

Original Article

Whole-blood platelet aggregation by *Porphyromonas gingivalis* in patients with peripheral arterial disease

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Objectives: To measure platelet aggregation promoted by *Porphyromonas gingivalis* (*P. gingivalis*) in whole blood, and to investigate the relation between *P. gingivalis* and peripheral arterial disease (PAD).

Methods and Results: Subjects were 30 patients who were diagnosed as having PAD (PAD Group), and 26 healthy adults without subjective symptoms or arteriosclerosis as a control (Control Group). PAD patients were classified depending on severity levels by Fontaine classification or toe pressure (TP). Twelve-minute changes of electrical impedance after adding *P. gingivalis* to whole blood was 10.2 ± 4.8 (range, 5.1-14.3) ohm in PAD Group, and 6.1 ± 5.6 (range, 0.2-10.8) ohm in Control Group. PAD Group showed significantly stronger whole-blood platelet aggregation by *P. gingivalis*. The patients with more severe PAD showed stronger whole-blood platelet aggregation by *P. gingivalis*. PAD Group had significantly higher serum IgG against *P. gingivalis* titers than Control Group. In PAD patients with teeth, there was a strong positive correlation between whole-blood platelet aggregation and IgG against *P.*

gingivalis titers.

Conclusions: Platelet aggregation promoted by *P. gingivalis* was significantly high in PAD patients, and was related to the deterioration of their symptoms even in whole blood, which was the environment closer to physiological conditions.

Key words: peripheral arterial disease; *Porphyromonas gingivalis*; whole-blood platelet aggregation

Introduction

Peripheral arterial disease (PAD) is a disease induced by stenosis due to the atheromatous plaque and occlusive thrombus formation due to rupture of the atheromatous plaque in the peripheral artery. PAD impairs blood flow in the lower limbs, which results in lower extremity ischemic ulceration or gangrene. As risk factors of atherothrombosis, smoking, gender (male) and aging have been reported in addition to lifestyle-related diseases (e.g., diabetes mellitus, hypertension, dyslipidemia). Furthermore, involvement of systemic chronic infection due to microorganisms such as periodontal pathogens, *Helicobacter pylori*, *Chlamydia pneumoniae*, and *Cytomegalovirus* has been recently reported in many studies¹⁻⁶. In 1998, Mendez et al. first reported that a periodontal disease was a significant independent risk factor of PAD⁷. Hung et al. show that a periodontal disease has 1.41-fold relative risk (95%CI=1.12-1.77) of lesion progression in PAD patients during 12-year follow up in their prospective cohort study⁸. These two epidemiological studies indicate that there is

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some relation between a periodontal disease and PAD.

A periodontal disease is defined as a chronic inflammatory disease of the tooth-supporting tissues induced by periodontal pathogens. Periodontal pathogens can easily invade the bloodstream by dental surgery and hygiene treatments, as well as chowing and tooth brushing. These bacteria attach to or invade the vascular endothelial cells, and deeply involves to the formation of the arteriosclerotic lesion⁹⁻¹². In fact, several investigators detect periodontal pathogens from the atherosclerotic plaque¹³⁻¹⁵. In our collaborative study with Section of Periodontology, Department of Hard Tissue Engineering, especially two kinds of periodontal pathogens, *Porphyromonas gingivalis* (*P. gingivalis*) and *Treponema denticola*, were detected with a high incidence from the arteriosclerotic plaque in PAD patients¹⁶. *P. gingivalis*, which is one of the causative microorganisms of a periodontal disease, has aggregation capacity of human platelets in platelet-rich plasma (PRP), and its mechanisms have been clarified in multiple studies^{17,18}. These studies suggest that platelet aggregation by *P. gingivalis* plays a considerable role in pathogenesis of PAD. However, to our knowledge, no studies have been performed by using whole blood.

In our study, whole-blood platelet aggregation by *P. gingivalis* was measured in PAD patients and healthy controls. First, we investigated whether *P. gingivalis* induced platelet aggregation in whole blood of healthy controls. Second, by comparing the two groups, the role of whole-blood platelet aggregation by *P. gingivalis* in PAD patients under the closer-to-*in-vivo* environment was investigated. Furthermore, IgG titers against *P. gingivalis* were measured in both groups, and its relation with platelet aggregation was statistically evaluated.

