Microbiome composition comparison in oral and atherosclerotic plaque from patients with and without periodontitis

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Abstract

Background There is no conclusive evidence regarding a causal relationship between periodontitis and atherosclerosis. In this study, we examined the microbiome in the oral cavity and atheromatous plaques from atherosclerosis patients with or without periodontitis to investigate the role of oral bacteria in the formation of atheromatous plaques. We chose four patients with and without periodontitis, who had undergone carotid endarterectomy. Bacterial samples were extracted from saliva on the tongue surface, from periodontal pocket (during the oral examination), and from the atheromatous plaques. We investigated the general and oral conditions from each patient and performed next-generation sequencing analysis for all bacterial samples. **Results** There were no significant differences between both groups concerning general conditions. However, the microbiome patterns of the gingival pocket showed differences depending on the absence or presence of periodontitis, while those of the saliva were relatively similar. The microbiome pattern of the atheromatous plaques was entirely different from that in saliva present on the tongue surface and gingival pocket, and oral bacteria were seldom detected. However, the microbiome pattern in atheromatous plaques was different in the presence or absence of periodontitis. **Conclusion** These results indicated that oral bacteria did not affect the formation of atheromatous plaques directly. However, the metabolic products of microbiome or the host inflammatory response might indirectly influence the composition of atheromatous plaques.

Introduction

Periodontitis is a predominant oral infectious disease in which an excessive immune response directed at the microbiome on the tooth surface destroys the periodontal tissue, forming periodontal pockets. The microbiome in these pockets includes pathogenic anaerobic bacteria that can form biofilms, which are inherently more resistant to antibacterial compounds and host immune components [1]. The mature biofilm causes further periodontitis progression because of the prolonged inflammation associated with the protracted immune response. Therefore, periodontitis has two main features: it is an infectious disease caused by microbiome imbalance and a chronic inflammatory disease caused by a dysregulated immune response.

These two characteristic features of periodontitis are shared by various systematic diseases, including diabetes, arteriosclerosis, cardiovascular diseases such as stroke and infective endocarditis, brain diseases, cancer, and non-alcoholic steatohepatitis, and increase the risks of preterm birth and low birth weight [2-9]. Arteriosclerosis includes atherosclerosis, in which an atherosclerotic plaque is formed on the blood vessel walls [10]. Briefly, the mechanism of plaque formation involves an endothelial dysfunction with various causes, such as hypertension, diabetes, obesity, and high cholesterol levels. All feature the accumulation of low-density lipoprotein (LDL) cholesterol between the intimal and medial endothelial surfaces. Monocytes that differentiate into macrophages ingest oxidized LDL cholesterol and die. Subsequently the cholesterol and lipid composition form an atherosclerotic plaque [11]. An association between atherosclerosis and periodontitis has been suggested in some epidemiology reports. Moreover, bacterial investigations aimed at detecting periodontal bacteria in the atherosclerotic plaque have been

conducted [12-14]. Although observational data support an association between periodontitis and atherosclerotic vascular disease, the data do not yet justify a causative relationship [15]. Multiple common factors that include diabetes, high blood pressure, dyslipidemia, and smoking habits affect the disease progression. However, there is little data concerning the direct involvement of periodontal bacteria in the development of atherosclerotic vascular disease [16, 17].

