Table 1. Detection patterns of mutans streptococci in layers of 3-day-old plaque taken from 10 subjects

	S. mutans										*S. sobrinus*									
Subject	A	B	C	D	E	F	G	H	I	J	A	B	C	D	E	F	G	H	I	J
DMFS	1	2	4	6	8	10	19	20	22	55	1	2	4	6	8	10	19	20	22	55
Layers																				
1	+	±	+	+	+	+	–	–	+	–	–	–	–	+	+	–	–	–	–	–
2	+	–	+	+	+	+	+	+	+	–	+	–	–	+	+	+	+	+	+	–
3	+	+	+	+	+	+	–	+	+	–	+	–	–	+	+	+	–	+	+	–
4	+	+	+	+	+	+	–	+	+	–	+	–	–	+	+	–	–	+	+	–
5	+	+	+	+	+	+	–	+	+	–	+	–	–	+	+	–	–	+	±	–
6	+	–	–	+	–	+	–	+	±	+	–	–	–	+	±	–	–	+	–	–
7	+	–	–	+	–	–	+	+	–	±	–	–	–	+	–	–	–	–	–	–
8	–	–	–	+	–	–	–	–	+	–	–	–	–	+	–	–	–	–	–	–
9	–	–	n.s.	+	–	n.s.	–	–	±	n.s.	–	–	n.s.	+	–	n.s.	–	–	–	n.s.
10	n.s.	+	n.s.	+	n.s.	n.s.	–	–	–	n.s.	n.s.	–	n.s.	±	n.s.	n.s.	–	–	–	n.s.

The number of plaque layers varied from 8 to 10 with the thickness of individual plaque, layer 1 being the outermost layer, layer 10 the innermost layer. n.s. = No sample available; + = detected; – = not detected; ± = detection difficult to judge.

brinus were detected were 56.5% (52/92) and 31.5% (29/92), respectively.

For the 7 subjects who had both species in their plaque, the distribution pattern of *S. mutans* in the plaque layers was compared to that of *S. sobrinus*. In every layer detected as mutans streptococci-positive, either only *S. mutans* or both species were detected. There were no layers in which *S. sobrinus* was clearly detected alone. Also there was a tendency for mutans streptococci to be frequently detected in the outer to middle layers of plaque, and these bacteria were seldom seen in the inner layers. The distribution pattern of mutans streptococci varied among the subjects, even though the results were obtained only from a small number of samples.

There was no obvious relationship between the pattern and distribution of mutans streptococci and the subjects' DMFS scores.

Discussion

The PCR using universal primers showed a positive band in every layer of dental plaque, indicating the existence of some bacteria throughout the plaque. Oral streptococci are aciduric facultative anaerobes and were therefore expected to be detected equally in both the outer aerobic and the inner anaerobic environments, irrespective of plaque structure. Nevertheless, the distributions of mutans streptococci-positive layers were limited, being seldom found in the inner plaque with the exception of subject D who had fully positive layers. The innermost layer corresponded to the bottom of the thick microbial deposit generated in the artificial stagnation site. It seemed to be difficult for mutans streptococci to survive in such an environment with a limited supply of nutrients such as sugar and with less pH drop. Thus, the levels of bacteria in the deeper layers in our devices might be below the detection limit of our microbiological method, even though it is claimed that this nested PCR could detect 100 and 10 fg DNA of *S. mutans* and *S. sobrinus*, respectively [Sato et al., 2003]. On the other hand, Dibdin and Shellis [1988] suggested that plaque formed in the presence of sucrose promoted porosity of the extracellular matrix. A fully positive pattern for mutans streptococci as seen in subject D's plaque or a dispersed pattern of *S. mutans* as seen in some samples might be the reflection of the subjects' sugar intake under the plaque formation.

The high prevalence rate of mutans streptococci found in various populations [Beighton et al., 1989; Fure and Zickert, 1990; Köhler et al., 1995; Milgrom et al., 2000; Okada et al., 2002] has been well documented, indicating that *S. mutans* was more widespread than *S. sobrinus*. In our depth-specific analysis, every mutans streptococci-positive layer was one in which either only *S. mutans* or both species were detected, suggesting that *S. mutans* has a wider habitat in plaque than *S. sobrinus* has. The results also revealed that mutans streptococci tended to be detected in the outer or middle plaque layers, demonstrating

the different distribution patterns of cariogenic bacteria between the subjects. Milgrom et al. [2000] revealed that *S. mutans* were found more frequently than *S. sobrinus* in the plaque on initial caries lesions as well as on sound enamel. Okada et al. [2002] reported that subjects that harbored both *S. mutans* and *S. sobrinus* had higher caries prevalence. In the present results, however, no obvious relationship was recognized between the distribution patterns of mutans streptococci throughout plaque and the subjects' DMFS scores. This might be due to the microbial difference between natural plaque and plaque formed in the device or due to the F component that accounted for almost all of the DMFS scores.

In the present study, layer-specific samples were prepared from newly formed 3-day-old plaque, with which more than half of the device's receptacles was filled. The plaque sample was formed with an uneven outer surface, tending to be thicker on or near the nylon edge of the device by incomplete filling of the ring. With the progress of sectioning from the plaque-saliva interface, the first layer that contained sections with toluidine blue-stained fragments was regarded as the outermost one. Therefore, the outermost layer might not exactly correspond to the outer plaque surface. However, the surface in a functional sense must correspond to the outer few layers, because the plaque layers that we dealt with were fairly thick. Our results, shown in table 1, demonstrated the effectiveness of this method, suggesting ecological features of mutans streptococci such as a frequent detection in the outer region, a wider habitat of *S. mutans* compared with *S. sobrinus* and so on. Therefore, any influence of this weakness, which stemmed from the unevenness of the outer plaque surface, would be limited to the evaluation of cariogenic bacteria within the plaque from their distribution patterns, so that in general this problem is not serious.

Although several analytical methods [Babaahmady et al., 1998; Milgrom et al., 2000; Rupf et al., 2001] have been used to identify the specific bacteria in plaque, there are only sparse data available concerning the three-dimensional plaque structure. In an attempt to clarify the etiology of periodontal disease, immunofluorescence studies have been carried out to locate the specific pathogens in the periodontal pockets, using sections obtained from biopsy samples of the tissue [Christersson et al., 1987; Noiri et al. 2001]. However, it would be difficult, using this technique, to ensure the location of the bacteria due to disturbances in the process of plaque sampling and preparation followed by staining. Moreover, fluorescence detection under the ordinary light microscope probably has inadequate resolution because of the short duration of the fluorescence. Fluorescence imaging by CLSM has also been applied to examine the heterogeneous structure of the plaque biofilms [Wood et al., 2000] and to clarify bacterial vitality relating to plaque structure [Auschill et al., 2001]. CLSM enabled us to examine the bulk specimens to identify several species by using different fluorochromes. There is, however, a great disparity of thickness between our sample and the thinner plaque for a CLSM observation. In addition, the autofluorescence of enamel may cause less accuracy of location of bacteria near the plaque-enamel interface.

On the other hand, our devices were snap-frozen in liquid nitrogen, lyophilized, then impregnated with methacrylate. Care must be taken to prevent distortion due to uneven drying. Serial sectioning of such an embedded undisturbed plaque sample ensures the validity of our depth-specific analysis of plaque from the outer surface towards the interior. Another advantage of our method is that it would be suitable for quantitative analysis. Although plaque mass or density is different from each layer or individual plaque, this difference is correctable. Plaque volume could be determined by measuring plaque area using image analysis, since section thickness is known. Quantitative differences of cariogenic bacteria have been detected in approximal plaque by an immunofluorescence study [Babaahmady et al., 1998]. It would be interesting to evaluate variations in plaque bacteria at different tooth sites, as well as at comparable sites from different teeth or different individuals. However, it would be difficult to apply the present method at sites on which the devices could not be attached, such as approximal and subgingival spaces.

Our depth-specific assay demonstrated variances in the pattern and distribution of mutans streptococci among our subjects, suggesting that it was important to evaluate their ecology within dental plaque. Further studies are needed to evaluate the bacterial pattern in plaque under various experimental conditions.

Acknowledgments

This work was supported by Grant-in-Aid for Scientific Research (C) No. 14571974 and AGU High-Tech Research Center Project from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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Int. J. Oral Maxillofac. Surg. 2004; 33: 584–592
doi:10.1016/j.ijom.2004.01.013, available online at http://www.sciencedirect.com

International Journal of
Oral & Maxillofacial Surgery

Research and Emerging Technologies
Pathology

PTC gene mutations and expression of SHH, PTC, SMO, and GLI-1 in odontogenic keratocysts

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K. Ohki, H. Kumamoto, R. Ichinohasama, T. Sato, N. Takahashi, K. Ooya:PTC gene mutations and expression of SHH, PTC, SMO, and GLI-1 in odontogenic keratocysts. Int. J. Oral Maxillofac. Surg. 2004; 33: 584–592.

