC-1 on isolated platelets ($P = 0.033$). Platelet activation was more pronounced in the patients with more severe periodontal disease, showing a severity-dependence. The levels of sCD40 ligand and of platelet-bound P-selectin were not increased.

Periodontitis is associated with increased platelet activation. Since platelet activation contributes to a pro-coagulant state and constitutes a risk for atherothrombosis, platelet activation in periodontitis may partly explain the epidemiological association between periodontitis and CVD.
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1. Introduction

Periodontitis is a chronic infectious disease of the supportive tissues of the teeth, which may lead to loss of teeth. It is one of the most common infections in humans, affecting in its most severe form, approximately 10% of the population [1]. Inflammation of periodontal tissues results in periodontal pocket formation and ulceration of the epithelial lining. In this way, periodontal pockets form ports of entry which may lead to transient bacteremias [2,3]. Regularly occurring bacteremias in periodontitis patients underlie chronic production of pro-inflammatory mediators like interleukin (IL)-1β, IL-6, C-reactive protein and tumor-necrosis factor (TNF)-α [4–6].

One of the features of systemic inflammation is an increase in the number of platelets and platelet activation [7]. Also periodontitis has been associated with elevated numbers of platelets [8]. Furthermore, platelet numbers decrease after periodontal therapy [9]. Interestingly, strains of the recognized periodontal pathogen *Porphyromonas gingivalis* (*P. gingivalis*), but also other dental plaque bacteria, such as *Streptococcus sanguis*, induce platelet activation and aggregation *in vitro* and in animal studies [10,11]. Activation of platelets leads to their release of pro-inflammatory mediators and exposure of pro-inflammatory receptors, resulting in platelet binding to leukocytes and endothelial cells [7]. These

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functions make platelets essential participants in both thrombotic and inflammatory reactions across the vasculature [12]. Platelet activation has been implicated in the development of atherosclerosis, atherothrombosis and subsequent coronary vascular and cerebrovascular diseases [13].

Epidemiological and intervention studies have associated periodontitis with atherosclerosis and cardiovascular diseases (CVD). The underlying mechanisms of this relationship are still obscure [14,15]. Nevertheless endotoxemia and in particular, systemic exposure to *P. gingivalis* and the severity of periodontal disease seem be important risk factors for CVD in periodontitis patients [16–19]. We hypothesize that platelet activation in periodontal patients may be an important link between periodontitis and CVD.

The aim of this study was to investigate whether periodontitis patients have a higher state of platelet activation compared to healthy controls.

2. Methods

All subjects were both verbally and written informed about the purpose of the study and had signed an informed consent. The Medical Ethical Committee of the Academic Medical Center of the University of Amsterdam approved the study. The present study consists of two parts. The study population of the first part has been derived from a previous cohort [20] and included 85 consecutive periodontitis patients and 35 healthy controls. On the basis of an extensive medical history by a written questionnaire and by interview, the following subjects were not included in the study: pregnant women and individuals who suffered from any given disease or chronic medical condition, apart from periodontitis, or had trauma or tooth extractions in the last 2 weeks, or received antibiotics within the last 3 months or any chronic medication. Concentrations of soluble(s) sP-selectin (sCD62P) and sCD40 ligand were determined in plasma samples collected from these periodontitis patients and controls. The results suggested that platelet activation was present in periodontitis. Therefore, the second study was initiated to further explore the possibility that platelets are activated in periodontitis. This time the focus was laid on measurements of platelet-bound activation markers, evaluated by means of flow cytometry of fresh platelets. Based on the P-selectin results in the first study (mean ± S.D.: 58.1 ± 26.1 ng/mL for controls, 82.9 ± 34.7 ng/mL for patients) the sample