Porphyromonas gingivalis (*Pg*) is the most prominent pathogen in periodontal disease. Many studies have addressed the association between *Pg* and atherosclerotic vascular disease in clinical and animal models. Some clinical analyses sought to detect the DNA of *Pg* in atherosclerotic plaques [18-21]. We also reported the significant correlation of plasma IgG titer to *Pg*, which are higher in patients with severe periodontitis, with the level of LDL cholesterol [22]. Furthermore, in a mice model, an association between periodontitis and atherosclerotic vascular disease was demonstrated using *Pg* [23]. Ford et al. [24] also reported that intraperitoneal immunizations with *Pg* enhance levels of serum antibodies to *Pg* and atherosclerosis. Hypercholesterolemia and subsequent arteriosclerosis occurs in apolipoprotein E knock-out mice (*ApoE*^{-/-}), which have been used to investigate the association between periodontitis and atherosclerosis. Lalla et al. [2] reported that oral infection with *Pg* accelerates early atherosclerosis in *ApoE*^{-/-} mice. Maekawa et al. [25] reported that chronic oral infection with *Pg* accelerates atheroma formation in *ApoE*^{shl} mice. However, the authors did not detect *Pg* DNA (16S-ribosomal RNA) by PCR in the aortic valve. Thus, how *Pg* induce atheroma formation is still unknown. The impact of the disruption of the normal microbiome on various diseases is unclear and a comprehensive analysis is necessary, since the microbiome could contribute to the formation of the atheroma microbiome.

Microbiome analysis has typically involved bacterial culture [26]. However, recent comprehensive bacterial analysis using the gene for 16S ribosomal RNA (rRNA) have been successful in detecting bacteria that are difficult to cultivate [27]. NGS has become a popular means of examining a large number and volume of samples [28]. In this study, the association between periodontitis and atherosclerosis in the context of the microbiome in the oral cavity and atherosclerotic plaque was investigated by NGS.

Results

General conditions of patients were evaluated based on age, disease history, body mass index, blood pressure, C-reactive protein, cholesterol, and HbA1c (Table 1). There were no significant differences between both groups in terms of age, sex, other disease such as diabetes, and markers of inflammation and cholesterol. We evaluated the periodontal condition for each patient group from oral examination and plasma IgG antibody titer test (Table 2). In the control group, the ratio of deep pocket probing depth (PPD) (> 4 mm) was 2.2 ± 2.1 %. The ratio of PPD in the periodontitis group (30.1 ± 6.4 %) was significantly higher. Serum IgG antibody titer was significantly higher in those with periodontitis than in control group for *Aggregatibacter actinomycetemcomitans* (*Aa*) Y4, *Aa* ATCC29523, *Aa* SUNY67, *Capnocytophaga ochracea* (*Co*) S3, and *Pg* FDC381.

The microbiome pattern of tongue surface was relatively similar between control samples and periodontitis samples (Fig. 1A). Among them, the ratio of *Filifactor* was significantly higher in periodontitis patients than in the respective controls (Fig. 1B).

The microbiome pattern of gingival pockets were notably different between the control and periodontitis samples (Fig. 2A). The ratio of *Rothia* and *Neisseria* were lower in periodontitis than in control samples. Conversely, the ratios of *Fusobacterium* and *Filifactor* were higher in periodontitis than in control samples (Fig. 2A, B). The ratio of *Desulfobulbus* was significantly higher in periodontitis samples than in controls (Fig. 2B).

The majority of the bacteria found in the atheromatous plaques microbiome belonged to the soil bacterial families *Burkholderiales*, *Bacillale*, and *Rhizobiales*. Their ratios were similar between periodontitis and control patients (Fig. 3). The ratio of *Sphingomonadales* was higher in periodontitis samples than in control samples.

Three gingival pocket bacteria from periodontitis (P2, P3, P4; red) and one control sample (H4; blue) were positioned in the center right side of the PCA graph (separated by a red solid circle), three gingival pocket bacteria from control samples (H1, H2, H3) and one gingival bacteria from periodontitis (P4) were located into the center of the plot (separated by a blue solid circle), respectively (Fig. 4). The two circular locations were sufficiently separated. Tongue surface bacteria from periodontitis samples were located in the center left side of the panel (P1, P2, P3, P4) (separated by a red dotted circle). This position was slightly toward to the right side than that of the control samples (H1, H2, H3, H4) (separated by a blue dotted circle). These two circular locations were comparatively closer. The bacteria in atheromatous plaques were located towards the lower middle region of the plot (separated by a green solid circle), and control and periodontitis atheroma samples could not be clearly distinguished between them. The atheromatous plaques bacteria were located far away from the oral samples (tongue surface and gingival pockets).