Abstract. The *Patched* (*PTC*) gene is responsible for basal cell nevus syndrome (BCNS) accompanied by multiple odontogenic keratocysts (OKCs), and its product plays a role in the Sonic hedgehog (SHH) signaling pathway involving smoothened (SMO) and GLI-1. To clarify the role of SHH signaling in OKCs, the expression of SHH, PTC, SMO, and GLI-1 and mutations of *PTC* were examined in 18 sporadic, 4 BCNS-associated OKCs and 7 control gingivae. SHH, PTC, SMO, and GLI-1 were detected in all OKC and gingiva samples by reverse transcriptase-polymerase chain reaction (RT-PCR). Immunoreactivity for SHH and GLI-1 was markedly higher in epithelial components than in subepithelial cells, while immunoreactivity for PTC and SMO was similar in epithelial components and subepithelial cells in OKCs. The positive rate of PTC and SMO expression in subepithelial cells of OKCs was significantly higher than that in gingivae. The positive rate of GLI-1 expression in subepithelial cells of BCNS-associated OKCs was significantly higher than that in primary OKCs. These results suggest that the SHH signaling might be involved in the pathophysiologic nature of OKCs. While mutations of the *PTC* gene could not be detected in 4 BCNS-associated OKCs by direct DNA sequencing, 3 of 5 primary and 4 of 4 recurrent OKCs had several mutations of this gene. These results suggest that *PTC* mutations are probably related not only to BCNS-associated OKCs but also to sporadic OKCs.

Key words: basal cell nevus syndrome (BCNS); *Patched* (*PTC*); Sonic hedgehog (SHH); Smoothened (SMO); GLI-1; odontogenic keratocyst (OKC).

Accepted for publication 7 January 2004

Available online 18 March 2004

Odontogenic keratocyst (OKC) is the most aggressive odontogenic cyst in the oral cavity[36]. The majority of patients are in their second or third decade of life. Most OKCs arise in the mandible, having a 3-fold higher frequency of OKCs than the maxilla[5,25], while the majority in the mandible was located in the third molar area often with extension into the ascending ramus[37]. Histologically, OKC is characterized by a lining of parakeratotic stratified squamous epithelium and a thin fibrous capsule, which occasionally contain islands of epithelium or separate daughter cysts[6,25]. OKC has a high recurrence rate, estimated to range between 30 and 60%[5,25]. OKC sometimes occurs in association with the basal cell nevus syndrome (BCNS)[5,14]. BCNS, also known as 'Gorlin syndrome' and 'nevoid basal cell carcinoma syndrome,' is a rare autosomal dominant disorder characterized by multiple basal cell carcinomas (BCCs), multiple OKCs, palmar or plantar pits (or both), ectopic calcification, such as calcified flax cerebri, and congenital skeletal anomalies, such as bifid, fused, splayed, or missing ribs[14]. Various low-frequency neoplasms, such as medulloblastomas, meningiomas, fibrosarcomas, ovarian fibromas, and cardiac fibromas, are also associated with BCNS[14].

0901-5027/060584 + 09 $30.00/0

The gene responsible for BCNS has been localized to chromosome 9q22.3-q31 by linkage analysis[11], and the human *Patched* (*PTC*) gene has been isolated from the candidate region[15,20]. The *PTC* gene, bearing strong homology to the *Drosophila* segment polarity gene *patched* (*ptc*), contains 23 exons spanning approximately 34 kbp and encodes a 1447 amino acid protein containing 12 transmembrane-spanning domains and 2 large extracellular loops[19,20,30,38]. Approximately 40% of BCNS cases have germ line mutations of the *PTC* gene. Several *PTC* mutations that are apparently not hereditary have been identified in lesions of BCCs, medulloblastomas, and OKCs[27,34,43,45,46]. In addition, deletions of 9q22.3-q31 were observed on loss of heterozygosity in many neoplasms related to BCNS and in sporadic BCCs and OKCs[12,13,29,34]. PTC product serves as a receptor for the secreted Sonic hedgehog (SHH) protein, and inhibits the signaling pathway by repressing the activity of Smoothened (SMO), another transmembrane membrane protein[1,30,38]. SMO also has a role in reception and transduction of the SHH signal[38]. SMO is responsible for triggering intercellular signaling and the subsequent activation of target genes such as GLI-1. In the absence of SHH, PTC interacts at the membrane with smoothened, rendering it inactive. However, when SHH binds to PTC, the inhibition of SMO signaling is released and downstream genes are transcriptionaly upregulated[44]. GLI-1 is a transcription factor that is thought to form a cytoplasmic complex and mediate SHH signaling from cytoplasm to nucleus[26]. Inherited or sporadic alterations in SHH signaling pathway genes have been implicated in a number of human birth defects, and aberrant activation of the SHH signaling pathway during adult life results in cellular proliferations manifested as cancer[44]. Thus, studying the SHH signaling pathway is essential to understanding the mechanisms of birth defects and cancer and ultimately may help to identify therapeutic targets in diseases involving the SHH signaling pathway[16].

Our previous study examined the proliferative activities, cell-cycle-related factors, and apoptosis-related factors in lining epithelium of solitary and BCNS-associated multiple OKCs. The results suggested that BCNS-associated OKCs might show different characteristics from solitary OKCs[22]. Genetic and immunohistochemical analysis of jaw cysts in *ptc* knockout mice revealed that down-regulation of ptc is associated with formation of the cysts[23]. In the present study, we examined the expression of SHH, PTC, SMO, and GLI-1 at mRNA and protein levels and investigated *PTC* gene mutations in primary, recurrent, and BCNS-associated OKCs. These results of mRNA expression and protein levels were compared with the findings in gingival tissue. On the basis of our findings, we discuss the role of the SHH signaling pathway in OKCs.

Materials and methods

The protocol for the present experiment was reviewed and approved by the Tohoku University Graduate School of Dentistry Research Ethics Committee.

Tissue samples

Specimens were surgically removed from 22 patients with OKC at the Department of Oral and Maxillofacial Surgery, Tohoku University Dental Hospital, and affiliated hospitals. The lesions were divided into several parts. The first part was fixed in 10% buffered formalin for several days and routinely processed for histological diagnosis according to the World Health Organization histological typing of odontogenic tumors[25]. The lesions comprised 13 primary, 5 recurrent, and 4 BCNS-associated OKCs. BCNS was diagnosed according to Gorlin's criteria, including at least two of the following findings: multiple basal cell carcinoma, any odontogenic keratocyst, palmar or plantar pits, ectopic calcification, or a family history of BCNS[9,14]. The second part of each lesion was immediately frozen and stored at −80 °C until RT-PCR analysis and direct DNA sequencing. The third part of each cyst was embedded in Tissue-Tek O.C.T. compound (Sakura Finetechnical, Tokyo, Japan), quick frozen, and stored at −80 °C for immunohistochemical examination. As control, seven normal gingiva samples were obtained at autopsy, performed at Tohoku University Medical Hospital.

RT-PCR

Total RNA from frozen samples (20 mg) of 11 primary OKCs, 3 recurrent OKCs, 4 BCNS-associated OKCs, and 5 normal gingivae was extracted with the use of a RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the recommendations of the manufacturer's protocol. Total RNA (2 μg) was reverse-transcribed with an Omniscript RT Kit (Qiagen) according to the manufacturer's instructions in a 20-μl volume. The complementary DNA (cDNA) (0.05 μg) was used as a template for amplification in a Mastercycler gradient (Eppendorf, Hamburg, Germany) with each primer set[8,15,39,40] as listed in Table 1. PCR was performed on 50 μl of a reaction mixture containing approximately 50 ng

Table 1. RT-PCR primers and antibodies

	Primer			Antibody		
	Sequence (5′-3′)	Annealing temperature (°C)	Product size (bp)	Clonality	Source	Dilution
SHH	F: GAAAG CAGAG AACTC GGTGG R: GGAAA GTGAG GAAGT CGCTG	57	170	Polyclonal (goat)	Santa Cruz Biotechnology, Santa Cruz, CA, USA	1:50
PTC	F: GTGGC TGAGA GCGAA GTTTC R: TTCCA CCCAC AGCTC CTC	60	163	Polyclonal (rabbit)	Santa Cruz Biotechnology	1:50
SMO	F: CTGGT ACGAG GACGT GGAGG R: AGGGT GAAGA GCGTG CAGAG	65	132	Polyclonal (goat)	Santa Cruz Biotechnology	1:50
GLI-1	F: CAGAG AATGG AGCAT CCTCC R: TTCTG GCTCT TCCTG TAGCC	60	413	Polyclonal (goat)	Santa Cruz Biotechnology	1:100
GAPDH	F: GGAGT CAACG GATTT GGT R: GTGAT GGGAT TTCCA TTGAT	62	206			

of template cDNA, 0.5 mol/l of each primer, and 25 µl of HotStarTaq Master Mix (Qiagen), according to the manufacturer's instructions. The conditions for amplification were optimized for primer pairs as follows: heat starting at 95 °C for 15 min; 45 cycles of denaturation at 94 °C for 30 s, annealing at 63 °C (for PTC and SHH), 65 °C (for SMO), 60 °C (for GLI-1), and 62 °C (for glyceraldehyde-3-phosphate dehydrogenase (GAPDH)) for 30 s, and elongation at 72 °C for 1 min; and final extension at 72 °C for 10 min. The RT-PCR amplified products were applied to 2% agarose gel, electrophoresed in 1 × TBE at 100 V for 30 min, stained with ethidium bromide, and visualized under ultraviolet (UV) light.

