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Periodontal pathogens in atheromatous plaques. A controlled clinical and laboratory trial

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Objective: A possible relationship between periodontitis and cardiovascular disease has been suggested. The aims of this controlled clinical study were: (i) to ascertain the presence of periodontal bacteria DNA [*Actinobacillus actinomycetemcomitans*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythensis* (formerly *Bacteroides forsythus*)] in carotid atheromatous plaques and (ii) to assess the concomitant presence of the same periodontal bacteria DNA, if any, in periodontal pockets and in carotid atheroma in the same patient.

Methods: A total of 52 patients scheduled for carotid endarderectomy were enrolled in this study. The test group consisted of 26 dentate patients; the control group included 26 edentulous patients. A complete periodontal examination, including radiographic orthopanoramic and subgingival plaque sample, was performed in the test population. Oral and X-ray examinations were performed in the control group. Atheromatous plaques were harvested during surgical procedure for each dentate and edentulous patient and then sent to the microbiological laboratory. Subgingival plaque samples and carotid specimens were examined using the polymerase chain reaction (PCR) technique by means of specific primers for periodontal bacteria. Amplification of extracted DNA was tested using human beta-globin specific-primers.

Results: Out of 52 endarterectomy samples, 12 (seven dentate, five edentulous patients) were excluded as negative to DNA amplification. In subgingival plaque samples of 19 test patients, *T. forsythensis* (79%), *F. nucleatum* (63%), *P. intermedia* (53%), *P. gingivalis* (37%) and *A. actinomycetemcomitans* (5%) were found. No periodontal bacteria DNA was detected by PCR in any of the carotid samples in either patient group.

Conclusion: The presence of periodontal bacteria DNA in atheromatous plaques could not be confirmed by this study and thus no correlation could be established between species associated with periodontal disease and putative bacteria contributing to atheromatous plaques.

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In the past decade a large amount of data suggested a possible relationship between periodontitis and cardiovascular disease (1–4). Several authors have reported significant associations between periodontal parameters (probing depth, clinical attachment level and tooth loss) and the extent and severity of the atherosclerosis (5). These data have gradually provided impetus to the infectious hypothesis of atherosclerosis, which suggests that localized infectious triggers may induce a chronic inflammatory response leading to the development and progression of atherosclerotic plaques (6). Many researchers focused their attention on a proposed ability of bacteria to colonize atheromatous plaques; several microrganisms, such as herpes virus (6), *Chlamydia pneumoniae* (7) and Cytomegalovirus (8), were identified in atheroma. More recently, Chiu (9), Haraszthy *et al.* (10) and Taylor-Robinson *et al.* (11) reported on the ability of periodontal pathogens to colonize atheromatous plaques in the carotid artery. Unfortunately, no information concerning the oral/periodontal condition and no data about periodontal infection were reported in these studies. The aims of this study were: (i) to ascertain the presence of periodontal bacteria DNA [*Actinobacillus actinomycetemcomitans*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Prevotella intermedia* and *Tannerella forsythensis* (formerly *Bacteroides forsythus*)] (12) in carotid atheromatous plaques in dentate and edentulous cardiovascular patients and (ii) to assess the concomitant presence of the same bacterial DNA, if any, in periodontal pockets and in carotid atheroma in the same dentate patient.

Material and methods

Study population

The study population consisted of consecutive patients scheduled for carotid endarterectomy at the Department of Vascular Surgery (University of Florence, Italy) during a 7-month period. The patients were identified as candidates for carotid endarterectomy according to the standard medical practices of the vascular surgery department. Patients who had undergone antibiotic treatment during the last 6 months and/or who had received periodontal or dental treatments in the last 6 months were excluded from the study. Informed consent was obtained from all the study participants before examination.

Fifty-two patients were enrolled in the study before hospital admission. The test group consisted of 26 dentate patients. The control group consisted of 26 patients edentulous for at least 2 years before examination. Information about smoking history, hypertension, diabetes and cardiovascular disease was obtained through clinical chartings and interviews. Oral and X-ray examinations (radiographic orthopanoramic) were performed on both the test and control patients.