Similar to the PCA results, bacteria from oral samples (tongue surface and gingival pocket) and atheromatous plaques were completely different (Fig. 5). However, 75% of atheromatous plaques bacteria from periodontitis were located at the lower part in the cluster and 75% of atheromatous plaques bacteria from controls were located at the upper part of the periodontitis samples.

We evaluated the correlation of the microbiome in atheromatous plaques between the control and periodontitis group. In both groups, the major bacteria of the network were *Agrobacterium*, *Delftia*, and *Rhizobium*. However, the network around *Cutibacterium* was different between control and periodontitis samples (Fig. 6).

Discussion

In this research, we performed a pilot study regarding a comprehensive microbiome analysis of the internal carotid artery stenosis in patients affected with and without periodontitis. We harvested bacteria samples from the tongue surface, gingival pockets, and atheromatous plaques from each patient and performed NGS analysis. The microbiome in the oral cavity was higher for the periodontal bacterial pathogens *Fusobacterium* and *Filifactor* in the periodontitis group compared to the control group. In particular, the ratio of *Filifactor* on the tongue surface was significantly higher in the periodontitis group in comparison to the control group. Conversely, the ratio of *Rothia* and *Neisseria* in gingival pockets, which are constituents of the normal bacterial flora in a healthy oral cavity, was lower in the periodontitis group than in the control group [29, 30]. Thus, remarkably, the ratio of normal bacteria in gingival pockets and on the tongue surface decreased, while the ratio of pathogenic bacteria increased.

As pathogenic factors for periodontitis, Red complex species (*Pg*, *T. denticola*, and *T. forsythia*) have been the focus of functional investigations [31]. Although there is no doubt regarding their relationship to periodontitis development, the microbiome does not contain just the pathogenic bacteria, but includes a mixture of various and diverse species of bacteria, to comprise the total population ultimately affecting the development of this disease [32]. It has been suggested that 17 novel bacteria including *Filifactor alosis* probably induce periodontitis, even though these bacteria were not previously thought to be periodontitis-specific pathogenic bacteria [33].

The normal bacterial flora in the oral cavity, which was previously disregarded as insignificant, is actually very crucial for periodontitis development or progression. In general, pathogenic bacteria, such as *Pg*, configure the microbiome with the normal bacteria flora [34]. If the balance of pathogenic and normal bacteria in the microbiome is lost for some reason, the microbiome increases its pathogenicity and induces the disease. Therefore, a comprehensive microbiome analysis is necessary to investigate normal as well as pathogenic bacteria composition.

To investigate the possibility that periodontal bacteria might contribute to atheromatous plaques formation directly on the vascular wall by hematogenous spread, NGS analysis was done using the atheromatous plaque samples. Previous reports established that *Pg* induces the expression of vascular cell adhesion molecule 1 from vascular endothelial cells, and promotes thrombus formation by macrophage invasion into blood vessels, resulting in platelet aggregation [35, 36]. Another report demonstrated that *Pg* infection accelerates the progression of atherosclerosis in a heterozygous apolipoprotein E-deficient murine model [23]. In the present study, oral bacteria were barely detectable in atheromatous plaques, regardless of the presence or absence of periodontitis. The patterns of the microbiome in atheromatous plaques were entirely different on the tongue surface and in gingival pockets. A prior study reported the detection of some oral bacteria in the atheromatous plaques [37]. However, other authors reported that *Pg* infection in an animal model induced atheromatous plaque formation, although this bacterium was actually not detected in the atheromatous plaques [38]. Our data enables us to conclude that it is unlikely that the oral bacteria spread hematogenously and directly induce the formation of atheromatous plaque on the aortic wall.