Table 1
Characteristics of the subjects participating in the first study

	Control ($n = 35$)	Periodontitis ($n = 85$)	P-value
Background characteristics			
Age	37.6 ± 9.1	44.7 ± 8.8	0.0001
Gender (males)	12 (34%)	41 (48%)	0.225
Ethnicity (Caucasian)	30 (86%)	70 (82%)	0.791
Smoking (smokers)	12 (34%)	40 (47%)	0.228
Education (<high school)	11 (31%)	46 (54%)	0.028
BMI (kg/m^2)	24.0 ± 3.5	25.4 ± 4.3	0.088
Blood pressure (mmHg)			
Systolic	114.3 ± 13.1	122.1 ± 19.1	0.034
Diastolic	73.6 ± 11.5	75.3 ± 11.5	0.465
Total cholesterol (mmol/L)	5.0 ± 0.9	5.5 ± 1.1	0.042
HDL (mmol/L)	1.5 ± 0.3	1.3 ± 0.4	0.106
LDL (mmol/L)	3.0 ± 0.9	3.5 ± 1.0	0.022
Triglycerides (mmol/L)	1.2 ± 0.6	1.5 ± 0.8	0.034
Systemic inflammatory markers			
Leukocytes (×10^9/L)			
Total	5.7 ± 1.2	7.1 ± 2.5	0.003
Neutrophils	3.2 ± 1.0	4.1 ± 1.6	0.001
Lymphocytes	1.9 ± 0.4	2.3 ± 0.7	0.005
Fibrinogen (g/L)	2.8 ± 0.5	3.1 ± 0.7	0.012
C-reactive protein (mg/L)	1.8 ± 1.8	2.9 ± 3.4	0.078
Dental characteristics			
No. of teeth present	28.2 ± 1.8	26.0 ± 3.4	0.001
No. of teeth with bone loss			
≥30%	0.0 ± 0.2	16.2 ± 6.6	
≥50%	0.0 ± 0.0	5.6 ± 5.0	

Values are mean ± S.D. or number (%) of subjects; P-values are from t-test or χ^2-test (or Fisher's exact test, where needed). BMI indicates body mass index.

size for the second study was estimated using $\alpha = 0.05$ and $\beta = 0.20$ (80% power). The sample size needed was 18 participants for each of the control and patient groups. Thus, a new study population was recruited, applying similar inclusion and exclusion criteria as in the first study. Subjects with platelet counts of >350 × 10^9/L (thrombocytosis) were excluded [13]. Eighteen consecutive periodontitis patients were included and for each patient an age-, gender-, race-, and smoking status-matched healthy control was recruited. After their initial inclusion two control subjects were excluded from analysis, because one subject had a platelet count >350 × 10^9/L and the other subject appeared to suffer from mild periodontitis. Both platelet-bound P-selectin and binding of PAC-1 on individual platelets were measured in this cohort by whole-blood flow cytometry. Statistical analysis of the data was performed with the SPSS package, Version 14 (SPSS, Chicago, IL, USA). Please see supplemental Methods for details of blood collection, platelet soluble markers measurements, flow cytometry and statistical analysis.

3. Results

3.1. Study population

Tables 1 and 2 summarize the background characteristics of the study populations of the first and second study, respectively. As shown in Table 1, several differences were present in the first study between groups, including age, educational level, systolic blood pressure, cholesterol, and triglycerides. As defined before [20], in the first study patients suffering from moderate ($n = 51$) or severe ($n = 34$) periodontitis were included. In the second part of our study, again moderate ($n = 10$) as well as severe ($n = 8$) periodontitis patients were included. Patients had significantly higher fibrinogen and platelet counts than controls (Table 2).

3.2. Soluble(s) P-selectin and CD40 ligand

The data in Table 3 are presented both as measured values (mean ± S.D.) and after correction by GLM (adjusted means

Table 2
Characteristics of the subjects participating in the second study

	Control ($n = 16$)	Periodontitis ($n = 18$)	P-value
Background characteristics			
Age	40.8 ± 10.8	42.8 ± 9.5	0.565
Gender (males)	5 (31%)	6 (33%)	1.000
Ethnicity (Caucasian)	12 (75%)	15 (83%)	0.681
Smoking (smokers)	4 (25%)	6 (33%)	0.715
Education (<high school)	3 (19%)	8 (44%)	0.152
BMI (kg/m^2)	25.9 ± 6.1	25.7 ± 2.9	0.886
Blood pressure (mmHg)			
Systolic	130.6 ± 18.5	127.6 ± 14.5	0.606
Diastolic	86.2 ± 10.0	86.5 ± 10.3	0.929
Total cholesterol (mmol/L)	4.9 ± 1.0	5.2 ± 1.2	0.486
HDL (mmol/L)	1.3 ± 0.3	1.2 ± 0.4	0.563
LDL (mmol/L)	3.2 ± 0.9	3.2 ± 0.8	0.903
Triglycerides (mmol/L)	0.9 ± 0.7	1.7 ± 2.5	0.194
Systemic inflammatory markers			
Leukocytes (×10^9/L)			
Total	5.7 ± 1.6	6.7 ± 2.0	0.123
Neutrophils	3.2 ± 1.1	3.9 ± 1.4	0.140
Lymphocytes	1.9 ± 0.6	2.1 ± 0.5	0.262
Fibrinogen (g/L)	3.0 ± 0.5	3.6 ± 0.5	0.002
C-reactive protein (mg/L)	2.4 ± 4.2	3.9 ± 5.2	0.340
Platelet count (×10^9/L)	241 ± 44	279 ± 52	0.029
Dental characteristics			
No. of teeth present	28.6 ± 1.4	26.6 ± 2.9	0.021
No. of teeth with			
≥30% bone loss	0.0 ± 0.0	17.1 ± 5.2	
≥50% bone loss	0.0 ± 0.0	6.2 ± 3.7	
Soluble platelet activation markers			
sP-selectin (ng/mL)	49.5 ± 13.6	56.9 ± 17.5	0.182
sCD40 ligand (pg/mL)	155 ± 114	217 ± 134	0.159