Immunohistochemical examination of SHH, PTC, SMO, and GLI-1 proteins

Serial frozen sections were cut at a thickness of 5 µm in a cryostat CM3000 (Leica, Bensheim, Germany), placed on glass slides, air-dried for 30 s at room temperature (22 °C), and fixed in acetone for 10 min at 4 °C. The slides were then rinsed for 15 min in 0.01 M phosphate buffer solution (PBS). After treatment with normal rabbit serum for 15 min to block non-specific binding, the sections were incubated with primary antibodies at 4 °C overnight. The applied antibodies are listed in Table 1. The standard streptavidin–biotin–peroxidase complex method was performed to bind the primary antibodies with use of a Histofine SAB-PO kit (Nichirei, Tokyo, Japan). Reaction products were visualized by immersing the sections for 1–5 min in 0.03% 3,3′-diaminobenzidine (DAB) solution containing 2 mM hydrogen peroxide. Nuclei were lightly counterstained with 1% methyl green. For control studies of the antibodies, the serial sections were treated with PBS and normal goat and rabbit IgG instead of the primary antibodies and were confirmed to be unstained.

Evaluation of immunostaining and statistical analysis

Immunohistochemical reactivity for SHH, PTC, SMO, and GLI-1 was evaluated and classified into three groups: (−) negative, (+) positive (less than 50% of epithelial cells or subepithelial fibroblasts stained), and (++) strongly positive (more than 50% of epithelial cells or subepithelial fibroblasts stained). The statistical significance of differences in the percentages of cases with different reactivity levels for SHH, PTC, SMO, and GLI-1 was analyzed by Pearson's chi-square test. *P* values less than 0.05 were considered to indicate statistical significance.

DNA extraction and direct DNA sequencing

Genomic DNA from frozen samples (25 mg) of 5 primary OKCs, 4 recurrent OKCs, and 4 BCNS-associated OKCs was extracted with a QIAamp DNA Mini Kit (Qiagen) according to the recommendations of the manufacturer's protocol. Each of the 20 exons (exon 2–19, 21, 22) comprising the *PTC* gene were separately amplified in a Mastercycler gradient (Eppendorf) with use of the oligonucleotide primers as described previously[15,28] (Table 2). PCR was performed on 50 µl of a reaction mixture containing approximately 50 ng of template DNA, 0.5 µmol/l of each primer, and 25 µl of HotStarTaq Master Mix (Qiagen) according to the manufacturer's instructions. The conditions for amplification were optimized for primer pairs as follows: heat starting at 95 °C for 15 min; 45 cycles of denaturation at 94 °C for 30 s, annealing at 57–60 °C for 10 s, and elongation at 72 °C for 1 min; and final extension at 72 °C for

Table 2. Primers for DNA sequencing

Exon	Sequence (5′-3′)	Annealing temperature (°C)	Exon size (bp)
2	F: GTGGC TGAGA GCGAA GTTTC	57	193
	R: TTCCA CCCAC AGCTC CTC		
3	F: CTATT GTGTA TCCAA TGGCA GG	60	200
	R: ATTAG TAGGT GGACG CGGC		
4	F: GAGAA ATTTT TGTCT CTGCT TTTCA	57	60
	R: CCTGA TCCAT GTAAC CTGTT TC		
5	F: GCAAA AATTT CTCAG GAACA CC	57	92
	R: TGGAA CAAAC AATGA TAAGC AA		
6	F: CCTAC AAGGT GGATG CAGTG	57	199
	R: TTTGC TCTCC ACCCT TCTGA		
7	F: GTGAC CTGCC TACTA ATTCC C	60	122
	R: GGCTA GCGAG GATAA CGGTT TA		
8	F: GAGGC AGTGG AAACT GCTTC	57	148
	R: TTGCA TAACC AGCGA GTCTG		
9, 10	F: GTGCT GTCGA GGCTT GTG	60	Exon 9: 132,
	R: ACGGA CAGCA GATAA ATGGC		exon 10: 157
11	F: GTGTT AGGTG CTGGT GGCA	60	98
	R: CTTAG GAACA GAGGA AGCTG		
12	F: GACCA TGTCC AGTGC AGCTC	60	245
	R: CGTTC AGGAT CACCA CAGCC		
	F: AGTCC CTGAT TGGGC GGAG	60	
	R: CCATT CTGCA CCCAA TCAAA AG		
13	F: GGCCT ACACC GACAC ACAC	60	403
	R: TTTTT TTGAA GACAG GAAGA GCC		
	F: GTCAG CAGAC TGATT CAGGT	57	
	R: AAGAT GAGAG TGTCC ACTTC G		
14	F: GACAG CTTCT CTTTG TCCAG	60	310
	R: ACGCA AAAGA CCGAA AGGAC GA		
15	F: AGGGT CCTTC TGGCT GCGAG	60	143
	R: TCAGT GCCCA GCAGC TGGAG TA		
16	F: AACCC CATTC TCAAA GGCCT CTGTT C	60	184
	R: CACCT CTGTA AGTTC CCAGA CCTCC		
17	F: AACTG TGATG CTCTT CTACC CTGG	60	281
	R: AAACT TCCCG GCTGC AGAAA GA		
18	F: TTTGA TCTGA ACCGA GGACA CC	60	138
	R: CAAAC AGAGC CAGAG GAAAT GG		
19	F: TAGGA CAGAG CTGAG CATTT ACC	57	143
	R: TACCT GACAA TGAAG TCG		
21	F: AACAG AGGCC CCTGA AAAAT	60	255
	R: GATCA CTTGG TGGGC AGG		
22	F: TCTAA CCCAC CCTCA CCCTT	57	538
	R: ATTGT TAGGG CCAGA ATGCC		
	F: AGAAA AGGCT TGTGG CCAC	60	
	R: TCACC CTCAG TTGGA GCTG		

10 min. Direct sequencing of the *PTC* gene exons was carried out by means of the above mentioned PCR primers, the PCR products purified on the GFX PCR DNA and a Gel Band Purification Kit (Amersham Biosciences, Little Chalfont, UK), and a Thermo Sequenase Cy5 Dye Terminator Sequencing Kit (Amersham Biosciences) according to the manufacturers' instructions. The sequencing reactions were for 45 cycles of 96 °C for 30 s, 60 °C for 10 s, and 72 °C for 60 s in a Mastercycler gradient (Eppendorf). Sequencing products were separated on denaturing 7% polyacrylamide gel (ReproGel Long Read; Amersham Biosciences), using an automated laser fluorescence sequencer (ALF express II DNA Sequencer; Amersham Biosciences). Sequencing data were analyzed with the use of an ALFwin Sequence Analyser Ver. 2.1 (Amersham Biosciences).

Genbank accession data

Nucleotide and amino-acid residue numbering is based on Genbank sequence U59464.

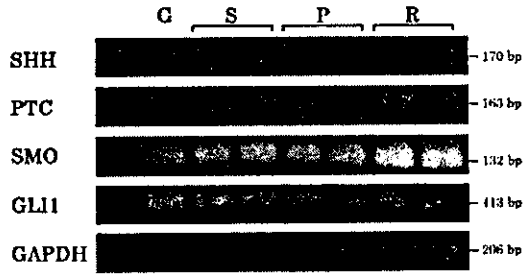

Fig. 1. SHH, PTC, SMO, and GLI-1 mRNA expression in odontogenic keratocysts (OKCs) and normal gingiva. (G: gingiva, S: BCNS-associated OKC, P: primary OKC, R: recurrent OKC). Reverse transcriptase-polymerase chain reaction (RT-PCR) products for SHH (170 bp), PTC (163 bp), SMO (132 bp), and GLI-1 (413 bp) were detected in all gingiva and OKC samples.

Results

Expression of SHH, PTC, SMO, and GLI-1 mRNA

To investigate the distribution patterns of SHH, PTC, SMO, and GLI-1 mRNA in OKCs, we examined 18 OKCs of different clinical characterization (11 primary OKCs, 3 recurrent OKCs, and 4 BCNS-associated OKCs) as well as 5 normal gingivae by RT-PCR analysis. RT-PCR products for SHH (170 bp), PTC (163 bp), SMO (132 bp), and GLI-1 (413 bp) were detected in all OKC and gingiva samples. Fig. 1 shows examples of SHH, PTC, SMO, and GLI-1 mRNA expression in OKCs and normal gingiva.

Immunoreactivity for SHH, PTC, SMO, and GLI-1

The results for immunohistochemical expression of SHH, PTC, SMO, and GLI-1 proteins are shown in Table 3. Fig. 2A–D shows typical staining of