Materials and Methods

Study population

Subjects were 30 patients (26 males and 4 females) who were diagnosed as having PAD at Tokyo Medical and Dental University Hospital between March and August 2009 (PAD Group). Diagnosis of PAD was made based on clinical symptoms, ankle brachial pressure index (ABI) measurement, and findings of duplex ultrasound and angiography. In addition, based on clinical symptoms, staging was performed using Fontaine classification. There were 20 patients who had grade II (claudication), 2 grade III (rest pain) and 8 grade IV (ischemic ulceration or necrosis). ABI was 0-0.83 (mean, 0.48), and toe pressure (TP) was 0-79

(mean, 33.0) mmHg. Fifteen patients had supra-inguinal occlusions, 11 had infra-inguinal occlusions, and 4 had combined lesions. All the patients received revascularization, and vascular bypassing was performed to 24 patients, and percutaneous transluminal angioplasty (PTA) to 6. Twenty-six healthy adults (20 males and 6 females) without subjective symptoms or arteriosclerosis who had normal ABI were used as a control (Control Group). The information about current health conditions and history of diseases, medication and smoking were collected from all the subjects through interviewing. The antiplatelet and/or anticoagulant treatment was discontinued in PAD Group 7 days before the blood samples collection. Current smokers gave up smoking 2 weeks before the blood samples collection. The subjects who had a malignant tumor, received dialysis chronically, took antiplatelet and/or anticoagulant drugs within 7 days, or received treatments for a periodontal disease within 6 months were excluded from the study. Characteristics of PAD and Control Groups are shown in Table 1. This study was approved by the ethical committee of Tokyo Medical and Dental University. Written informed consent was obtained from all the subjects.

Blood sampling

Venous blood of 12 ml was collected from all the subjects. To 5 ml of the blood, 0.5 ml of 3.8% sodium citrate was added as an anticoagulant agent, and gently shaken to mix them completely. The sample was immediately used for measurement of whole-blood platelet aggregation. Residual 7 ml of the blood was placed in an EDTA-2Na blood vacuum tube, and centrifuged at 2500 rpm for 10 minutes at 4°C. This was used to measure IgG titers against *P. gingivalis*.

Incubation of *P. gingivalis*

P. gingivalis ATCC 33277 strain provided by Section of Periodontology, Department of Hard Tissue Engineering, was incubated under the anaerobic environment (10% CO₂, 10% H₂, 80% N₂) at 37°C. As a culture medium, a tripticase soy (TS) blood agar plate added 5-mg/l hemin and 50-μg/l vitamin K₁ was used. For whole-blood platelet aggregation, *P. gingivalis* incubated under the above conditions for 2 days was used. The number of *P. gingivalis* was measured by direct microscopic counting procedure using the Petroff-Hausser counting chamber, and the concentration was adjusted to be 10⁷-10⁸ cells/ml.

Table 1. Characteristics in peripheral arterial disease (PAD) and Control Groups

Characteristic	PAD patients	Control subjects
Number of subject	30	26
Age (Mean)	72.2	32.3
Gender (Male/Female)	26/4	20/6
Hypertension (%)	25 (83)	2 (8)
Diabetes mellitus (%)	16 (53)	0 (0)
Dyslipidemia (%)	10 (33)	0 (0)
Ischemic heart disease (%)	11 (37)	0 (0)
Smoking (%)	26 (87)	10 (38)
Past (%)	21 (70)	4 (15)
Current (%)	5 (17)	6 (23)

A positive history of hypertension was defined as the use of antihypertensives and/or a systolic pressure above 140 mmHg and/or diastolic pressure above 90 mmHg.

A positive history of diabetes mellitus was defined as a history of diagnosed diabetes, use of insulin and/or hypoglycemic medication, and/or a fasting blood glucose level higher than 7 mmol/L and/or a hemoglobin A_{1c} level above 0.065.

A positive history of dyslipidemia was defined as the use of cholesterol lowering medication or a serum total cholesterol level above 5.68 mmol/L and/or a serum triglyceride level of more than 1.58 mmol/L.

