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Detection and Localization of Periodontopathic Bacteria in Abdominal Aortic Aneurysms

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Objectives. *We examined a possible link between periodontal disease and abdominal aortic aneurysm (AAA) by studying resected aneurysmal specimens from AAA patients for the presence of periodontitopathic bacteria.*
Design. *Prospective case control study.*
Material and methods. *Thirty-two AAA patients were enrolled in the study. Periodontitis was classified according to the probing depth of preriodontal pocket. Thirty-two aneurysmal walls, 16 mural thrombi, 5 atherosclerotic occlusive aorta and 5 control arterial tissue, were examined for 7 periodontal bacteria using polymerase chain reaction (PCR) method. The localization of the bacteria in the aneurysmal/atherosclerotic wall was determined by thromboendarterectomy.*
Results. *All patients had periodontal disease, and most cases were severe. PCR examination of the aneurysmal specimens showed that 86% were positive for periodontal bacterial DNA. No bacteria were detected in the control specimens. The bacteria were found in both the intimal/medial layer and the adventitial layer of the aneurysmal wall but only in intimal/medial layer of the atherosclerotic occlusive aorta.*
Conclusion. *Periodontopathic bacteria were present in a high percentage of specimens of diseased arteries from AAA patients and were found throughout the whole aneurysmal wall. These bacteria may play a role in the development of AAAs and/or contribute to weakening the aneurysmal wall.*

Keywords: Periodontitis; Abdominal aortic aneurysm; Periodontopathic bacteria; Polymerase chain reaction.

Introduction

Risk factors for nonspecific abdominal aortic aneurysm (AAA) include smoking, hypertension, hyperlipidemia, male sex, and advanced age. However, these factors do not completely account for the development of AAA. Several studies have suggested a link between atherosclerosis and microbial infections. Chronic systemic inflammatory diseases, including infections with *Chlamydia pneumoniae*, *Helicobacter pylori*, and *cytomegalovirus*, and chronic dental infections (such as periodontitis) have been hypothesized to contribute to the systemic development of arteriosclerosis.[1–7] Because atherosclerotic disease and aneurysmal disease have similar risk factors, periodontal disease may also be involved in the development of aneurysmal disease, perhaps by means of degeneration of the arterial wall by periodontal bacteria. In Japan, the prevalence of periodontal disease is still higher than that in other developed countries. Thus, periodontal disease remains an important public health concern.

The aim of this study was to examine a possible link between periodontitis and AAA. If such a link exists, it could have important implications, especially with respect to the prevention and treatment of vascular disease. The present study examined whether periodontal bacteria-specific DNA were present in specimens of diseased arterial wall and mural thrombus removed from patients during AAA repair. We also investigated the localization of periodontopathic bacteria within the aneurysmal wall and atherosclerotic occlusive wall.

Materials and Methods

Patients and specimens

Patients who were referred to our institution for surgical AAA repair between April 2001 and June

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2003 were considered for enrolment in the study. Those found to have inflammatory or infective AAA were excluded from further analysis; only those with nonspecific AAAs were included. Preoperatively, angiography, ultrasonography, and computed tomography (CT) were performed to evaluate the arterial lesion. Patients with AAA larger than 4 cm in maximal transverse diameter measured by CT scanning underwent surgical repair. In total, 32 patients (27 males and 5 females; mean age, 73 years; range, 52–80 years) were enrolled in the study. The patients' data (risk factor for vascular disease and periodontal disease) were obtained from the chart review.

All patients underwent a complete blood count, blood chemistry analysis, coagulation test, measurement of acute-phase reactants (C-reactive protein and fibrinogen), and assessment of the risk factors for AAA and/or periodontal disease, which included hypertension and hyperlipidemia in relation to AAA, diabetes mellitus in relation to periodontal disease, and smoking in respect to AAA and periodontal disease. A positive history of hypertension was defined as the use of antihypertensive medication and/or a systolic pressure above 140 mmHg and/or diastolic pressure above 90 mmHg. A positive history of hyperlipidemia was defined as the use of cholesterol lowering medication and/or a high level of serum total cholesterol (5.68 mmol/l and above) and/or a fasting plasma triglyceride level of more than 1.58 mmol/l. A positive history of diabetes was defined as a history of diagnosed diabetes, use of insulin or hypoglycemic medication, or a fasting blood glucose level higher than 7 mmol/l or a haemoglobin A_{1c} level above 0.07.