The co-occurrence analysis of the microbiome in atheromatous plaques revealed that the most significant bacteria were *Agrobacterium*, *Delftia*, and *Rhizobium*, which constituted the network in both groups. Although these are soil bacteria, they were also previously detected in atheromatous plaques [39]. Another significant bacterium, *Cutibacterium*, configured the network in the control group. The relationship with *Cutibacterium* was different among the control and periodontitis groups. This bacterium is categorized as a normal bacterium present on the skin and in the gut, although it was also detected in atheromatous plaques [40]. *Cutibacterium* reportedly can cause sarcoidosis, sepsis, and infective endocarditis, and heat-killed *Cutibacterium* render mice very susceptible to lipopolysaccharide (LPS) toxicity. *Cutibacterium* also promotes the production of cytokines, such as interleukin-12, interferon-gamma, and Toll-like receptor 4 [41]. Presently, it is conceivable that LPS produced by periodontal bacteria activated *Cutibacterium* in the blood vessels, which then form the atheromatous plaques. In this scenario, the difference of the network in atheromatous plaques between the control and periodontitis samples might be caused by LPS that is spread hematogenously, as well as by the chronic inflammatory effect. Recently, it was reported that the production of trimethylamine-N-oxide, which promotes atherosclerosis, depends upon the metabolism of the intestinal microbiome [42]. Some previous studies and the present data indicate that the loss of microbiome balance in the human body affects the development of atherosclerosis. Periodontitis has a great effect on the microbiome configuration in the oral cavity and promotes the formation of various metabolic products. However, this detailed mechanism of atherosclerosis development remains largely unknown. In a further study, we intend to investigate the relationship between periodontitis and atherosclerosis.

Conclusion

The ratio of oral bacteria in atheromatous plaques was remarkably low, and the microbiome pattern in the atheromatous plaques was entirely different from that found in the oral microbiome. In other words, oral bacteria did not directly induce the atheromatous plaque configuration. However, the microbiome pattern and the correlation of the microbiome in atheromatous plaques were different between the controls and periodontitis samples. Thus, metabolic products of the microbiome, or the host's inflammatory response, might indirectly affect the atheromatous plaque configuration.

Materials And Methods

Ethics statement

This study was approved by the ethics committee of Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences and Okayama University Hospital (Authorization Number: 1603–059) and Brain Attack Center Ota Memorial Hospital (Authorization Number: 121). All enrolled patients provided written informed consent for the use of their resected tissue and oral samples.

Participants

The study focused on 12 patients who visited Brain Attack Center Ota Memorial Hospital between April 2016 and March 2018, and who were diagnosed with internal carotid artery stenosis. The patients were $\geq$ 40 years of age, underwent carotid endarterectomy, had more than ten teeth, and consented to participate.

Samples

We harvested atheromatous plaques from the carotid artery walls extracted during carotid endarterectomy. Bacteria in the gingival pocket were collected using absorbent paper points (United Dental Manufactures Inc., Johnson City, TN, USA). The three paper points were inserted into patients' gingival pockets and were harvested after one minute. Each item was put them into an Eppendorf tube containing PBS. Later, bacteria from the tongue surface were collected using forensic swabs (Sarstedt AG & Co. Nümbrecht, Germany) by wiping the dorsum portion of each tongue several times. Each swab was placed in a tube and immediately stored at -80°C until DNA purification. Blood samples were collected from each patient and serum was prepared as previously described [43].

Oral examination

Periodontal examinations were performed to evaluate the average pocket probing depth (PPD) and rate of bleeding on probing (BOP) for each teeth of each patient. The patients were then divided into three groups according to the Japanese Society for Periodontology Clinical Practice Guideline for the Periodontal Treatment [44]. Briefly, patients with <5% of deep PPD (>4 mm) were defined as periodontally healthy (control, n = 4; H1-H4), patients (n = 4) with 6-23% deep PPD were defined as mild periodontitis and were excluded from further analysis, and patients with >24% of deep PPD were designated as severe periodontitis (periodontitis, n = 4; P1-P4).