A complete periodontal examination (pocket depth, clinical attachment level, plaque index, bleeding on probing in six points/tooth) was performed in the test group by a single periodontist (CF). A sample of subgingival plaque was collected from the deepest pocket of each dentate patient. After a careful supragingival scaling, a sterile paper tip point was inserted in the pocket and maintained *in situ* for 10 s. Subsequently the paper tip point was inserted in an individual vial and sent to the laboratory.

The surgical procedures for carotid endarterectomy were performed 3 weeks after the oral examinations. Atheromatous plaques were harvested from all patients during surgery. The specimens were placed in a vial with 10 ml of sterile saline solution, free of previous contaminating DNA, and frozen at −20°C. Both subgingival plaques samples and surgical specimens were sent to the laboratory (LAMMB, Department of Molecular Biology, University of Siena).

Laboratory procedure

Subgingival plaque samples and carotid specimens were analysed by the study microbiologist (GC). DNA was extracted from the subgingival plaque samples according to Contreras *et al.* (13). The transport vials containing the atheromatous specimens were opened only in the laminar air flow safety cabinet at the microbiology laboratory. All the specimens were kept at −20°C until processing. Care was taken to maintain aseptic handling of tissue samples. Approximately 100 mg of tissue was harvested from the middle of the atherosclerotic plaque and then homogenized and subjected to DNA extraction. A standard protocol for DNA extraction was used based on proteinase-K digestion and the use of cetyltrimethylammonium bromide (CTAB) to remove complex polysaccharides that may inhibit polymerase chain reaction (PCR) amplification. The extracted DNA was measured by spectroscopy. The amounts of DNA extracted ranged from 30 ng to 100 ng for each endarterectomy specimen. The human beta-globin test was used as the control for inhibiting the amplification (14).

Primers design

Subgingival plaque and carotid specimens were examined using a PCR method for periodontal bacteria. The primers developed were characterized using the Oligo version 6 program (MedProbe, Oslo, Norway). The primers for *P. gingivalis* were designed on the collagenase gene sequence (GenBank Accession number AB006973). The primer OG49 (AAGGACTGAATATGCGCGCCCGATC) was complementary to the sequence 131–155; the primer Og50 (GAGGTGATAATTCGCTCTCGG TCCCTACATCT) was complementary to the sequence 1275–1306. The annealing temperature was 60°C and the length of the predicted product of the first amplification was 1176 bp. The primers for *A. actinomycetemcomitans* were designed on the *glyA* gene and the *lKtC* gene (GenBank Accession number Z23269). The primer OG57 (AGCGGACGTGAA AGAACTTGC) was designed using the *glyA* gene of strain *JP2* (GenBank Accession number Z23269) and was complementary to the sequence 1317–1337. OG58 (GCAATAGGAA CCCCATCTCTCAT) was designed using *lKtC* gene of the strain *JP2* (GenBank accession number M27399)

complementary to the sequence 258–280. The primers developed to detect *P. intermedia* OG51 (GTGCTTGCAC ATTCTGGACGTCGAC) and OG-52 (CGTCTGCAATTCAAGCCCGG GTAAG), *F. nucleatum* OG41 (GGC CACAAGGGGACTGAGACA) and OG42 (TTTAGCCGTCA CTTCTTC TGTTGG), *T. forsythensis*, OG45 (GTCGGACTAATACCTCATAAAA CA) and OG46 (TCGCC CATT GACCAATATT) were designed using the 16S small subunit ribosomal RNA gene sequences. All of the primer sequences were compared with the GenBank to ensure their specificity.