Aneurysmal wall and thrombus specimens were obtained during surgical AAA repair. Two patients with splanchnic artery aneurysms and one patient with arteriovenous malformation were included in this study as control. Control specimens were defined as those without atherosclerotic findings macroscopically and microscopically in the arterial wall, which were the external iliac arteries from two AAA patients, visceral arteries from two splanchnic artery aneurysm patients, and popliteal artery from a patient with arteriovenous malformation. To localize periodontal bacterial DNA within the arterial wall, five resected aneurysmal wall samples were divided into the intimal/medial layer and adventitial layer by thromboendarterectomy. The different layers were analysed by PCR. To compare the bacterial localization between aneurysmal and atherosclerotic disease, five patients with atherosclerotic occlusive aorta were included in this study. Five atherosclerotic occlusive aorta specimens were obtained during aorto-bifemoral bypass and were studies by the same method as mentioned above.

Periodontal examination

Preoperatively, all patients including 32 AAA patients, five patients with atherosclerotic occlusive aorta and control patients were examined for periodontal disease by a periodontal specialist and were classified into four groups according to the probing depth of periodontal pocket. Patients with a periodontal pocket less than 2 mm were considered to periodontally healthy. A pocket of 2–5 mm indicated moderate periodontitis, and a pocket deeper than 5 mm was defined as severe periodontitis. Another grade was edentulous. Oral specimens (saliva and/or subgingival bacterial plaque) were taken at the same time and stored at −80 °C until use. The periodontal specialist was blind to clinical systemic findings of these patients (AAA, other arterial disease or control).

Polymerase chain reaction (PCR) analysis for periodontopathic bacteria

All aneurysmal, atherosclerotic and thrombus specimens obtained during operation and oral samples (saliva and/or plaque) obtained preoperatively, were examined by PCR analysis for seven most important periodontal pathogens: *Porphyromonas gingivalis*, *Treponema denticola*, *Prevotella intermedia*, *Campylobacter rectus*, *Tannerella forsythensis* (formerly, *Bacteroides forsythus*), *Prevotella nigrescens* and *Actinobacillus actinomycetemcomitans*. PCR analysis were performed by a technician who was blind to clinical findings of patients (AAA, other arterial disease or control), sampling sites and periodontopathic grade. Immediately after resection, the specimens were frozen and stored at −80 °C under sterile conditions. Just before PCR analysis, the samples were quickly thawed in a 37 °C water bath for 5 min. Then the samples were homogenized and RNA extracted using a High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) according to the instruction manual. The 16S rDNA-specific primers and reaction condition used here were described in detail by Ashimoto et al. before.[8] Briefly, 50 μl of PCR reaction mixtures contained 5 μl of samples, 5 μl of 10× PCR buffer (Promega, Madison, WI), 2.0 unit *Taq* DNA polymerase (Promega, Madison, WI), and 0.2 mM of each of deoxyribonucleotides (Promega, Madison, WI), 1.0 μM of each primer, and 1.5 mM $MgCl_2$ for *P. gingivalis*, *T. forsythensis*, *T. denticola*, *C. rectus*, 1.0 mM $MgCl_2$ for *A. actinomycetemcomitans*, *P. intermedia*, *P. nigrescens*.The temperature profiles included an initial denaturation step at 95 °C for 2 min, followed by 36 cycles of 95 °C (*P. gingivalis*, *T. forsythensis*, *T. denticola*,

C. rectus) or 95 °C (*A. actinomycetemcomitans*, *P. intermedia*, *P. nigrescens*) for 30 s (denaturation), 60 °C (*P. gingivalis*, *T. forsythensis*, *T. denticola*, *C. rectus*) or 55 °C (*A. actinomycetemcomitans*, *P. intermedia*, *P. nigrescens*) for 1 min (annealing), 72 °C for 1 min (*P. gingivalis*, *T. forsythensis*, *T. denticola*, *C. rectus*) or 2 min (*A. actinomycetemcomitans*, *P. intermedia*, *P. nigrescens*) (extension), and a final extension step of 72 °C for 10 min. Oral samples were analyzed using the same method.

Pathological assessment

Resected specimens of the aneurysmal and atherosclerotic walls and mural thrombi were fixed by immersion in 10% neutral-buffered formalin and embedded in paraffin. Subsequently, 4-μm sections were cut, stained with haematoxylin and eosin (HE) and Verhoeff-van Gieson stain, and examined microscopically.