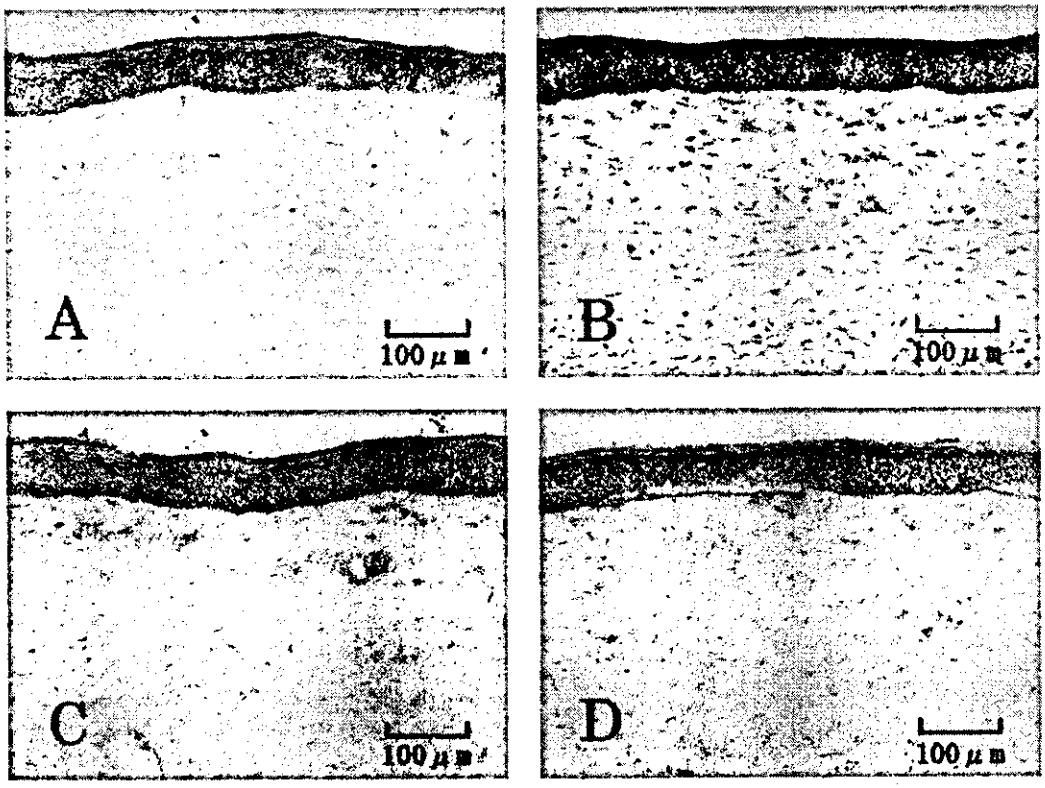

Fig. 2. Immunohistochemical expression of SHH (A), PTC (B), SMO (C), and GLI-1 (D) in odontogenic keratocyst. SHH, PTC, SMO, and GLI-1 were detected in the cytoplasm of basal to superficial epithelial cells. The positive rate of PTC expression in the cytoplasm of subepithelial fibroblasts was higher then that of SHH, SMO, and GLI-1.

Table 3. Immunohistochemical expression of SHH, PTC, SMO, and GLI-1 in gingivae and odontogenic keratocysts

			SHH			PTC			SMO			GLI-1		
			-	+	++	-	+	++	-	+	++	-	+	++
Gingiva (n = 7)		Covering epithelium	0 (0%)	1 (14%)	6 (86%)	0 (0%)	1 (14%)	6 (86%)	0 (0%)	4 (57%)	3 (43%)	0 (0%)	5 (71%)	2 (29%)
		Connective tissue	4 (57%)	3 (43%)	0 (0%)	3 (43%)	4 (57%)	0 (0%)	2 (29%)	5 (71%)	0 (0%)	7 (100%)	0 (0%)	0 (0%)
OKC	Total (n = 22)	Lining epithelium	0 (0%)	0 (0%)	22 (100%)	0 (0%)	2 (9%)	20 (91%)	0 (0%)	4 (18%)	18 (82%)	0 (0%)	6 (27%)	16 (73%)
		Connective tissue	4 (18%)	18 (82%)	0 (0%)	0 (0%)	4 (18%)	18 (82%)	0 (0%)	14 (64%)	8 (36%)	12 (55%)	8 (36%)	2 (9%)
	Primary-OKC (n = 13)	Lining epithelium	0 (0%)	0 (0%)	13 (100%)	0 (0%)	1 (8%)	12 (92%)	0 (0%)	2 (15%)	11 (85%)	0 (0%)	5 (38%)	8 (62%)
		Connective tissue	2 (15%)	11 (85%)	0 (0%)	0 (0%)	2 (15%)	11 (85%)	0 (0%)	8 (62%)	5 (38%)	10 (77%)	3 (23%)	0 (0%)
	Recurrent-OKC (n = 5)	Lining epithelium	0 (0%)	0 (0%)	5 (100%)	0 (0%)	1 (20%)	4 (80%)	0 (0%)	2 (40%)	3 (60%)	0 (0%)	0 (0%)	5 (100%)
		Connective tissue	2 (40%)	3 (60%)	0 (0%)	0 (0%)	1 (20%)	4 (80%)	0 (0%)	4 (80%)	1 (20%)	2 (40%)	2 (40%)	1 (20%)
	BCNS-OKC (n = 4)	Lining epithelium	0 (0%)	0 (0%)	4 (100%)	0 (0%)	0 (0%)	4 (100%)	0 (0%)	0 (0%)	4 (100%)	0 (0%)	1 (25%)	3 (75%)
		Connective tissue	0 (0%)	4 (100%)	0 (0%)	0 (0%)	1 (25%)	3 (75%)	0 (0%)	2 (50%)	2 (50%)	0 (0%)	3 (75%)	1 (25%)

Immunohistochemical expression: (−) negative, (+) positive, (++) strongly positive. Statistical significance: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Table 4. PTC gene mutations in odontogenic keratocysts (OKCs)

Patient	Diagnosis	Mutations		
		Exon	Nucleotide change	Amino acid change
1	Primary OKC	12	C1686T (silent mutation)	Ala562Ala
2	Primary OKC	22	G3840T (silent mutation)	Ser1280Ser
		22	G3852T (missense mutation)	Gln1284His
		22	C3859T (missense mutation)	His1287Asn
		22	C3905A (missense mutation)	Ser1302His
3	Primary OKC	22	G3852T (missense mutation)	Gln1284His
		22	C3875T (missense mutation)	Ser1292Phe
		22	C3905A (missense mutation)	Pro1302His
4	Primary OKC	No mutation		
5	Primary OKC	No mutation		
6	Recurrent OKC	4	635insG (frameshift mutation)	
		4	638insG (frameshift mutation)	
7	Recurrent OKC	10	1371delG (frameshift mutation)	
		22	T3944C (missense mutation)	Leu1315Pro
8	Recurrent OKC	10	1371delG (frameshift mutation)	
9	Recurrent OKC	10	1371delG (frameshift mutation)	
		21	3641delC (frameshift mutation)	
		21	3661insC (frameshift mutation)	
		21	C3728G (missense mutation)	Ala1243Gly
10	BCNS	No mutation		
11	BCNS	No mutation		
12	BCNS	No mutation		
13	BCNS	No mutation		

SHH, PTC, SMO, and GLI-1 proteins in one OKC. SHH, PTC, SMO, and GLI-1 were detected in the cytoplasm of basal to superficial epithelial cells and some subepithelial fibroblasts in both normal gingivae and OKCs. The positive rate of PTC expression in the cytoplasm of subepithelial fibroblasts was higher then that of SHH, SMO, and GLI-1 in OKCs. There was no distinct difference in SHH reactivity between gingivae and OKCs, or among primary, recurrent, and BCNS-associated OKCs. The positive rate of PTC expression in subepithelial tissues of gingivae was significant lower then that of total ($P < 0.001$), primary ($P < 0.01$), recurrent ($P < 0.05$), and BCNS-associated OKCs ($P < 0.05$). The positive rate of SMO expression in subepithelial fibrous connective tissues of OKCs was significantly higher than that in normal gingivae ($P < 0.05$). The positive rate of GLI-1 expression in the subepithelial fibrous connective tissues of BCNS-associated OKCs was significantly higher than that in normal gingivae ($P < 0.05$), and subepithelial GLI-1 expression differed significantly between primary and BCNS-associated OKCs ($P < 0.01$).

Mutations of the *PTC* gene

PTC gene mutations were examined in 5 primary, 4 recurrent, and 4 BCNS-associated OKCs by direct DNA sequencing. Mutations in the coding region of the *PTC* gene were found in 3 primary and 4 recurrent OKCs, whereas no mutation was found in 2 primary and 4 BCNS-associated OKCs (Table 4). Each mutation was confirmed by reverse sequencing. These putative disease-associated mutations were distributed throughout the gene. Eight of the 13 sequence alterations were single nucleotide changes (Fig. 3A), resulting in 6 missense mutations and 2 silent mutations. Six missense mutations were detected in exons 21 and 22, containing 2 C → T and 1 T → C transition, and 1 G → T, 1 C → A, and 1 C → G transversion. We also detected a C → T transition at nucleotide 1686 in exon 12 and a G → T transversion at nucleotide 3840 in exon 22, resulting in silent mutation. Five frameshift mutations were detected (Fig. 3B and C). These single nucleotide deletions or insertions, consisting of G insertion in exon 4, C insertion in exon 21, G deletion in exon 10, and C deletion in exon 21, resulted in the induction of premature stop codons at several bp downstream of the mutation sites. Locations of these mutations on PTC protein are summarized in Fig. 4. In addition to these disease-associated mutations, several variants were designated "polymorphism" on the basis of their presence in unaffected individuals or the finding that the underlying sequence changes did not alter the encoded amino acids.