Measurement of whole-blood platelet aggregation

To measure whole-blood platelet aggregation, Chrono-Log Whole Blood Impedance Aggregometer (WBA) Model 591 (Chrono-Log Corp., U.S.A.) was used. With WBA, platelet aggregation is measured as changes of electrical impedance levels. Since cellular segregation is not required, WBA is thought to be simpler and easier method than optical measurements¹⁹. In addition, since the whole blood samples reserve platelet activators (agonists) such as prostacyclin and thromboxaneA₂ (TXA₂), WBA has been used to prove the effect of antiplatelet drugs in many studies as well as to evaluate the platelet function in various diseases^{19,21}. Phosphate buffered saline (PBS) of 500 µl was added to the 500 µl whole blood mixed with sodium citrate. The sample was incubated at 37°C for 5 minutes. After confirming its stability at baseline by 1-minute monitoring, 50 µl of *P. gingivalis* (10⁴-10⁵ cells) was added, and the changes of electrical impedance levels were measured for subsequent 12 minutes. All the charts showing changes over time were saved. All the measurements were performed within one hour after blood sampling, and were repeated 3 times per a sample.

Measurement of IgG titers against *P. gingivalis*

Seven milliliters of venous blood placed in an EDTA-2Na blood vacuum tube were centrifuged at 2500 rpm for 10 minutes at 4°C. The separated serum was immediately stored at -80°C until analysis. IgG titers were

measured using a previously described enzyme-linked immunosorbent assay (ELISA) method²². Since the subjects without teeth could be already infected by *P. gingivalis*, their data were excluded from the analysis.

Statistical Analysis

Excel-Toukei 2008 (Social Survey Research Information Co., Ltd.) was used for statistical analysis. Data were expressed as mean ± SD (minimum value - maximum value). Mann-Whitney U test was used, and p values of less than 0.05 were considered to be statistically significant. The absolute values of the correlation coefficient of not less than 0.7 were regarded to show the strong correlation.

Results

Characteristics of participants

Characteristics of PAD and Control Groups are shown in Table 1. The number of the blood platelets was 22.1 ± 6.8 (11.8-40.0) × 10⁴ cells/µl in PAD Group, and 20.2 ± 4.4 (12.6-32.1) × 10⁴ cells/µl in Control Group. There was no significant difference between two groups, and other blood test did not indicate any significant difference, either.

Whole-blood platelet aggregation reaction by *P. gingivalis*

Representative changes over time of electrical imped-

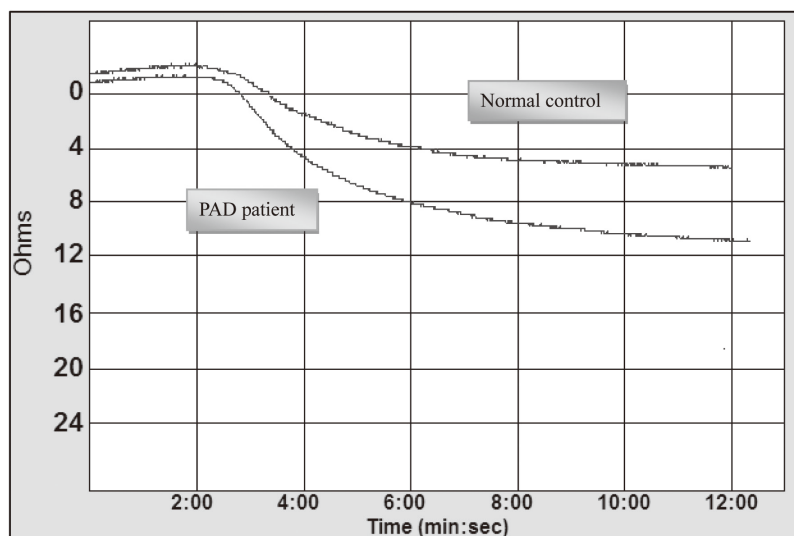


Figure 1 : Whole-blood platelet aggregation by *Porphyromonas gingivalis* (*P. gingivalis*)
Time-course changes of electrical impedance (ohm) after adding *P. gingivalis* to whole blood are shown. The changes of electrical impedance within 12 minutes after adding *P. gingivalis* (0 min) was recorded as whole-blood platelet aggregation reaction.