Positive controls

P. gingivalis, *T. forsythensis, P. intermedia* and *F. nucleatum* gene targets were cloned into pGemT-easy plasmids (Promega, Milan, Italy) and used as positive controls. *A. actinomycetemcomitans* CCUG 37005 was cultured on Shaedler Anaerobe Agar (Boehringer Mannheim, Gmbh, Germany) incubated in a chamber for 7 days at 37°C in anaerobic atmosphere created with AnaeroGen System (Sigma, St Louis, MO, USA). Colonies obtained from cultures were suspended in 500 μl of sterile water. DNA was extracted and quantified both by spectrophotometer and electrophoresis and used as the positive control.

PCR conditions

The PCR protocol consisted of a preliminary denaturation step (95°C for 5 min), 35 cycles of annealing (annealing temperature of each pair of primers for 1 min), extension (72°C for 1 min and 40 s) and denaturation (95°C for 1 min) and a final elongation (5 min at 72°C).

Analysis of PCR products

A 10 μl aliquot of amplified samples from a PCR tube was electrophoresed through a 1% agarose gel (Sigma) for 30 min. After 20 min of ethidium bromide staining (0.5 μg/ml), the amplification products were visualized and photographed under a UV light transilluminator (Bio-Rad Laboratories, Hercules, CA, USA).

Detection limit

In order to determine the PCR detection limit, plasmids were extracted from *Escherichia coli* by QIAprep Spin6 according to the manufacturer's instructions. Plasmid DNA was quantified by a spectrophotometer using 10-fold serial dilutions. Dilutions ranged from 10^8 to 10 copies/ml and 1 μl for each one was used in PCR to determine the sensitivity of the methods. DNA extracted from biological sample dilutions were spiked into beta-globin positive atheromatous DNA samples for the evaluation of the lower PCR detection limit.

Results

A total of 52 patients (26 dentate and 26 edentulous) were enrolled in the study. Twelve out of 52 patients (seven in test group and five in control group) were excluded because they were negative to DNA amplification using human beta-globin specific-primers. The test group (dentate patients) consisted of 19 patients (14 males and five females) with a mean age of 71.37 ± 6.14 years. Eleven patients were smokers. The mean tooth loss was 13.0 ± 6.25, mean clinical attachment level was 4.69 ± 1.58, mean pocket depth was 2.87 ± 0.82, mean full mouth plaque score was 75.95% ± 26.81 and mean full mouth bleeding score was 58.85% ± 27.15. The DNA of at least one of the probed bacteria was detected in each subgingival sample. The DNA of *T. forsythensis* was detected in 79% of patients, *F. nucleatum* in 63%, *P. intermedia* in 53%, *P. gingivalis* in 37% and *A. actinomycetemcomitans* in 5% of samples (Table 1). No statistically significant association between the periodontal pathogens was found. The control group (edentulous patients) comprised 21 patients (15 males and six females); the mean age was 73.33 ± 6.11. Anamnesis was generally positive for periodontal disease, as the patients reported that their teeth were frequently lost with elevated mobility (single patient results not shown). For the edentulous patient group no PCR for oral pathogens was performed, as were no comparable sites for sampling.

Table 1. Results of polymerase chain reaction detection in subgingival plaque samples

Bacterial species	Frequency of detection
Tanarella forsythiensis	15/19 (79%)
Fusobacterium nucleatum	12/19 (63%)
Prevotella intermedia	9/19 (53%)
Porphyromonas gingivalis	8/19 (37%)
Actinobacillus actinomycetemcomitans	1/19 (5%)

The carotid specimens of all 40 patients revealed evidence of severe atherosclerosis; the plaques showed a calcified core with frequent circumferential fatty deposits. No DNA of periodontal bacteria was detected by PCR in any of the carotid samples in either patient group. The analysis of the detection limit of the PCR-based technique demonstrated the effectiveness of this procedure. Irrespective of the pathogen (*P. gingivalis*, *T. forsythensis*, *P. intermedia*, *F. nucleatum* and *A. actinomycetemcomitans*), the methodology allowed for the amplification of 10 genome equivalents. When using spiked clinical samples the detection limit was found to be 100 genome equivalents, which is equivalent to 1000–5000 bacteria per gram of tissue sample.

