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Oral Microbiology

Chlamydia pneumoniae together with collagenase-2 (MMP-8) in periodontal lesions

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OBJECTIVE: Dental infections may be associated with subsequent atherosclerosis. In this regard we wanted to study if traces of *Chlamydia pneumoniae* can be found from deep periodontal pockets characterized with elevated collagenase-2 [matrix metalloproteinase (MMP)-8] levels in gingival crevicular fluid (GCF). *Chlamydia pneumoniae* has not previously been found to infect the oral cavity.

SUBJECTS AND METHODS: Subgingival samples of dental plaque were collected from 31 teeth in 12 adult periodontitis patients by means of sterile curettes and examined for *C. pneumoniae* using a quantitative PCR technique. GCF samples were also collected and assayed by an immunofluorometric assay (IFMA) for MMP-8.

RESULTS: *Chlamydia pneumoniae* RNA was demonstrated in a sample from one of the patients studied. Periodontal treatment eliminated the *C. pneumoniae* from the patient's subgingival dental plaque as well as reduced GCF MMP-8 level.

CONCLUSIONS: Our findings suggest that *C. pneumoniae*, which is not normally thought to be involved in periodontitis, can be found in dental plaque.
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Keywords: *Chlamydia pneumoniae*; MMP-8; periodontal lesions; dental plaque

Introduction

Evidence from controlled clinical studies shows that infection with *Chlamydia pneumoniae* and dental infections may be associated with subsequent atherosclerosis (Danesh, 1999; Valtonen, 1999). However, *C. pneumoniae* has not so far been found to infect oral tissues. Tran *et al* (1997), using a 16S rRNA-based identification method, did not find the organism in subgingival dental plaque samples from 50 adult patients with advanced periodontal disease. However, *C. pneumoniae* has been identified in the oropharynx. During epidemics it has been found to be responsible for up to 9% of cases of pharyngitis in adults (Huovinen *et al*, 1989). We therefore sought *C. pneumoniae* in subgingival samples of dental plaque from adults with deep periodontal pockets. Deep periodontal pockets were also assayed for their levels of matrix metalloproteinase (MMP)-8, a tissue key destructive host-cell derived proteinase reflecting periodontal disease, in the gingival crevicular fluid (GCF) samples (Chen *et al*, 2000; Mäntylä *et al*, 2003).

Material and methods

The subjects of the study were generally healthy adults suffering from moderate – severe periodontitis (Mancini *et al*, 1999) and they were patients of the Institute of Dentistry of the University of Helsinki, Finland. All patients gave informed consent to participation in the study. Approval for the study was provided by the ethical committee of the Dental Institute of the University of Helsinki. Periodontal status was determined by measuring gingival pocket depths, and attachment levels/loss of periodontal supporting tissue, assessing amounts of bleeding on probing, and calculating a plaque index. Subgingival samples of plaque were collected from patients meeting the following criteria:

1 No history of antibiotic administration within the previous 6 months.
2 No history of treatment for periodontitis within the previous 6 months.
3 At least five sites with a periodontal probing depth ≥5 mm and radiographic signs of bone loss (Table 1).

Patients were diagnosed as having moderate periodontitis if there were fewer than 10 pockets >5 mm in

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Table 1 Clinical characteristics of 12 adult periodontitis patients, occurrence of *Chlamydia pneumoniae* in subgingival dental plaque samples from these patients, and gingival crevicular fluid collagenase-2 (MMP-8) levels in the patients