Values are mean ± S.D. or number (%) of subjects; P-values are from t-test or χ^2-test (or Fisher's exact test, where needed). BMI indicates body mass index.

Table 3
Plasma levels of the soluble platelet activation markers sP-selectin and sCD40 ligand in the subjects in the first study

	Control ($n=35$)	Periodontitis ($n=85$)	P-value
sP-selectin (ng/mL)			
Mean ± S.D.[a]	58.1 ± 26.1	82.9 ± 34.7	0.0002
Adj. mean (CI)[b]	61.2 (49.6–72.8)	81.6 (74.5–88.7)	0.005
# subjects ≥68 ng/mL[c]	11 (31.4%)	50 (58.8%)	0.009
sCD40 ligand (pg/mL)			
Mean ± S.D.[a]	624 ± 564	794 ± 647	0.180
Adj. mean (CI)[b]	636 (408–863)	789 (648–930)	0.278
# subjects ≥609 pg/mL[c]	15 (42.8%)	46 (54.1%)	0.317

[a] Raw data and P-value from t-test.
[b] Adjusted (Adj.) means, confidence intervals (CI) and P_{GLM} from a general linear model (GLM) correcting for age, educational level, systolic blood pressure, total cholesterol and triglycerides.
[c] Tested with χ^2-test and the P_{χ^2}-values are given.

and confidence intervals). Plasma levels of sP-selectin were significantly elevated in periodontitis patients (Table 3). Also after adjusting for potential confounders (age, educational level, systolic blood pressure, cholesterol, and triglycerides), these differences remained highly significant. The median value of sP-selectin (68 ng/mL) for the total study population was selected as cut-off point for the frequency distribution analysis. There were more periodontitis patients with sP-selectin above the median than controls.

Plasma levels of sCD40 ligand were somewhat higher in periodontitis patients, but not significantly different from controls (Table 3). The study population median for CD40 ligand was 609 pg/mL, which was used as cut-off point for the frequency distribution analysis. The distribution of subjects with sCD40 ligand values above the population median was not different between periodontitis patients and controls. Overall, the plasma concentrations of sP-selectin and sCD40 ligand were highly associated (Fig. 1). Also after adjusting for the same covariates as in the GLM, the correlation remained strong (overall partial correlation coefficient $r_{adj}=0.496$, $P_{adj}<0.001$); this correlation was not found within the control subjects ($r_{adj}=0.352$, $P_{adj}=0.056$), but was very strong within periodontitis patients ($r_{adj}=0.566$, $P_{adj}<0.001$).

In a stepwise linear regression analysis, the severity of periodontitis (i.e. moderate or severe) was the strongest predictor of sP-selectin levels, with severe periodontitis patients having higher sP-selectin than moderate periodontitis patients ($\beta=0.258$, $P=0.010$), while all of the background characteristics were not significantly contributing to the sP-selectin. sCD40 ligand values were not predicted by disease severity or by any of the background characteristics of the patients.

3.3. Platelet-bound P-selectin and binding of PAC-1

In the second study the fraction (%) of platelets binding anti-P-selectin was not significantly different between patients and controls (Fig. 2A). The % of platelets expressing P-selectin was not significantly correlated with the number of teeth with ≥50% bone loss ($r=0.293$, $P=0.093$). Furthermore, the amount of surface expressed P-selectin, as reflected by the mean fluorescence intensity (MFI), was comparable in patients and controls (Fig. 2B). The P-selectin MFI was not correlated with the number of teeth with ≥50% bone loss ($r=0.168$, $P=0.342$).