ance levels after adding *P. gingivalis* in each group are shown in Fig. 1. This PAD patient shown in Fig. 1 was a 70-year-old man. He was classified in grade II using Fontaine classification (TP=30 mmHg). He had a history of hypertension, and the number of the blood platelets was 28.1×10^4 cells/ μ l. In both cases, there was a time lag of approximately 2 minutes between the adding of *P. gingivalis* and the start of reaction. In addition, all the reaction achieved nearly equilibrium 10 minutes after the start of reaction. Twelve-minute changes of electrical impedance after adding *P. gingivalis* to whole blood were 10.0 ± 2.3 (6.6-13.0) ohm in PAD Group, and 6.1 ± 2.9 (0.3-9.5) ohm in Control Group. PAD Group showed significantly stronger whole-blood platelet aggregation by *P. gingivalis* than Control Group (Fig. 2). The past medical history and the number of the blood platelets were compared with the following two groups in PAD patients: strong whole-blood platelet aggregation (>12 ohm; n=10) group and weak whole-blood platelet aggregation (<8 ohm; n=8) group. The number of the blood platelets was 25.2 ± 8.5 (11.8-40.0) $\times 10^4$ cells/ μ l in strong platelet aggregation group, and 19.8 ± 4.2 (13.5-25.7) $\times 10^4$ cells/ μ l in weak platelet aggregation group. There was no significant difference between two groups about both the past medical history and the number of the blood platelets.

PAD patients were classified depending on disease severity, and levels of whole-blood platelet aggregation were compared. There were 20 patients who had

grade II, 2 grade III and 8 grade IV. The changes of electrical impedance were 9.1 ± 2.3 (6.6-13.0) ohm in patients with grade II, and 11.4 ± 1.5 (9.0-13.0) ohm in grades III and IV (Fig. 3).

When classifying PAD patients into two groups of high TP (>50 mmHg; n=9) and low TP (<50 mmHg; n=21) groups, whole-blood platelet aggregation was 7.6 ± 0.9 (6.6-8.8) ohm in the high TP group, and 10.3 ± 2.2 (6.6-13.0) ohm in the low TP group (Fig. 4). The patients with more severe PAD showed stronger whole-blood platelet aggregation.

Serum IgG titers against *P. gingivalis*

Serum IgG titers against *P. gingivalis* were significantly higher in PAD Group (14.5 ± 15.8 (0-52.0)) than in Control Group (2.6 ± 3.8 (0-10.8)) (Fig. 5). The details of the 8 PAD patients whose serum IgG titers were especially high, 5 patients were grade II, one patient was grade III, 2 patients were grade IV using Fontaine classification. Only one patient had high TP (>50 mmHg), and other 7 patients had low TP (<50 mmHg).

Whole-blood platelet aggregation and IgG titers

The correlation between whole-blood platelet aggregation and IgG titers was investigated in 17 PAD patients with teeth. There was a strong positive correlation with a correlation coefficient of 0.7 (Fig. 6).

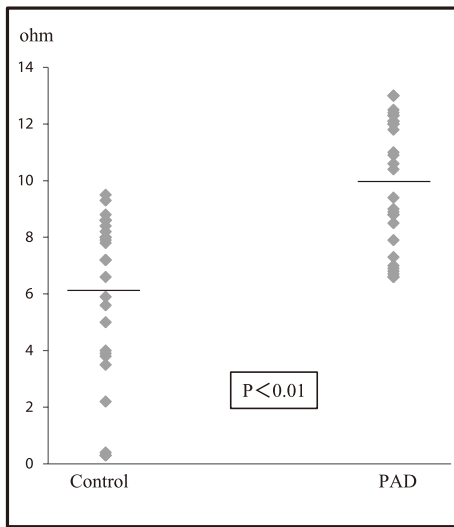


Figure 2 : Comparison of whole-blood platelet aggregation between peripheral arterial disease (PAD) and Control Groups
Whole-blood platelet aggregation by *Porphyromonas gingivalis* (*P. gingivalis*) was compared between PAD Group (n=30) and Control Group (n=26). PAD Group showed significantly stronger whole-blood platelet aggregation by *P. gingivalis* than Control Group ($P < 0.01$).

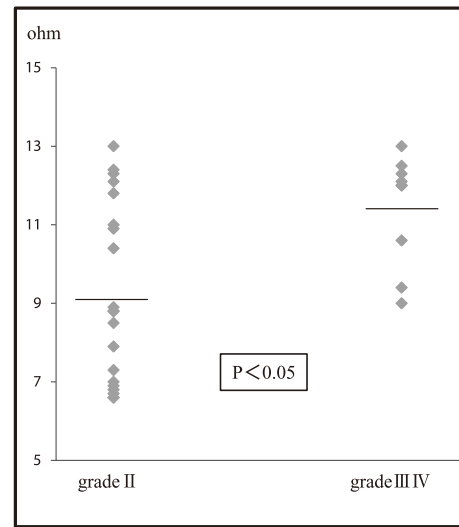


Figure 3 : Comparison of whole-blood platelet aggregation by peripheral arterial disease (PAD) severity (1)
Whole-blood platelet aggregation was compared between the following two groups divided based on Fontaine classification: grade II group (n=20) and grades III and IV group (n=10). Whole-blood platelet aggregation was significantly stronger in patients with more severe symptoms ($P < 0.05$).