DNA purification

APs were extensively minced using a scalpel and suspended in phosphate buffered saline (PBS). The collected material from paper points and swabs were resuspended using PBS. One milliliter of each resuspended bacterial sample was transferred to 2 ml Lysing Matrix B tubes (MP Biomedicals, Santa Ana, CA, USA) containing 0.1 mm silica beads and 500 μ l ATL buffer (Qiagen, Hilden, Germany). The contents of each tube were homogenized using FastPrep 24 (MP Biomedicals) for 45 s at 6.5 m/s. Bacterial DNA was extracted using the QIAamp DNA Microbiome Kit (Qiagen) according to the manufacturer's instructions. The quality and quantity of the DNA were verified using the NanoDrop 2000

spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and the PicoGreen dsDNA assay kit (Life Technologies, Grand Island, NY, USA).

Library preparation, sequencing, and analysis of 16S rRNA

The V3 and V4 regions of the 16S rRNA was amplified using the forward primer 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG, and the reverse primer 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC. Thermal cycling conditions were 98°C for 3 min; 25 cycles of 98°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and a final extension at 72°C for 5 min. After PCR clean-up step using AMPure XP Beads (Beckman Coulter, Inc., Brea, CA, USA), a second PCR was performed to add sequencing adapters and dual-index barcodes to the amplicon target to distinguish amplicons from each sample using the same reaction conditions with only eight cycles instead. After the PCR clean-up step, the quality and quantity of the amplicon were verified using KAPA library quantification kit (KAPA Biosystems Inc., Wilmington, MA, USA). Aliquots (5 µL) of the diluted amplicon from each library were combined to form pooling libraries. Six pM of pooling libraries with PhiX was sequenced using the MiSeq® system (Illumina Inc., San Diego, CA, USA). The obtained sequence was analyzed using the CLC Microbial Genomics Module of the CLC Genomics Workbench (CLC bio, Aarhus, Denmark). Briefly, we extracted 5911 bacteria having sequences that passed both those being retained and operational taxonomic units (OTUs) selected based on a 97% similarity with a minimum of 10 reads representing each OTU [45]. Principal component analysis (PCA) and clustering analysis were performed using R statistical software [39]. We also performed co-occurrence analysis for the 13 highly detected operational taxonomic units from control and periodontitis samples using the Quantitative Insights Into Microbial Ecology approach [46].

Plasma IgG antibody titer test against periodontal bacteria

Plasma IgG antibody titer against periodontal bacteria was determined as described previously [22, 43, 47, 48]. Bacterial antigens used were sonicated preparations of *Aggregatibacter actinomycetemcomitans* (Aa) Y4, Aa ATC29523, Aa SUNY67, *Eichenerra corrodens* (Ec) FDC1073, *Fusobacterium nucleatum* (Fn) ATCC25586, *Prevotella intermedia* (Pi) ATCC25611, *P. nigrescens* (Pn) ATCC33563, *Capnocytophaga ochracea* (Co) S3, *Porphyromonas gingivalis* (Pg) FDC381, Pg SU63, *Treponema denticola* (Td) ATCC35405, and *Tannerella forsythia* (Tf) ATCC43037. The sera from five healthy participants without periodontitis (24-29 years of age) were pooled and used to calibrate the analyses. Standard titration curves were prepared using serial dilutions of this pooled control serum. The absorbance of each sample after reaction was defined as an ELISA unit (EU), with 100 EUs corresponding to a 1: 3,200 dilution of the calibrator sample [43]. According to the formula for clinical use, the mean $\pm$ 2 standard deviations of the controls, based on the reported dataset of IgG titers to individual pathogens among five healthy individuals, was defined as the standard value of 1.