Age (years)	*Sex*	*Degree of Periodontitis*	*AL (mm) before treatment*	*PD (mm) before treatment*	*PD (mm) after treatment*	Chlamydia pneumoniae *(count) before treatment*	Chlamydia pneumoniae *(count) after treatment*	*MMP-8 level (μg/l) before treatment*	*MMP-8 level (μg/l) after treatment*
40	M	Severe	ND	4	<4				
						1952	1990	ND	ND
33	M	Severe	9	7	3,5			1687	833
			10	7	2	3 173 343[a]	1410	6172	2662
37	M	Severe	ND	6	5				
			ND	6	5	2740	1548	ND	ND
32	F	Moderate	4.5	6	3			2141	594
			0	4	2,5	2729	1337	2233	864
35	F	Moderate	6	5	4			7514	3160
			1.5	5	3	2694	2066	427	251
			1.5	5	3			246	1061
31	F	Severe	5	6	<4				
			5	7	5	2413	2547	5	474
			6	8	6			362	2786
65	F	Severe	ND	5	5				
			ND	7	4	3325	2335	ND	ND
			ND	6	5				
40	F	Moderate	ND	5	4				
			7	6	5,5	1680	2653	362	258
			6	6	3,5			20	99
			5	5	5			159	448
50	M	Severe	ND	6	6				
			ND	6	7	28 441	2223	ND	ND
			ND	7	5				
47	F	Severe	ND	6	5				
			4	5	4,5	2604	2138	0	606
			0	5	1			11	340
49	F	Severe	ND	5	<4				
			6	5	2	2251	3263	160	196
51	F	Severe	9	10	6			3753	411
			4	6	3	2250	1963	1966	1474
			4	6	3			1749	625
42.5[b]			4.9[b]	5.8[b]	4.2[b]			1609[b]	952[b]

AL, attachment loss; PD, probing depth; ND, not determined.
[a]positive, [b]mean.

depth and as having severe periodontitis if there were more than 10 pockets >5 mm in depth (Mancini *et al*, 1999). Only one patient was a non-smoker. Periodontal treatment consisted of scaling and root planing (SRP). Instruction relating to oral hygiene was given. Samples for microbiological investigation (Nieminen *et al*, 1995; Mellanen *et al*, 1996) and GCF samples for determination of MMP-8 (a marker for severe periodontitis) were taken before and after (4 weeks) SRP therapy (Chen *et al*, 2000; Mäntylä *et al*, 2003). Briefly, supragingival plaque was removed, the site was isolated and dried prior GCF sample collection (Mäntylä *et al*, 2003), and thereafter the subgingival plaque sample was taken with curette. The plaque samples from different periodontal pockets were pooled to give one sample per patient (Nieminen *et al*, 1995; Mellanen *et al*, 1996).

Subgingival plaque samples were collected from 31 teeth in 12 adult periodontitis patients, by means of sterile curettes, from one to four periodontal pockets ≥4 mm deep before therapy, and examined for *C. pneumoniae* by means of PCR. Supragingival plaque had previously been removed from the teeth with a curette. Gingival margins were dried with air and isolated from saliva with cotton rolls. All samples of subgingival plaque from different periodontal pockets were pooled in 1 ml of saline to give one sample per patient. DNA analyses were undertaken on the samples by means of the QIAamp DNA Mini Kit® (Qiagen, Hilden, Germany), following the instructions of the manufacturer. A hot-start technique was used with *C. pneumoniae OMP*1 gene probes. Europium-labelled probes were used in hybridization of PCR products. Counts of 50 000 or over were regarded as positive, counts of less than 50 000 as negative (Rintamäki *et al*, 2002).

Results

In one patient the count for *C. pneumoniae* was positive before periodontal treatment by SRP. This patient did not differ from the other patients as regards quantitative results related to microbiological or enzymatic markers (MMP-8) of periodontal disease (Table 1). The count in this patient was negative after SRP treatment.

Following culture of pooled subgingival plaque samples from untreated periodontal pockets the following

mean percentages of bacteria associated with periodontitis were found: *Porphyromonas gingivalis* 30%, *Prevotella intermedia* 11.2%, *Actinobacillus actinomycetemcomitans* 1.0%, *Bacteroides forsythus* 4.1%, *Campylobacter rectus* 9.6%, *Peptostreptococcus micros* 5.6%. Mean GCF concentration of MMP-8 was 1609 $\mu g/l$ before SRP, 952 $\mu g/l$ after, but in some GCF samples expressing ≤ 400 $\mu g/l$ MMP-8 increases in their MMP-8 concentrations could be observed (Table 1). However, all periodontitis sites expressing >1000 $\mu g/l$ MMP-8 in GCF MMP-8 responded to SRP (Table 1).