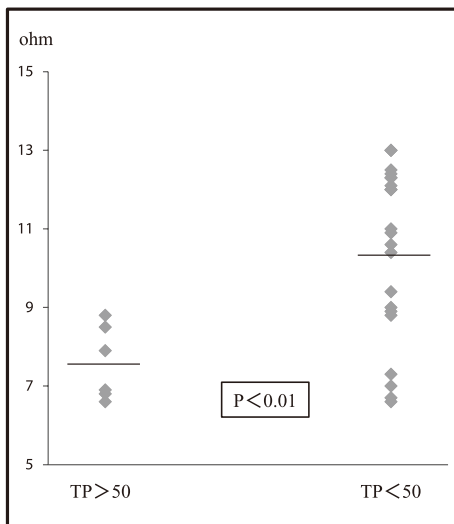


Figure 4 : Comparison of whole-blood platelet aggregation by peripheral arterial disease (PAD) severity (2)
Whole-blood platelet aggregation was compared between the following two groups divided based on toe pressure (TP): high TP group (> 50 mmHg; n=9) and low TP (< 50 mmHg; n=21) groups. Whole-blood platelet aggregation was significantly stronger in patients with worse test results ($P < 0.01$).

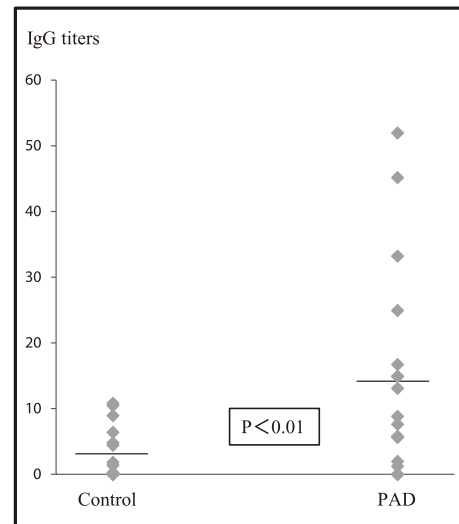


Figure 5 : Comparison of IgG titers against *Porphyromonas gingivalis* (*P. gingivalis*) between peripheral arterial disease (PAD) and Control Groups
Serum IgG titers against *P. gingivalis* were compared between PAD Group (n=17) and Control Group (n=23). Serum IgG against *P. gingivalis* titers were significantly higher in PAD Group than in Control Group ($P < 0.01$).

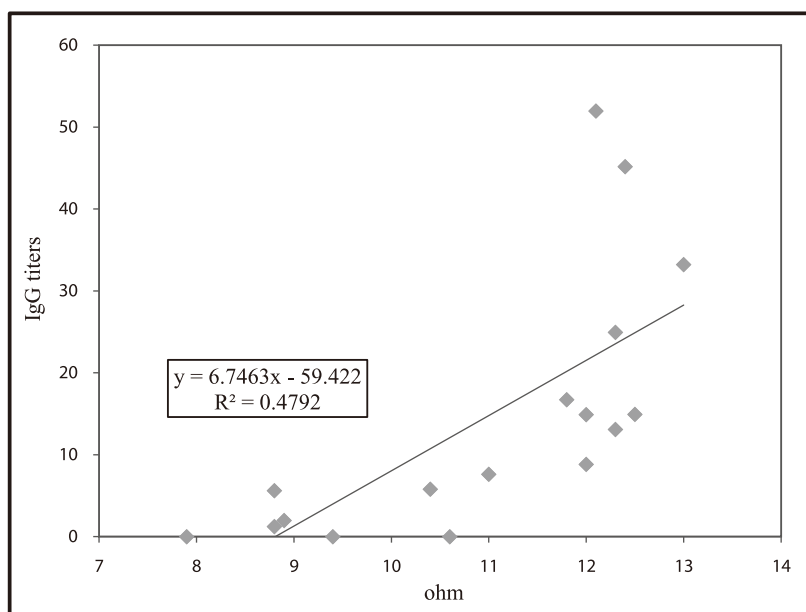


Figure 6 : Correlation between whole-blood platelet aggregation and IgG titers in peripheral arterial disease (PAD) patients with teeth
The correlation between whole-blood platelet aggregation reaction by *P. gingivalis* and IgG against *P. gingivalis* titers was investigated in 17 PAD patients with teeth. There was a strong positive correlation with a correlation coefficient of 0.7.