Discussion

The SHH signaling pathway plays an important role in mammalian embryonic development of structures such as the neural tube, axial skeleton, limbs, lungs, skin, and hair follicles[2,7,17,31,35]. Expression of genes involved in the SHH signaling pathway has been confirmed temporally and spatially during early tooth development, suggesting a role in early tooth germ initiation and subsequent epithelial–mesenchymal interactions. SHH expression was localized to the epithelial thickenings at epithelial thickening stage of tooth development. SHH transcripts were found to be restricted to cells that will form the enamel knot. At the early bud stage, the localized expression of SHH was intensified. By the cap stage, the enamel knot has fully formed and SHH expression clearly marks this structure. PTC and GLI-1 expressions were restricted to the mesenchyme underlying the tooth thickening. At the early bud stage, PTC and GLI-1 were expressed uniformly in the odontogenic epithelium such as dental papilla and dental epithelium, and mesenchyme. At the cap stage of tooth development, PTC and GLI-1 were expressed in a region of the tooth germ that appeared to surround the area of the enamel knot that was marked by SHH expression. SMO was found to be ubiquitously expressed throughout tooth development. With the passage of stage, the expression of SMO was slightly more specific, being more strongly expressed in the epithelial component, but absent from the enamel knot. These features suggest that SHH signaling is involved in both lateral (epithelial-mesenchymal) and planar (epithelial-epithelial) pathways during tooth development[17]. PTC, SMO, and GLI-1 mRNA is up-regulated in BCCs, although tumor-adjacent epidermis shows low expression of these molecules[33,40]. In our previous study, normal gingival tissues were positive for ptc, shh, and smo proteins in *ptc* knockout mice[23]. In the present study, SHH, PTC, SMO, and GLI-1 were detected in all normal gingivae at both mRNA and protein levels, suggesting that gingival tissue might be affected by these molecules.

SHH signaling is emerging as one of the most important regulators of oncogenic transformation[16]. The *PTC* gene

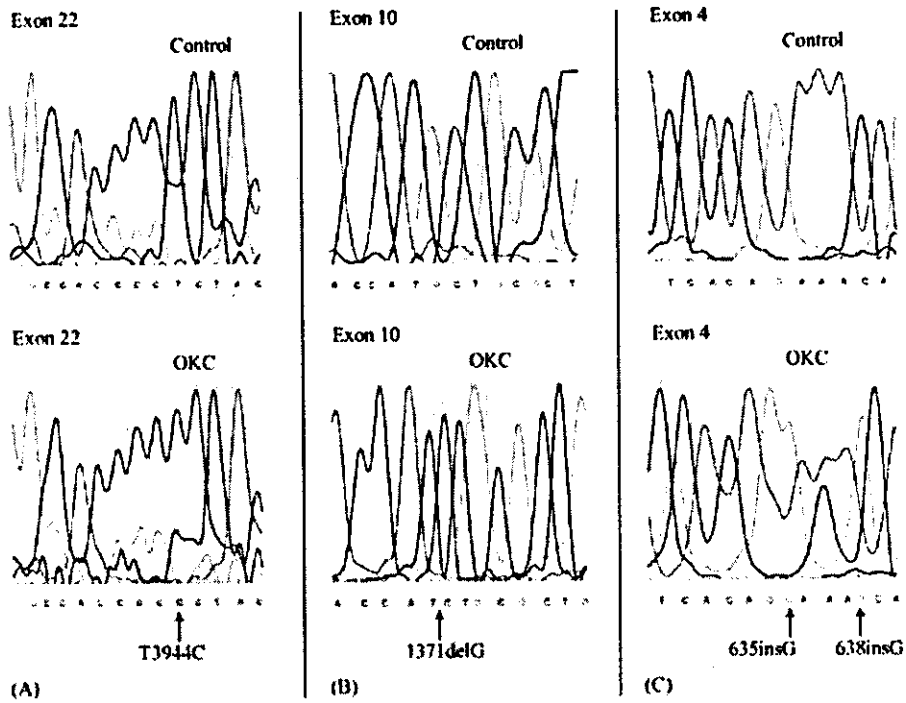

Fig. 3. PTC gene mutations in odontogenic keratocysts (OKCs). (A) Direct DNA sequencing of exon 22 (upper: control, lower: OKC) T to C transitional change at nucleotide 3944, that resulted in leucine to proline substitution at codon 1315. (B) Direct DNA sequencing of exon 10 (upper: control, lower: OKC). A single nucleotide of G was deleted at position 1371, which was expected to result in a stop codon after a run of 33 novel amino acids downstream. (C) Direct DNA sequencing of exon 4 (upper: control, lower: OKC). A single nucleotide of G was inserted at position 635 and 638, which was expected to result in a stop codon after a run of seven novel amino acids downstream.

functions as a tumor suppressor, and *SHH*, *SMO*, and *GLI-1* mutants have been shown to function as oncogenes[8,10,38,46]. Mutational inactivation of *PTC* leads to overexpression of the mutant transcript owing to failure of a negative feedback mechanism[13,41]. Expression studies with *in situ* hybridization and RT-PCR have shown PTC overexpression in BCCs as compared with normal skin[32,41]. Previous studies have demonstrated that SMO is also overexpressed in BCC, whereas SHH is expressed in only some BCCs[21,40]. In the present study, SHH, PTC, SMO, and GLI-1 were detected at mRNA and protein levels in all OKCs. The expression of SHH and GLI-1 was more marked in lining epithelium than in subepithelial cells, while PTC and SMO were similarly expressed in both epithelial and subepithelial cells. These features suggest that SHH signaling is transmitted via epithelial-subepithelial interactions in the cyst walls of OKCs. Our previous study revealed that ptc expression was decreased in lining epithelium of mandibular cysts in *ptc* knockout mice[23]. In the present study, expression of PTC, SMO, and GLI-1 in subepithelial cells was greater in OKCs than in normal gingiva. These findings suggest that OKCs show different characteristics from normal gingival tissues with respect to SHH signaling, and such differences might be involved in the formation of OKCs. Our previous study clarified differences in proliferative activity and apoptotic factors between sporadic and BCNS-associated OKCs[22]. In the present study, GLI-1 expression in subepithelial fibroblasts was slightly higher in BCNS-associated OKCs than in sporadic OKCs. These results suggest that the characteristics of SHH signaling differ between BCNS-associated OKCs and sporadic OKCs.

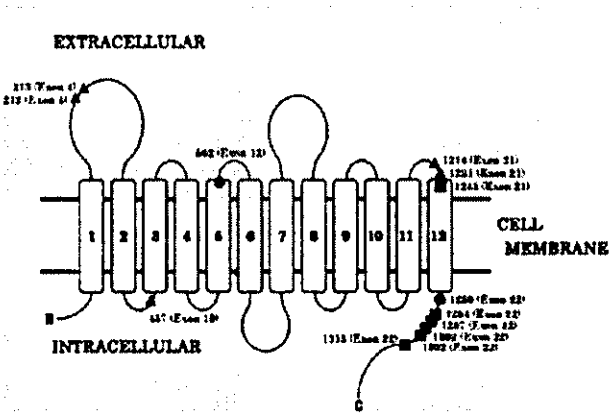

Fig. 4. Location of mutations on PTC protein. (▲: frameshift mutation, ■: missense mutation, ●: silent mutation).

PTC gene mutations are found not only in BCNS but also in sporadic tumors, such as BCC, trichoepithelioma, and medulloblastoma[34,42,45]. Between 20 and 30% of sporadic BCCs show somatic *PTC* mutations, and 68% show

loss of heterozygosity (LOH) on chromosome 9q, the PTC locus[11]. *PTC* mutations and LOH at the PTC locus have been identified in OKCs arising in BCNS and in sporadic OKCs[27,29,47]. In the present study, we identified 13 mutations of the *PTC* gene in three primary OKCs and four recurrent OKCs. These findings suggest that abnormality of the *PTC* gene is essential for the development of OKCs. Generally, exogenous mutagens can cause specific hot spots of somatic mutations in human cancers: for example, the oncogene of *K-RAS* and the tumor suppressor gene of *TP53*[4,18]. Previous studies have shown that *PTC* mutations are dispersed throughout the entire coding sequence of the gene without any apparent hot spots in BCNS-associated lesions[15,20,43,46]. In the present study, mutations of the *PTC* gene were also distributed in a variety of exons. PTC protein possesses several major domains, including the large extracellular loops that interact with SHH ligand and the large C-terminal intracellular domain that may interact with the intracellular protein of SMO protein[3,13,24]. In the present study, most *PTC* mutations of OKCs were detected in the first large extracellular loop or the large C-terminal intracellular domain, suggesting that these mutations inhibit SHH signaling in OKCs. Homozygous *ptc* knockout mice ($ptc^{-/-}$ mice) die in utero during early organogenesis, while $ptc^{+/-}$ mice show a number of developmental abnormalities and a high incidence of tumors, similar to human BCNS[23]. We detected no alteration of the *PTC* gene in BCNS-associated OKCs. These results suggest that the BCNS with germline mutations might cause chromosomal deletion and express another stable allele in OKC tissues.

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Oral Microbiology Immunology 2004: 19: 379–385
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Oral Microbiology and Immunology

Detection frequency of periodontitis-associated bacteria by polymerase chain reaction in subgingival and supragingival plaque of periodontitis and healthy subjects

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Mayanagi G, Sato T, Shimauchi H, Takahashi N. Detection frequency of periodontitis-associated bacteria by polymerase chain reaction in subgingival and supragingival plaque of periodontitis and healthy subjects.
Oral Microbiol Immunol 2004: 19: 379–385. © Blackwell Munksgaard, 2004.