Results

No patient had leukocytosis or an elevated level of C-reactive protein. The patients' risk factors for AAA and periodontal disease are shown in Table 1. All five male patients (mean age: 62 years, range 52–71 years) with atherosclerotic occlusive aortic disease were current smokers. One of these patients had a history of hypertension and another hyperlipidemia. The mean transverse maximum diameter of the AAAs repaired was 6.7 cm (range, 4–8 cm). All the 32 AAA patients were found to have periodontitis, and most cases were severe or edentulous (Table 2). The patients with atherosclerotic occlusive aorta were also found to have moderate or severe periodontal disease (moderate: 2, severe: three cases).

Table 1. Characteristics of 32 patients (mean age, 72 years) with abdominal aortic aneurysm

Characteristics	Abdominal aortic aneurysm
Sex: M/F	27 (84)/5 (16)
Risk factor for aneurysmal disease	
Hypertension	16 (50)
Hyperlipidemia	11 (34)
Risk factor for aneurysmal disease and periodontal disease	
Smoking	27 (84)
Past	18 (25)
Current	9 (59)
Risk factor for periodontal disease	8 (25)
Diabetes mellitus	3 (9)

Values are number (%) of specimens. The past smoking is defined as breaking free from cigarettes for more than 2 years.

The pathological examinations of the resected specimens showed lymphocytic responses but no leukocytosis or other evidence of infection. The microscopical findings were compatible with AAA with the evidence periodontopathic bacteria.

Results of the PCR analysis of aneurysmal wall, mural thrombus and oral samples of AAA patients are shown in Table 3 and Fig. 1. There were 28 patients (88%) positive for periodontopathic bacteria in oral samples. *P. gingivalis* was the most frequently detected (81%) in oral samples followed by *T. forsythensis* (72%), *T. denticola* (59%), *P. intermedia* (41%), *C. rectus* (34%), *P. nigrescens* (19%) and *A. actinomycetemcomitans* (3%). From the 28 patients whose oral samples were positive for periodontopathic bacteria, bacterial DNA were found in the aneurysmal wall specimens of 24 patients (84%), with *P. gingivalis* and *T. denticola* being the most frequently detected species (85 and 63%, respectively). Periodontal bacterial DNA was also observed in 14 of the 16 mural thrombus specimens examined (88%). In spite of high positive rate in oral samples (72%) of *T. forsythensis*, *T. forsythensis* were detected in only five patients (16%) in aneurysmal wall and not detected in the mural thrombus. Bacterial DNA was not detected in any control specimens. Analysis of different aneurysm wall layer after thromboendarterectomy showed bacterial DNA (four of the seven pathogens studied) in both the intimal/medial and the adventitial layers. In contrast, we could not detect periodontopatal bacterial DNA in the adventitial layer of the atherosclerotic arterial wall sample (Table 4).

Discussion

In 1989, Mattila et al.[9] described a controlled study that revealed a strong association between dental disease and acute myocardial infarction due to atherosclerosis. In 1998, Mendez et al.[5] reported a study which found that periodontal disease was a significant independent risk factor for peripheral vascular disease. All the patients in that study had moderate to severe periodontal disease, as indicated by periodontal attachment loss or edentulous condition. The degree of attachment

Table 2. Grade of periodontal disease in 32 patients with abdominal aortic aneurysm

Grade of disease	Abdominal aortic aneurysm
Periodontally healthy [normal]; probing depth, <2 mm	0
Moderate periodontitis; probing depth >2 mm but <5 mm	7 (22)
Severe periodontitis; probing depth >5 mm	20 (63)
Edentulous	5 (15)

Table 3. Presence of periodontal bacteria in oral samples, and the positive ratio of periodontal bacteria in arterial (aneurysmal) wall and mural thrombus to positive finding of those in oral samples

Type of bacteria	Oral sample ($n=32$)	Aneurysmal wall	Mural thrombus
All bacteria studied	28 (88)	24/28 (86)	14/16 (88)
Porphyromonas gingivalis	26 (81)	22/26 (85)	12/15 (80)
Treponema denticola	19 (59)	12/19 (63)	3/10 (30)
Prevotella intermedia	13 (41)	4 /13 (31)	0/7 (0)
Campylobacter rectus	11 (34)	5/11 (45)	1/7 (14)
Tannerella forsythensis	23 (72)	5/23 (22)	0/13 (0)
Prevotella nigrescens	6 (19)	1/6 (17)	0/3 (0)
Actinobacillus actinomycetemcomitans	1 (3)	0/1 (0)	0