Statistical analysis

The statistical analysis was performed using the Mann-Whitney *U* Test. A *P*-value of 0.05 was considered significant and was determined using SPSS Ver. 23 (SPSS Inc., Chicago, IL, USA) for all the experimental results.

Abbreviations

NGS: Next-generation sequencing; LDL: low-density lipoprotein; Pg: Porphyromonas gingivalis; Aa: Aggregatibacter actinomycetemcomitans; Co: Capnocytophaga ochracea; rRNA: ribosomal RNA; PPD: pocket probing depth; BOP: rate of bleeding on probing; PCA: principal component analysis

Declarations

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Authors' contributions

DI performed samples collection, conducted the experimental works, interpreted the results, and wrote the draft manuscript. KY supervised all experiments and edited the manuscript. KM performed the oral examination and sample collection. MT performed co-occurrence analysis. SO, MS, YS operated patients and collected samples. MT and ST performed NGS analysis. ZA performed English editing for the manuscript. TK, KO, TY gave professional advises. ST was responsible for the study, supervised and edited the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All datasets present in the research article were submitted as supplementary data.

Ethics approval and consent to participate

This study was approved by the ethics committee of Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences and Okayama University Hospital (Authorization Number: 1603–059) and Brain Attack Center Ota Memorial Hospital (Authorization Number: 121).

Consent for publication

We obtained contents for publication from participants.

Competing interests

The authors declare that they have no competing interests.

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Tables

Due to technical limitations, tables 1 and 2 are only available as a download in the supplemental files section.

Figures

Figure 1

Figure 1

Characterization of microbiome on the tongue surface The average ratio of the bacteria on the tongue surface from control and periodontitis patient samples is presented. (A) Bacterial genera are indicated. (B) Thirteen bacterial species were highly detected by NGS analysis. * indicates $P < 0.05$; Mann-Whitney U Test.

Figure 2

Figure 2

Characterization of microbiome in gingival pockets The average ratios of the bacteria in gingival pockets from control and periodontitis samples are shown. (A) Bacterial genera are indicated. (B) Thirteen bacteria that were highly detected from the NGS analysis. * indicates $P < 0.05$; Mann-Whitney U Test.

Figure 3

Figure 3

Characterization of microbiome in atheromatous plaques The average ratio of the bacteria in gingival pocket from our patients (control and periodontitis samples) is depicted and the order of the bacteria is shown. Thirteen bacteria were highly expressed from the NGS analysis.

Figure 4

Figure 4

Comparison of PCA results between control and periodontitis samples The PCA results from each sample are identified tagged as follows: tongue surface: □; gingival pocket: ●; atheromatous plaques: △; controls: blue color; periodontitis: red color. Blue solid circle: three gingival pocket bacteria from control samples and one gingival pocket from periodontitis. Red solid circle: three periodontitis gingival bacteria pocket and one healthy gingival pocket sample. Red dotted circle: four tongue surface from periodontitis

samples. Blue dotted circle: four tongue surface from control samples. Green solid circle: four atheroma plaque samples from controls and four atheroma samples from periodontitis.

Figure 5

Figure 5

Comparison of clustering analysis results between control and periodontitis samples The clustering analysis results from each sample were tagged as follows: T: tongue surface; P: gingival pocket; A: atheromatous plaques. Blue text represents control samples and red text specifies periodontitis samples

OTU: Operational Taxonomic unit
 OTU_1: *Agrobacterium*
 OTU_2: *Cutibacterium*
 OTU_3: *Delftia*
 OTU_4: *Rhizobium*

Figure 6

Figure 6

Co-occurrence analysis of microbiome in atheromatous plaques Co-occurrence analysis for the 13 highly detected operational taxonomic units (OTUs) from control and periodontitis samples using the Quantitative Insights Into Microbial Ecology approach. correlation coefficient > 0.4. Abbreviations are: OTU_1: *Agrobacterium* OTU_2: *Cutibacterium* OTU_3: *Delftia* and OTU_4: *Rhizobium*

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