Discussion

Use of a quantitative PCR-technique resulted in *C. pneumoniae* being identified in only one subject. In a study by Tran *et al* (1997) *C. pneumoniae* was not found in dental plaque from any of the 50 patients involved. Regarding the sensitivity and specificity of our and the PCR method of Tran *et al* (1997) following points are noteworthy. Sensitivity of our hot start *C. pneumoniae OMP*1 gene PCR and liquid hybridization assay with Europium-labelled probe is about 1 *C. pneumoniae* elementary body (Rintamäki *et al*, 2002). The sensitivity measurement has been repeated several times with consistent results (Rintamäki *et al*, 2002). The sensitivity was achieved only when 50 PCR cycles were used; if number of cycles was less, sensitivity decreased. Tran *et al* used 36 PCR cycles in their 16S rRNA-based PCR identification method and amplicons were detected in 1.5% agarose gel electrophoresis stained with ethidium bromide. When we compared our liquid hybridization PCR to PCR with gel detection, the sensitivity of PCR with liquid hybridization with Europium-labelled probes was clearly higher than that with gel detection (Rintamäki *et al*, 2002). This may explain the difference between our study and that by Tran *et al* (1997). Specificity of our assay has been tested with *C. trachomatis, S. pneumoniae* and several other bacteria strains. The primer HB1 and HB2 are genus specific and the specificity of the assay is obtained by *C. pneumoniae* specific probe. Several applications have been published using gene sequences from different target genes and the sensitivity has been increased by the hybridization (Dowell *et al*, 2001; Boman and Hammerschlag, 2002). The time resolved fluorescence (TRF) technology is a reliable method for demonstration of some viruses and bacteria (Hukkanen and Vuorinen, 2002; Rintamäki *et al*, 2002). In addition, nowadays real-time PCR technique combines rapid amplification and quantitative product detection via specific hybridization in one step (Apfalter *et al*, 2003). Interestingly, the sensitivities of PCR methods for *C. pneumoniae* seem to vary hugely (Boman, Gaydos and Quinn, 1999; Apfalter *et al*, 2002). Isolated *C. pneumoniae* DNA was used by preparing two different positive dilutions for every assay. The negative control was included after every five samples. Optimal hybridization was confirmed by using two different synthetic target molecules. Our method was among the best in international comparison of PCR methods (Rintamäki *et al*, 2002).

Our findings suggest that mouths of patients with severe periodontitis can harbour *C. pneumoniae*. The patient harbouring *C. pneumoniae* aged 33 evidently had severe postjuvenile aggressive periodontitis and in this respect differed from the other patients. Furthermore, this finding may eventually indicate possible differences in the microflora and in this respect requires further studies. Overall, the anaerobic bacteria detected in our patients were typical of those found in deep periodontal pockets (Nieminen *et al*, 1995).

It is known that infections associated with progressive periodontitis destroy periodontal tissue and if not treated teeth can be lost. This destruction results in high levels >1000 $\mu g/l$ of collagenase-2 (MMP-8) in the GCF of severe postjuvenile or aggressive periodontitis patients (Mancini *et al*, 1999; Chen *et al*, 2000; Mäntylä *et al*, 2003). SRP result in levels of MMP-8 in the GCF reverting to those seen in periodontally healthy sites (Mancini *et al*, 1999; Chen *et al*, 2000; Mäntylä *et al*, 2003). The only case positive for *C. pneumoniae* seen in our study also responded to SRP. However, some periodontitis sites can be non-responsive to SRP and this can be reflected as even increases in GCF MMP-8 levels to which also post-treatment (SRP) wound healing may contribute (Said *et al*, 1999; Kiili *et al*, 2002).

Chlamydia pneumoniae is not normally thought to be involved in periodontitis. The presence of *C. pneumoniae* in deep periodontal pockets reported here therefore needs to be repeated on a larger scale to address it's contribution to potential relationship between periodontal and cardiovascular diseases.

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