Values are number (%) of specimens. Bacteria are detected by polymerase chain reaction assay.

loss in their investigation was substantially higher than the average level in Japan.[10] In the light of these findings regarding a link between dental and vascular disease, we investigated the possible association between periodontal disease and AAA by studying specimens obtained at AAA repair for the presence of periodontopathic bacteria.

Previously several studies have used PCR method to detect various infectious microorganisms in patients with vascular disease. *C. pneumoniae*, cytomegalovirus,

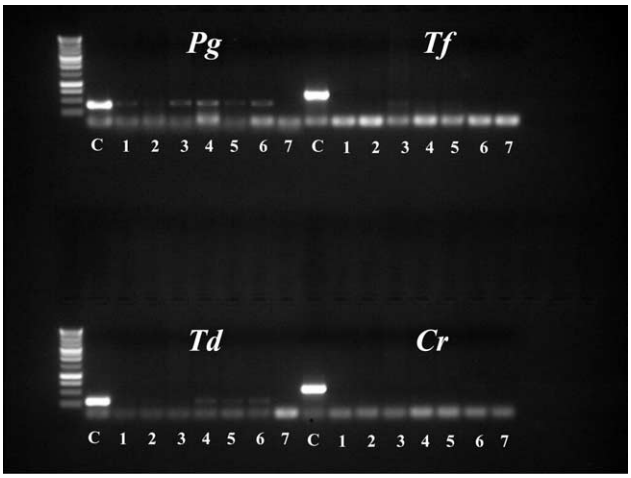

Fig. 1. Polymerase chain reaction assay with amplified bands of periodontal bacteria-specific 16S ribosomal RNA. Lanes 1 to 7 represent results with a specimen of surgically excised diseased arterial (aneurysmal) wall from a patient with abdominal aortic aneurysm. Lane C is the positive control. Positive bands are present in lanes 1 to 6 for *Porphyromonas gingivalis*, in lanes 4 to 6 for *Treponema denticola*, and in lane 3 only for *Tannerella forsythensis*. There is no positive band for *Campylobacter rectus*.

and *H. pylori* were found in atherosclerotic plaque, especially that from carotid arteries.[1] Chiu[1,11] observed multibacterial infections in carotid plaque. Identification of *C. pneumoniae* in atherosclerotic lesions of coronary arteries by electron microscopy, PCR, and immunocytochemistry (ICC) employing *C. pneumoniae*-specific monoclonal antibody was reported in 1992.[12] Subsequently, *C. pneumoniae* was also found in abdominal aortic tissue.[13–15]

Periodontopathic bacteria were first detected in atherosclerotic lesions in 1999.[11] In patients with periodontitis, subgingival plaque organisms can be introduced into the bloodstream many times a day through chewing and toothbrushing. Thus, the oral cavity or periodontal pockets may represent a large reservoir of gram-negative pathogenic microorganisms that could interact with cardiovascular tissues.[16] Recently, periodontal pathogens have been found in atherosclerotic lesions in peripheral arteries as well as in the abdominal aorta.[17,18]

In the current study, we used PCR method to determine whether seven species of periodontal bacteria were present in specimens of diseased arteries and oral samples from patients with AAA and atherosclerotic occlusive aorta. Poor periodontal conditions were observed in AAA patients studied compared to the average level of Japanese general population.[10] High detection frequencies of periodontopathic bacteria were found in both the oral and arterial samples from AAA patients. We observed no periodontopathic bacteria in control arterial wall specimens. Periodontal diseases were suggested to be associated with AAA to some degree. Two species, *P. gingivalis* and *T. denticola*, were frequently detected among aneurysmal wall, mural thrombus and oral samples. In spite of the detection of *T. forsythensis* in the saliva of 72% of patients, this organism was found in only five aneurysmal walls (16%). Several studies on the detection of oral bacteria from AAA lesions have been reported recently. Martin et al.[19] reported a case of aortic aneurysm caused by *A. actinomycetemcomitans* with endocarditis showing high fever, which was a mycotic (infected) aneurysm. In the present study we excluded mycotic aneurysms. da Silva et al.[20] reported an association between AAA and several bacteria including periodontopathic bacteria. These authors reported a different groups of pathogens including *Propionibacterium acnes*, *Propionibacterium granulosum*, *Actinomyces viscosus*, *Actinomyces naeslundii*, and *Eggerthella lenta*. Okuda et al.[18] also used PCR to determine whether the periodontal pathogens *P. gingivalis*, *T. denticola*, *T. forsythensis*, and *A. actinomycetemcomitans* were present in atherosclerotic lesions in patients with AAA. However, only *T. denticola* were