The aim of this study was to compare the detection frequencies of 25 bacterial species in subgingival and supragingival plaque of 18 untreated periodontitis subjects and 12 periodontally healthy subjects. Genomic DNA was extracted from subgingival and supragingival plaque samples, and bacterial detection was performed by polymerase chain reaction of the 16S rRNA genes. Fourteen bacteria showed no relationship with periodontitis, and 11 of these 14 species were frequently detected (≥50%) in subgingival plaque in both periodontitis and healthy subjects. Nine bacteria such as *Eubacterium saphenum, Prevotella intermedia,* and *Treponema denticola* seemed to be related to periodontitis; their detection frequencies in subgingival plaque samples were higher in periodontitis than in healthy subjects, but these differences were not statistically significant by multiple comparisons ($0.002 \leq P < 0.05$). Two species (*Mogibacterium timidum* and *Porphyromonas gingivalis*) were detected significantly more frequently in subgingival plaque of periodontitis subjects than of healthy subjects ($P < 0.002$), with *P. gingivalis* being detected only in periodontitis subjects, suggesting that these two species are closely related to periodontitis. There were no significant differences in the detection frequencies of the 25 bacteria between subgingival and supragingival plaque, suggesting that the bacterial flora of supragingival plaque reflects that of subgingival plaque.

Key words: 16S rRNA genes; periodontitis; polymerase chain reaction; subgingival plaque; supragingival plaque

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Accepted for publication June 30, 2004

The composition of dental plaque microbiota in the human oral cavity is diverse and complex. It has been previously estimated by culturing and molecular biological methods that more than 600 species of bacteria inhabit the human oral cavity (13, 25, 27, 37, 49). The initiation and progression of periodontitis is thought to be caused by several species of these bacteria accumulating in subgingival periodontal pockets. *Porphyromonas gingivalis, Tannerella forsythia* (*Tannerella forsythensis*, formerly *Bacteroides forsythus*), and *Treponema denticola* are widely regarded as major periodontal pathogens (38), and numerous etiologic investigations of periodontal disease have therefore targeted these species. *Campylobacter rectus, Eikenella corrodens, Fusobacterium nucleatum, Micromonas micros* (formerly *Peptostreptococcus micros*), *Treponema amylovorum,*

Treponema maltophilum, Treponema medium, Treponema socranskii, Treponema vincentii, Mogibacterium timidum (formerly *Eubacterium timidum*), *Capnocytophaga ochracea*, and *Capnocytophaga sputigena* have also been recognized as pathogens of periodontitis based on their presence and relative numbers in healthy vs. diseased sites (18, 24, 48, 51). *Actinobacillus actinomycetemcomitans, Prevotella tannerae, Prevotella intermedia, Prevotella nigrescens, Centipeda periodontii*, and *Selenomonas sputigena* have been found in subgingival lesions of periodontitis patients (1, 23, 25, 36). *Dialister pneumosintes, Slackia exigua* (formerly *Eubacterium exiguum*), *Eubacterium saphenum, and Porphyromonas endodontalis* have also been considered to be associated with periodontitis (10, 18, 46).

Although all 25 bacterial species mentioned above are thought to be related to periodontitis, they have not been considered in the same study. Therefore, firstly, we undertook a comprehensive investigation of the 25 bacteria in subgingival and supragingival plaque using polymerase chain reaction (PCR) of the 16S rRNA genes. Secondly, we evaluated the relationship between periodontitis and the detection frequency of these bacteria. Thirdly, we compared the bacterial detection frequencies in subgingival plaque with the frequencies in supragingival plaque.

Material and methods

Subject population

Eighteen patients with periodontitis (mean age 63 ± 10.4 years; range 41–77 years) and 12 periodontally healthy subjects (mean age 27 ± 1.8 years; range 22–29 years) were randomly selected for this study. They had not received periodontal treatment or antimicrobial therapy for at least 6 months and were free of systemic diseases. Informed consent was obtained from each subject. Probing depths were measured in all teeth at six sites per tooth in each subject, and the teeth with the deepest probing depths were chosen as the target sites of sampling. The deepest probing depths were <4 mm (range 2.0–3.0 mm; mean 2.4 ± 0.5 mm) in periodontally healthy subjects (n = 12) and ≥4 mm (range 4.0–10.0 mm; mean 6.2 ± 2.1 mm) in subjects with periodontitis (n = 18).

Collection of samples

Supragingival plaque samples were taken with sterile explorers. For subgingival plaque samples, each of the target teeth was isolated with cotton rolls and air-dried after thorough removal of supragingival plaque with sterile cotton pellets. The samples were then collected using sterile periodontal pocket probes. In addition, alveolar mucosal samples (7 of 12 healthy subjects, 6 of 18 periodontitis subjects) were collected at the same sampling sites by swabbing 10 times with sterile swabs. All samples were immediately suspended in 1 ml of sterile distilled water and stored at −20°C before extraction of genomic DNA.

DNA extraction and nested PCR detection

After thawing, samples were centrifuged at 7740 × *g* for 5 min and the supernatants removed. Genomic DNA was then extracted from the pellets using an InstaGene Matrix Kit (Bio-Rad Laboratories, Richmond, CA) according to the manufacturer's instructions.

In the first amplification, the 16S rRNA genes were amplified by PCR with universal primers 27F and 1492R (19, 34, 35) and Taq DNA polymerase (HotstarTaq Master mix; Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The primer sequences were: 27F, 5′- AGA GTT TGA TCC TGG CTC AG -3′; and 1492R, 5′- TAC GGG TAC CTT GTT ACG ACT T -3′. PCR mixtures were 5 μl of genomic DNA and 95 μl of reaction mixture containing 1.5 mM $MgCl_2$. PCR amplification was performed in a PCR Thermal Cycler MP (TaKaRa Biomedicals, Ohtsu, Shiga, Japan) programmed for 15 min at 95°C for initial heat activation, 35 cycles of 1 min at 94°C for denaturation, 1 min at 60°C for annealing, and 1.5 min at 72°C for extension, and 10 min at 72°C for final extension. The predicted PCR product with the universal primers was 1505 bp in length.

The 25 bacteria were identified by amplification of the first PCR amplification products using species-specific primers (Table 1) based on 16S rRNA gene sequences. PCR mixtures were 1 μl of the first PCR amplification mixture and 24 μl of reaction mixture containing 1.5 mM $MgCl_2$. PCR amplification was performed in a PCR Thermal Cycler MP (TaKaRa Biomedicals) programmed for 15 min at 95°C for initial heat activation, 35 cycles of 1 min at 94°C for denaturation, 1 min at 55°C for annealing, and 1.5 min at 72°C for extension, and 10 min at 72°C for final extension. The predicted sizes of PCR products with species-specific primers are listed in Table 1.

The PCR products were separated on 2% agarose gels (High Strength Analytical Grade Agarose, Bio-Rad Laboratories) in Tris-borate EDTA buffer (100 mM Tris, 90 mM borate; 1 mM EDTA, pH 8.4) stained with ethidium bromide and photographed under ultraviolet light. A 100-bp DNA Ladder (Invitrogen Corp., Carlsbad, CA) was used as a molecular size marker.

Data analysis

Fisher's exact probability tests were applied to compare the detection frequencies of the bacterial species between the healthy and periodontitis subjects, and between supragingival and subgingival plaques to evaluate significance. *P*-value was adjusted from 0.05 to 0.002 based on the Bonferroni correction for multiple comparisons.

Results

The detection frequencies of target bacteria in subgingival and supragingival plaque are shown in Fig. 1. The bacterial species were listed in order of the differences (*P*-values) in their detection frequencies in subgingival plaque between healthy and periodontitis subjects. There were no significant differences ($P \geqslant 0.05$) in the detection frequencies of the first 14 bacteria between healthy and periodontitis subjects. Eleven of the 14 species (*F. nucleatum, C. rectus, E. corrodens, P. nigrescens, C. ochracea, T. maltophilum, S. exigua, M. micros, T. forsythia* (*T. forsythensis*), *C. sputigena*, and *C. periodontii*) were frequently detected in subgingival plaque samples (≥50%) of both periodontitis and healthy subjects. *A. actinomycetemcomitans, T. amylovorum*, and *T. vincentii* were infrequently detected in both periodontitis and healthy subjects (Fig. 1).

Of the last 11 species in Fig. 1, the detection frequencies of nine bacteria (*E. saphenum, P. intermedia, T. denticola, P. tannerae, T. medium, D. pneumosintes, T. socranskii, P. endodontalis*, and *S. sputigena*) seemed to be higher in subgingival plaque samples from periodontitis subjects than in healthy subjects, although these differences were not statistically significant by multiple comparisons ($0.002 \leq P < 0.05$, Fig. 1). The detection frequencies of *M. timidum* and *P. gingivalis* of periodontitis subjects were significantly higher ($P<0.002$) than those of healthy subjects. In addition, *P. gingivalis* was not detected in the subgingival plaque of healthy subjects (Fig. 1).