Discussion

In our study, platelet aggregation by *P. gingivalis* was evaluated in PAD patients under the closer-to-*in-vivo* environment by using whole blood. First, we confirmed that platelet aggregation was induced by reacting whole blood with *P. gingivalis*, as with other platelet aggregators, in Control Group (Figs. 1 and 2). Further, as shown in Fig. 2, PAD Group showed significantly stronger aggregation than Control Group. Age was significantly higher in PAD Group than in Control Group (Table 1). However, it is reported that whole-blood platelet aggregation induced by specific platelet aggregators such as collagen, arachidonic acid and ADP is not correlated with age or smoking^{23,24}. Therefore, it was thought that whole-blood platelet aggregation by *P. gingivalis* was scarcely affected by age or smoking. It is reported that whole-blood platelet aggregation is significantly stronger in hypertension patients without complications²⁵, but it has not been clarified yet that this reaction is primary or induced by hypertension²⁶. To our knowledge, there is no report on the correlation between whole-blood platelet aggregation and other risk factors such as diabetes mellitus and dyslipidemia. Influence of the factors other

than age and smoking on our measurement results could not be completely denied, although it was confirmed that whole-blood platelet aggregation by *P. gingivalis* was significantly higher in PAD patients, which was closer to the physiological conditions than PRP.

Fitzgerald et al. describe the mechanism that various bacteria such as *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *P. gingivalis* activate platelets and induce platelet aggregation in cardiovascular-related infectious diseases including infective endocarditis²⁷. Lourbakos et al. report that cysteine proteinase of gingipain-R, which is produced and secreted on the surface or outside of the bacterial body by *P. gingivalis*, has platelet activation ability in their study using washed platelets²⁸. However, Naito et al. thereafter prove that platelet aggregation by *P. gingivalis* is induced not by gingipain-R, but by binding of Hgp44 adhesin on the bacterial surface with Fc γ R II a receptors on platelets through IgG¹⁷. It was speculated that platelet aggregation in whole blood was induced by the same mechanism as that in PRP. Our study showed that platelet aggregation was induced by reacting whole blood with *P. gingivalis*. Aggregation was stronger in PAD Group, and there was a strong positive correlation between whole-blood platelet aggregation and IgG titers

in PAD patients with teeth. Our results indicated that PAD patients with a periodontal disease were under the conditions that platelet aggregation was easily accelerated due to increased IgG titers.

Within an organism, when over the certain number of *P. gingivalis* invaded the bloodstream, those that escaped from phagocytosis by leukocytes accelerated platelet aggregation; which might results in the involvement of *P. gingivalis* in arterial thrombus formation. As shown in Figs. 3 and 4, the patients with more severe PAD showed significantly stronger whole-blood platelet aggregation by *P. gingivalis*. It is reported that, in acute chest pain patients without elevation of troponin or changes on an electrocardiogram, Glycoprotein IIb/IIIa (GP IIb/IIIa)-positive platelet-derived-microparticles (PMPs) which is recently used as a platelet activation marker is a sensitive indicator²⁹. This result indicated that stimulation of platelet activation might be involved in the more serious events in patients with coronary artery diseases. PMPs are significantly increased even in PAD patients³⁰. Similarly, stimulation of platelet activation was thought to have a significance for patients with severe PAD. PAD patients repeating bacteremia by *P. gingivalis* had elevation of IgG titers in addition to progress of arteriosclerotic lesion, and were under the conditions that activation and aggregation of platelets were easily accelerated. Accordingly, they might have a high possibility to deteriorate ischemic symptoms due to occlusion or stenosis of the arteries.

Conclusions

In this study, it was confirmed that whole-blood platelet aggregation by *P. gingivalis* was significantly strong in PAD patients even under the closer-to-*in-vivo* environment, and also that serum IgG titers against *P. gingivalis* were significantly higher in PAD group than Control group. There was a strong positive correlation between whole-blood platelet aggregation and IgG against *P. gingivalis* titers, and *P. gingivalis* seemed to be involved in deterioration of symptoms.

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