Table 4. Localization of periodontal bacteria in arterial wall in abdominal aortic aneurysm and aortic atherosclerosis

Type of bacteria	Abdominal aortic aneurysm ($n=5$)		Aortic atherosclerotic occlusive artery ($n=5$)	
	Intimal/medial layer	Adventitial layer	Intimal/medial layer	Adventitial layer
All bacteria studied	5	5	4	0
Porphyromonas gingivalis	5	4	4	0
Treponema denticola	5	4	3	0
Campylobacter rectus	3	2	1	0
Prevotella intermedia	0	0	0	0
Tannerella forsythensis	0	0	0	0
Prevotella nigrescens	0	0	0	0
Actinobacillus actinomycetemcomitans	0	0	0	0

Values are number of specimens. Bacteria are detected by polymerase chain reaction assay; localization within the arterial wall is done with use of thromboendarterectomy.

detected in their study. The difference between their findings and ours might be due to the fact that Okuda et al. studied formalin-fixed, paraffin-embedded specimens, whereas we examined fresh-frozen samples.

The findings of periodontal pathogens in AAA biopsies could be interpreted as a secondary phenomenon with transient bacteremia leading to invasion of already formed aortic aneurysm. Alternatively the bacteria could be instrumental in the development of AAA. Such an association might involve an inflammatory response or direct specific effects of the periodontal bacteria on host tissues. For example, *P. gingivalis* infection accelerated the progression of atherosclerosis in an ApoE-deficient murine model.[7] The periodontopathic bacteria could be introduced into the bloodstream many times a day through chewing and toothbrushing. It was possible that these bacteria then adhered to the vascular endotherial cell, especially those with initial injury such as atherosclerotic changes. Because only genetic materials, which appeared to be inactive, were recognized in our study, we could not determine whether the pathogens we detected were alive or in a chronic-latent or dead form. Further studies employing an animal model are required.

P. gingivalis was the most prevalent periodontopathic bacteria in the specimens from our AAA patients. An association between periodontal disease due to *P. gingivalis* infection and atherosclerosis was previously reported.[7,21] Fimbria, a cell membrane component of *P. gingivalis*, was found to be important to adhere to and invade vascular endothelial cells and thus accelerate progression of atherosclerosis by means of either direct infection[7,21,22] or thrombus formation.[23]

We found periodontal bacteria in both the intimal/-medial layer and the adventitial layer of the aneurysmal wall in patients with AAA, but could not detect periodontal bacteria in the adventitial layer of the atherosclerotic arterial wall (Table 4). Studies using immunohistochemistry (IHC) often observed *C. pneumoniae* in foam cells within atheromatous plaque and in macrophages in the intima, media, and adventitia layers.[24] Okuda et al.[18] found *T. denticola* in foam cells and the interstitium between smooth muscle cells by IHC. *T. denticola* was detected in foam cells, which were identified as macrophages and/or smooth muscle cells by double-labelling IHC.[18,24] *P. gingivalis* is able to invade the deeper structures of connective tissues of human gingiva by degrading epithelial cell junction complexes.[25] Periodontal pathogens could invade the intimal, medial, and adventitial layers of the arterial wall by degenerating intercellular matrix in the aneurysmal aortic wall.

In summary, a markedly higher prevalence of periodontitis was found in the AAA patients investigated compared to that in general Japanese population. Periodontal bacterial DNA was found in the resected aneurysmal wall or mural thrombus from 86% of these patients and in all layers of the aneurysmal wall. In the present study, we prospectively evaluated an association between periodontal disease and non-specific AAA by using PCR analysis. At present it is not clear whether the periodontopathic bacteria accelerate the growth or weakening of the aortic wall or whether they are secondary colonizers of the aneurysm.

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