Table 1. Target bacteria and their species-specific primers

Species	Sequence (5'-3')	Size	References
Actinobacillus actinomycetemcomitans	CTC AGA GAT GGG TTT GTG CC AGA TTC ACT CCC CAT CGC TG	273 bp	(47)
Campylobacter rectus	TTT CGG AGC GTA AAC TCC TTT TC TTT CTG CAA GCA GAC ACT CTT	598 bp	(1)
Capnocytophaga ochracea	AGA GTT TGA TCC TGG CTC AG GAT GCC GTC CCT ATA TAC TAT GGG G	185 bp	(4)
Capnocytophaga sputigena	AGA GTT TGA TCC TGG CTC AG GAT GCC GCT CCT ATA TAC CAT TAG G	185 bp	(4)
Centipeda periodontii	AGA GTT TGA TCC TGG CTC AG TTA CAA AGG ATT ATT CGC CC	450 bp	(36)
Dialister pneumosintes	TTC TAA GCA TCG CAT GGT GC GAT TTC GCT TCT CTT TGT TG	1105 bp	(6)
Eikenella corrodens	CGA TTA GCT GTT GGG CAA CTT ACC CTC TGT ACC GAC CAT TGT AT	410 bp	(9)
Eubacterium saphenum	TCT ACT AAG CGC GGG GTG A A CCC GAT TAA GGG TAC	430 bp	(12)
Fusobacterium nucleatum	GAA GAA ACA AAT GAC GGT AAC AAC GTC ATC CCC ACC TTC CTC CT	705 bp	(31)
Micromonas micros	TCG AAC GTG ATT TTT GTG GA TCC AGA GTT CCC ACC TCT	1074 bp	(29)
Mogibacterium timidum	AAG CTT GGA AAT GAC GC CCT TGC GCT TAG GTA A	524 bp	(12)
Porphyromonas endodontalis	GCT GCA GCT CAA CTG TAG TC CCG CTT CAT GTC ACC ATG TC	672 bp	(2)
Porphyromonas gingivalis	GCG TAT GCA ACT TGC CTT AC GTT TCA ACG GCA GGC TGA AC	518 bp	(47)
Prevotella intermedia	CGT GGA CCA AAG ATT CAT CGG TGG A CCG CTT TAC TCC CCA ACA AA	259 bp	(26)
Prevotella nigrescens	GTG TTT CAT TGA CGG CAT CCG ATA TGA AAC CA CGT CTC TGT GGG CTG CGA	828 bp	(26)
Prevotella tannerae	CTT AGC TTG CTA AGT ATG CCG AG CTG ACT TAT ACT CCC G	550 bp	(50)
Selenomonas sputigena	AGA GTT TGA TCC TGG CTC AG TC AAT ATT CTC AAG CTC GGT T	478 bp	(36)
Slackia exigua	GCC AAG CGG CCT CGT CGA AG C GGC TTT AAG GGA TTC GCT CG	697 bp	(12)
Tannerella forsythensis	AAA ACA GGG GTT CCG CAT GG C ACC GCG GAC TTA ACA GC	426 bp	(22)
Treponema amylovorum	AGA GTT TGA TCC TGG CTC AG C ACG CCT TTA TTC CGT GAG	193 bp	(48)
Treponema denticola	TAA TAC CGA ATG TGC TCA TTT ACA T TCA AAG AAG CAT TCC CTC TTC TTC TTA	316 bp	(1)
Treponema maltophilum	AGA GTT TGA TCC TGG CTC AG CT ATT GTG CTT ATT CAT CAG GC	438 bp	(48)
Treponema medium	CAC TCA GTG CTT CAT AAG GG CG GCC TTA TCT CTA AGA CC	856 bp	(33)
Treponema socranskii	AGG TAG ACA GCG GGA AAG GA AA CCC AAC ACC TCA CGG CA	902 bp	(32)
Treponema vincentii	GTC TCA ATG GTT CAT AAG AA CAA GCC TTA TCT CTA AGA CT	856 bp	(33)

There were no significant differences in the detection frequencies of the 25 bacteria between subgingival and supragingival plaque (Fig. 1). The detection frequencies in alveolar mucosal plaque samples were generally lower than those in the respective subgingival and supragingival plaque samples (Fig. 2).

The percent agreement of detection frequencies of the 25 bacteria was also evaluated. In subjects with periodontitis, the percent agreement of subgingival vs. supragingival plaque was higher (mean 81%, range 50–100%) than that of alveolar mucosal plaque vs. subgingival plaque (mean 63%, range 17–100%) and alveolar mucosal plaque vs. supragingival plaque (mean 60%, range 33–83%). This trend was also observed in healthy subjects (data not shown).

Discussion

In this study, the detection frequencies of 25 bacteria in subgingival, supragingival, and alveolar mucosal plaque of untreated periodontitis subjects and periodontally healthy subjects were obtained by nested PCR based on 16S rRNA genes. Since the subject's age is one of the most important risk factors of periodontitis, the periodontitis subjects were first divided into two groups according to age (41–64 years and 65–77 years). However, as the detection frequencies of the 25 bacterial species were quite similar in subgingival plaque samples from the two groups (data not shown), the groups were combined into one in the present study.

The first 14 bacteria in Fig. 1 showed no significant differences in detection frequencies in subgingival plaque between healthy and periodontitis subjects ($P \geqslant 0.05$); 11 of these bacteria were detected at high frequencies (≥50%) in both periodontitis and healthy subjects (Fig. 1). The detection rates of *C. rectus* and *E. corrodens* have been reported to be unrelated to the progression of attachment loss (28). Moreover, *C. rectus, E. corrodens, C. ochracea*, and *C. sputigena* have been frequently

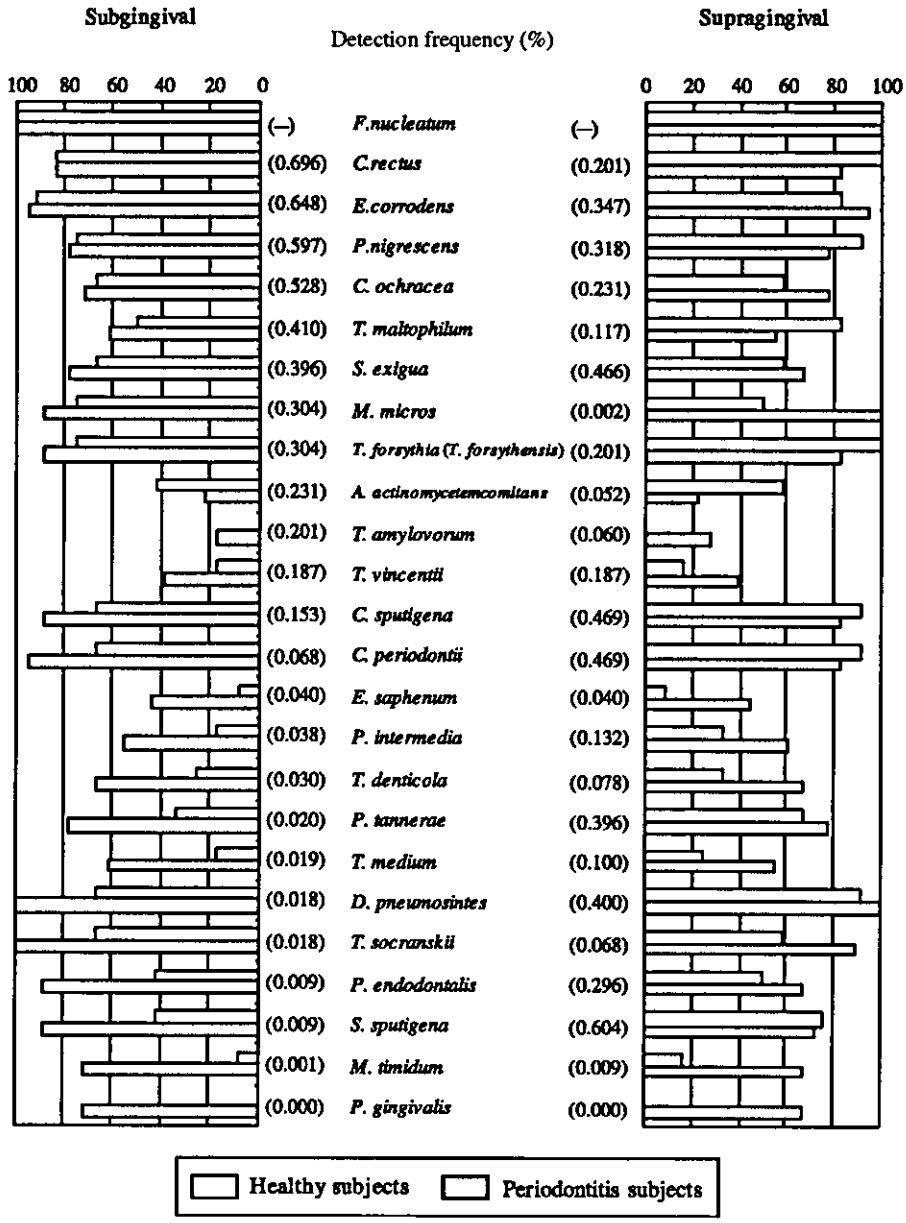

Fig. 1. Detection frequencies of target bacteria in subgingival and supragingival plaque. *P*-values of differences between healthy and periodontitis subjects are given in parentheses. Bacterial species are listed in order of *P*-values.

detected in plaque samples from healthy children (14). The results obtained in this study are therefore in accordance with those of previous studies, suggesting that these species are among the commensal bacteria of the oral cavity. *F. nucleatum* and *T. forsythia* (*T. forsythensis*) were also frequently detected in both healthy and periodontitis subjects in this study, suggesting that their relationship with periodontitis is uncertain. The *F. nucleatum* species consists of several subspecies (subspecies *nucleatum*, *polymorphum*, *fusiforme*, and *vincentii*) (7), and the *T. forsythia* (*T. forsythensis*) species has been reported to have *prtH* genetic subtypes (42). Thus, the pathogenicity of *F. nucleatum* and *T. forsythia* (*T. forsythensis*) probably varies depending on the strain (8, 30, 42). Further study will be required to determine the subspecies and subtypes of *F. nucleatum* and *T. forsythia* (*T. forsythensis*) specific to periodontitis. *A. actinomycetemcomitans* was detected at the adult periodontitis sites in less than 25%, which was lower than at healthy sites in this study (Fig. 1). This is consistent with a recent study (11), although this species has been considered to be associated with juvenile and adult periodontitis (17, 20, 52, 53). This discrepancy could be due to differing pathogenicity of the various serotypes and genotypes of *A. actinomycetemcomitans* (3, 43).