In contrast, the fraction of platelets binding PAC-1, i.e. the antibody specifically binding to the fibrinogen-binding (activated) conformation of the platelet glycoprotein IIb–IIIa complex, was elevated in periodontitis patients compared to controls (Fig. 2C, $P=0.033$). This % of PAC-1 binding platelets remained elevated ($P_{GLM}=0.030$) in patients

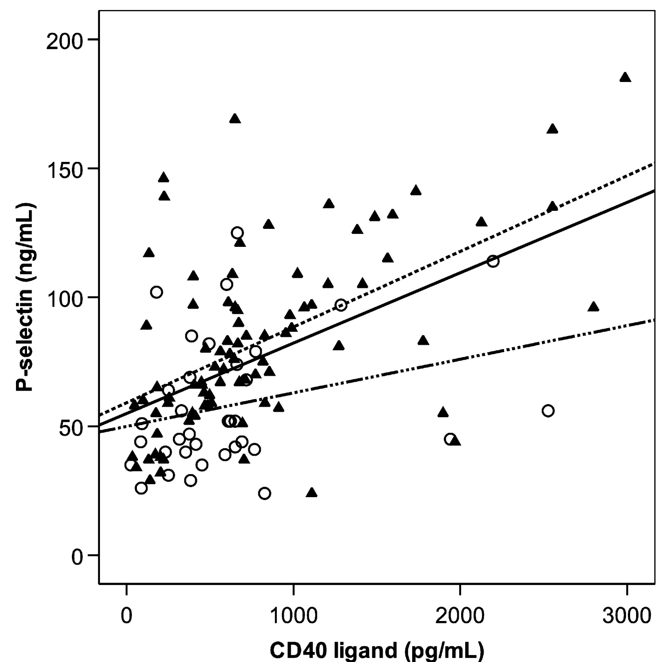

Fig. 1. sP-selectin and sCD40 ligand plasma levels in the first study in patients (▲) and controls (◯). Each symbol represents one subject. There was an overall correlation between sP-selectin and sCD40 ligand (continuous line, Pearson's correlation coefficient $r=0.500$, $P<0.001$), originating mainly from patients (dotted line, $r=0.547$, $P<0.001$) and not from controls (dashed–dotted line, $r=0.281$, $P=0.102$).

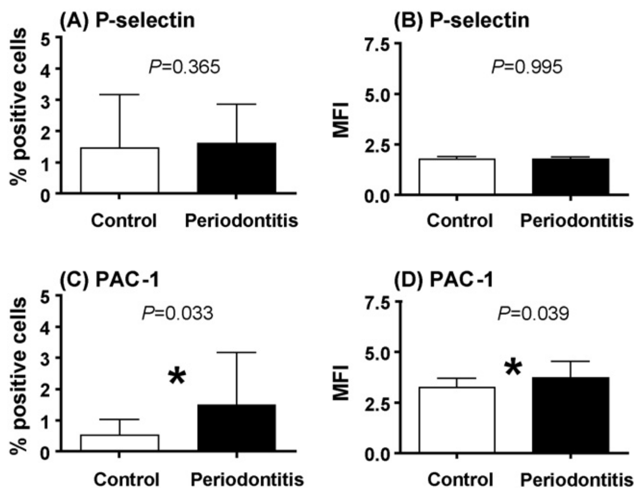

Fig. 2. Flow cytometric determination of platelets exposing P-selectin: (A) % of positive cells and (B) mean fluorescence intensity (MFI) of P-selectin; binding of PAC-1: (C) % of positive cells and (D) MFI of PAC-1, in patients (n = 18) and controls (n = 16) in the second study. Bar graphs represent mean ± S.D.

after correcting for non-matched background characteristics (educational level, BMI, systolic blood pressure, cholesterol, triglycerides). Moreover, in patients, the fraction of platelets binding PAC-1 was significantly correlated with the number of teeth with ≥50% bone loss (r = 0.388, P = 0.023). Also the extent of binding of PAC-1, as reflected by MFI, was increased in patients compared to controls (Fig. 2D, P = 0.039) and showed a severity-dependence (number of teeth with ≥50% bone loss, r = 0.377, P = 0.028). The PAC-1 MFI was still significantly increased in periodontitis patients after correcting for non-matched background characteristics (P_{GLM} = 0.032).

4. Discussion

The present study investigated whether periodontitis is associated with increased activation of platelets. The results of our study indicated for the first time increased platelet activation in periodontitis patients compared to healthy controls. This was based on elevated levels of sP-selectin, which we found to be positively associated with periodontitis severity. These results were for us the first indication that human periodontitis *in vivo* may indeed be associated with platelet activation. In the second study, using flow cytometry, and thus enabling the direct detection of single activated platelets in whole blood, increased platelet activation was demonstrated, confirming the results of the first study.