Discussion

Different studies have suggested a possible association between periodontitis and the extent and severity of cardiovascular diseases (1–5). However, complete evidence has not been established in this field and other studies which questioned this association have been published (15–17).

Experimental studies have demonstrated the ability of such periodontal pathogens to interact with the endothelial surface (18) and to induce smooth cell proliferation (19) and the local release of inflammatory cytokines (20). Therefore the presence of periodontal bacteria in human atherosclerotic plaques (9–11) may play a role in

the initiation, development and progression stage of atherosclerosis.

The present case-control study was first aimed to ascertain the presence of periodontal bacteria DNA in carotid atheroma in dentate and edentulous patients. Periodontal examination in the test group demonstrated a high rate of patients with chronic periodontitis associated with high mean age, poor oral hygiene and smoking history.

A total of 52 carotid samples were collected and analysed for DNA of periodontal pathogens (*P. gingivalis*, *T. forsythensis*, *P. intermedia*, *F. nucleatum* and *A. actinomycetemcomitans*) by PCR. Twelve out of the 52 carotid samples were excluded from the analysis because they were negative to the human beta-globin test used as the control for inhibition of the DNA amplification. The reason for this may be explained by the fact that certain reagents such as porphyric components may hinder the results of the amplification (21).

Subgingival plaques from 19 dentate patients (corresponding to dentate patients with carotid samples positive to human beta-globin test) revealed the presence of at least one of the periodontal species. No statistically significant association among pathogens was reported. This microbial profile reflects the findings described in specific studies that investigated the composition of subgingival plaque in much larger sample of patients (22, 23).

In neither the dentate nor edentulous patient groups was DNA of periodontal pathogen detected in the carotid atheromatous plaques.

Our data do not concur with the results published by Chiu (9) (immunocytochemical investigation – 42% of carotid plaques positive for *P. gingivalis*), Haraszthy *et al.* (10) (PCR-amplified 16S rDNA and DNA species-specific probes, 30% positive for *T. forsythensis*, 26% for *P. gingivalis*, 18% for *A. actinomycetemcomitans*, 14% for *P. intermedia*), Taylor-Robinson *et al.* (11) (PCR-amplified 16S rDNA and universal primers, 22% positive for *A. actinomycetemcomitans* and 9% for *P. intermedia*) and Ishiara *et al.* (24) (PCR-amplified 16S rRNA, 21.6% positive for *P. gingivalis*, 23.3% for *A. actinomycetemcomitans*, 5.9% for *T. forsythensis*). These studies identify at least one, but often multiple, periodontal pathogen in atheromatous samples, whereas we have no evidence of periodontal bacteria DNA in our sample collection.

A possible reason for this difference could be the methodology used in different laboratories. PCR procedures may extensively vary for extraction procedures, primers designs and reaction conditions. The primers used in this PCR study differ from those applied in the previous studies. The primers for *P. gingivalis* and *A. actinomycetemcomitans* were designed using specific gene sequences; those for *T. forsythensis*, *P. intermedia* and *F. nucleatum* were developed using the 16S small subunit ribosomal RNA gene sequences. The efficiency of our primers was confirmed on clinical samples spiked with plasmid DNA. An alternative hypothesis, even if less probable, could be that the prevalence of periodontal bacteria DNA in atheromatous lesions differs due to epidemiological reasons (disease stage, nutrition, geographic factors, ethnicity, etc.). However, the presence of bacterial DNA in atheromatous plaques still remains a controversial issue. An example is a multicenter PCR comparison trial for the detection of *C. pneumoniae* in endarterectomy specimens, in which the positivity rate varied between 0 and 60% using different test methods on the same atherosclerotic plaques (25).

In conclusion, the results of this study do not support the previous findings that reported a frequent presence of periodontal pathogens in carotid atheroma lesions. Our data, therefore, tend to exclude a direct correlation between the detection of periodontal bacteria DNA in oral lesions and its concomitant presence in carotid atheroma.

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