Of the remaining 11 species, the detection frequencies of nine bacteria in subgingival plaque were higher in periodontitis subjects than in healthy subjects, but this difference was not

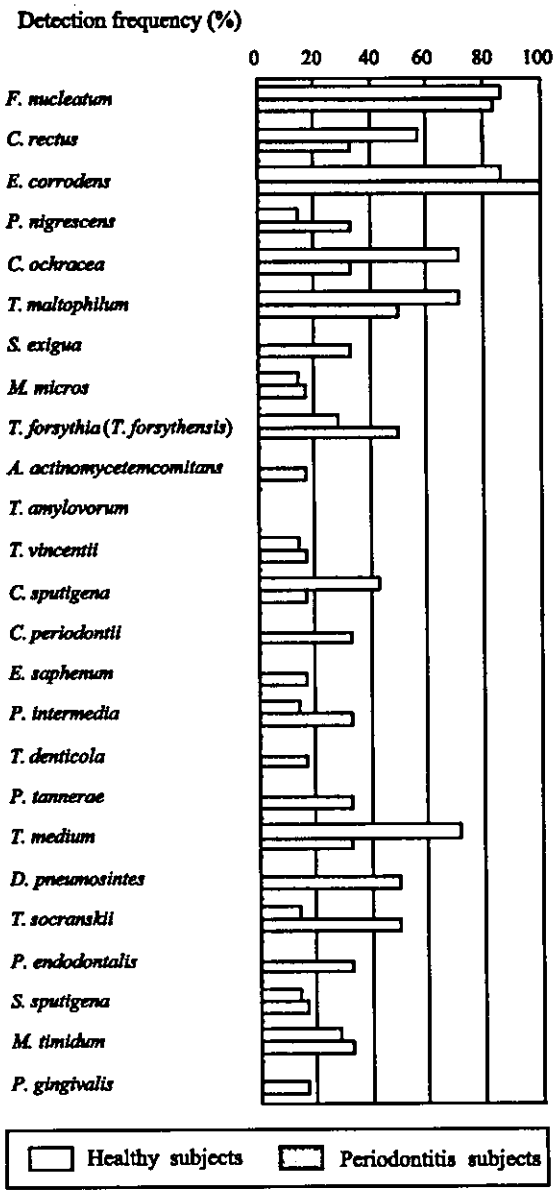

Fig. 2. Detection frequencies of target bacteria in alveolar mucosal plaque. Bacterial species are listed in the same order as in Fig. 1.

statistically significant ($0.002 \leq P < 0.05$) (Fig. 1). Two species (*M. timidum* and *P. gingivalis*) were detected significantly more frequently ($P < 0.002$) in subjects with periodontitis than in healthy subjects *P. gingivalis* was detected only in the periodontitis subjects. This is further evidence of an association of these two species with periodontitis, as reported previously for *P. gingivalis* (3, 18, 41, 51). *M. timidum* has also been isolated from deep periodontal pockets, supporting such an association (24, 45).

The detection frequencies of the bacteria in subgingival and supragingival plaque were similar in this study. A few studies have also suggested that the microbiota of subgingival and supragingival plaque were similar (16, 24, 51). Supragingival plaque may play an important role in bacterial invasion to subgingival sites, providing a reservoir of bacteria (51).

The recently proposed concept of 'biofilm' implies that bacteria establish a kind of supportive community (5). Based on this concept, bacterial species in dental plaque cooperate to make their environment anaerobic through oxygen consumption in their sugar and amino acid metabolic pathways (15, 21, 44) and maintain neutral pH via acid-neutralization by amino acid metabolism (39, 40), thus providing an environment where obligate anaerobic and acid-sensitive periodontopathic bacteria, such as *P. gingivalis*, are able to survive and grow. This may explain why *P. gingivalis* was detected at supragingival sites, which often become acidic and aerobic, in this study. From this point of view, the bacteria, frequently detected both in supragingival and subgingival plaque and at both healthy and periodontitis sites, are not individually pathogenic in the oral cavity, but rather may influence the initiation and progression of periodontitis when present together with periodontopathic bacteria.

The detection frequencies of bacteria in alveolar mucosa (Fig. 2) were generally lower than those in subgingival and supragingival plaque (Fig. 1), and the agreement rate of these bacteria at alveolar mucosa vs. subgingival (63%) or supragingival (60%) sites was also lower than those at subgingival vs. supragingival sites (81%). This suggests that alveolar mucosa has a microbiota composition that apparently differs from that of subgingival and supragingival plaque. This may be due to unique environmental factors of alveolar mucosa such as oxygen exposure and mobility, which result in less suitable environments for the target bacteria used in this study.

In conclusion, of 25 bacteria, 14 species showed no relationship with periodontitis, and 11 out of these 14 species were frequently detected (≥50%) in subgingival plaque in both periodontitis and healthy subjects. Nine bacteria seemed to be related to periodontitis, although their higher detection frequencies in subgingival plaque were not statistically significant by multiple comparisons. Only two species (*M. timidum* and *P. gingivalis*) in subgingival plaque were detected significantly more frequently in subjects with periodontitis than in healthy subjects, suggesting that the two species are closely related to periodontitis. In addition, the similarity of detection frequencies of the bacteria between subgingival and supragingival plaque suggests that analysis of supragingival plaque microbiota is useful in estimating the microbial composition of subgingival plaque.

Acknowledgments

This study was supported in part by Grants-in-Aid for Scientific Research (B)

(Nos. 13557187 and 16390611 to H.S.; Nos. 14370687 and 16390601 to N.T.); for Exploratory Research (No. 15659500 to N.T.); and for Young Scientists (B) (No. 14771000 to T.S.) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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Eur J Oral Sci 2005; 113: 34–40
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European Journal of Oral Sciences

Expression of type IV collagen and laminin at the interface between epithelial cells and fibroblasts from human periodontal ligament

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Shimonishi M, Sato J, Takahashi N, Komatsu M. Expression of type IV collagen and laminin at the interface between epithelial cells and fibroblasts from human periodontal ligament. Eur J Oral Sci 2005; 113: 34–40. © Eur J Oral Sci, 2005

The present study was undertaken to examine whether synthesis of type IV collagen and laminin around the epithelial rests of Malassez (ERM) requires direct contact between cells from ERM and periodontal ligament fibroblasts. Human periodontal ligament (HPDL) explants produced outgrowths containing both ERM cells and fibroblasts when cultured in a modified serum-free medium. The interface between ERM cells and fibroblasts was examined using phase-contrast microscopy (PCM) and scanning electron microscopy (SEM). Expression of type IV collagen and laminin was studied by immunohistochemistry and *in situ* hybridization. It was observed that ERM cells grew underneath fibroblasts or attached to them. At the interface, type IV collagen and laminin and their respective mRNAs were abundant in both ERM cells and fibroblasts, while these proteins and mRNAs showed little if any staining in cells further away from the interface. Hence, these findings indicate that synthesis of type IV collagen and laminin is induced by direct interaction between ERM cells and periodontal ligament fibroblasts.

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Key words: type IV collagen; laminin; epithelial rests of Malassez; periodontal ligament fibroblasts

Accepted for publication September 2004

The epithelial rests of Malassez (ERM) are normal consistent constituents of the periodontal ligament (PDL) and persist throughout the life of a tooth as a network of cell clusters connected to each other and possibly also to the junctional epithelium of gingiva (1, 2). Although their functions are still unknown, it has been suggested that they play a role in maintaining the PDL space (3), and they are perhaps also associated with cementum formation and cementum repair (4–7). Increased proliferation of the epithelial-rest cells has been reported to occur after surgical trauma or infection (8, 9).

In general, epithelial cells are separated from the underlying connective tissue by a basement membrane, which is composed of a mixture of matrix components mainly including type IV collagen (10), laminin (11, 12), and heparin sulfate proteoglycan (10, 13). It has been shown that extracellular matrix molecules are essential for cell adhesion, migration, differentiation, and growth, and that production of the extracellular matrix is involved in the interaction between the epithelial and connective tissue cells of the periodontium (14–19). Similarly, the ERMs are surrounded by a continuous basement membrane (20, 21). In an *in vitro* ultrastructural study, BRUNETTE *et al.* (22) found material reminiscent of a basal lamina, apparently synthesized by epithelial cells cultured from monkey periodontal ligament, in an area where the epithelial cells were in contact with collagen-like material that may have been produced by fibroblast-like cells. HOU *et al.* (23) have suggested that human PDL fibroblasts adjacent to the mouse epithelial root sheath cells may exhibit enhanced intracellular fluorescence for collagen I and fibronectin.

The aim of the present study was to determine whether a basement membrane could develop *in vitro* under conditions that allowed epithelial cells and fibroblasts derived from human PDL to interact directly with each other under chemically defined conditions. Since extracellular matrix components are essential for the formation of a basement membrane between two cell types, we evaluated the synthesis extracellular matrix proteins and their corresponding mRNA to elucidate the effect of the interaction between epithelial cells and fibroblasts from human PDL.

Material and methods

Cell culture

Freshly extracted third molars from 34 patients between 17 yr and 28 yr of age were obtained from the Department