Although the levels of sCD40 ligand showed a tendency to be elevated in periodontitis patients and also correlated with the levels of sP-selectin, this increase did not reach statistical significance. CD40 ligand is a glycoprotein found on a large variety of cells including B-cells, T-cells, basophiles, eosinophiles, and epithelial cells [21]. Platelets carry preformed CD40 ligand that becomes exposed on the cell surface during platelet activation [22]. In a subsequent cleavage step, the soluble form sCD40 ligand is generated. It has been estimated that 95% of the circulating sCD40 ligand is of platelet origin [23]. Nevertheless, throughout the literature differences have been reported with regard to the extent of various platelet activation markers, which is due to the relative contributions of activation and inactivation pathways that differ between clinical conditions [24].

We demonstrated for the first time that binding of PAC-1, a recognized measure of platelet activation, was increased in periodontitis. Not only did patients have an increased fraction of platelets binding PAC-1, but also the extent of binding of PAC-1 to individual platelets was increased compared to controls. Moreover, the proportion of platelets binding PAC-1 correlated with the periodontal disease severity, indicating a severity dependent relationship. These results further suggest that platelet activation indeed occurs in periodontitis. PAC-1 binds to the ligand-binding conformation of the (activated) glycoprotein (GP) IIb/IIIa, the most abundant receptor on the platelet surface. It has been shown that binding of fibrinogen to (activated) GP IIb/IIIa can be a more sensitive marker of platelet activation than exposure of P-selectin [24]. Furthermore, it has been demonstrated in an animal study that activated platelets, expressing membrane-bound P-selectin, become P-selectin negative in a proportion >95% within 2 h after activation [25], but continue to circulate in blood and be responsive to platelet-activating agents. The P-selectin released from activated platelets is found in plasma as sP-selectin. Possibly, this latter phenomenon explains why in periodontitis patients we found increased sP-selectin, while platelet-bound P-selectin was not increased.

It should be recognized that not only platelets but also endothelial cells are a potential source of sP-selectin [26]. However, there are reasons to assume that in the present cohort elevation of sP-selectin originates mainly from platelets: (1) plasma levels of von Willebrand factor [20], a marker of endothelial activation, were not associated with sP-selectin (r = −0.005, P = 0.953). (2) We found a significant association between plasma levels of sP-selectin and sCD40 ligand, i.e. two markers of platelet activation, in the present study. (3) FACS analyses performed in the second study showed platelet activation. (4) The Michelson et al. study showed that activated platelets shed their membrane-bound P-selectin, which is to be found in plasma as sP-selectin [25]. We suggest that these observations support our notion that the elevated sP-selectin plasma levels in the present study are of platelet, rather than of endothelial origin, and therefore are likely to represent platelet activation.

We speculate that a higher incidence of bacteremias and dissemination of bacterial products and inflammatory cytokines in periodontal patients compared to healthy controls provide an explanation for the current findings [3–6]. Activated platelets release an arsenal of potent inflammatory and mitogenic substances into the local microenvironment, thereby altering chemotactic and adhesive properties of

endothelial cells [27,28]. These molecules acting together, accelerate inflammatory processes, enhance cell recruitment, and make platelets crucial participants in activation and proliferation of the endothelium. The higher number of platelets as well as activated platelets may contribute also to the pro-coagulant state in periodontitis, a condition reported in periodontitis on the basis of elevated PAI-1 [20]. We believe that the subtle platelet activation occurring in periodontitis helps explaining the epidemiological observations of increased risk for coronary heart disease in subjects with periodontal disease compared to subjects with no periodontal disease [14]. A similar association is found between periodontitis and stroke, especially in individuals younger than 65 years of age [14]. In subjects at risk for myocardial infarction or stroke due to an extensive atherosclerosis process, a preexistent state of platelet activation and a pro-coagulant state will add to the blood clot formation at time of atherosclerotic plaque rupture [29,30].

Randomized controlled clinical trials studying the effects of treatment of periodontitis on platelet activation may provide further insight on the contribution of this chronic infectious condition to atherothrombosis. One intervention study showed that treatment of patients with periodontitis is followed by an improvement of endothelial function [15]. Although the biological basis of this improvement is unknown, one may speculate that reduced platelet activation and its concurrent pro-coagulant phenotype may be part of the explanation. Within our department, a study has been initiated to evaluate whether periodontal therapy indeed affects platelet activation and pro-thrombotic phenotype in periodontitis patients.

In conclusion, we provide evidence that periodontitis is associated with increased platelet activation. Our findings suggest that also via platelet activation, periodontitis may constitute a risk for CVD.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.atherosclerosis.2008.05.035.

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