をみる前に来院しなくなる，いわゆるドロップアウトの例は多い。我々の研究結果では，初診時にSF-36の社会生活機能（SF）が比較的高いのに，こころの健康（MH）が，低値の場合にドロップアウトする危険性が高い傾向が示されている。これらのことから，患者の主観的健康感を評価することは，患者主体の治療効果判定に有用であると考えられる。しかし，SF-36には個人の主観的健康感の変化を追跡できるほどの感度がないため，今後より精度の高い口臭患者に特異的なQOL評価尺度の開発が望まれる。

6．まとめ

岩手医科大学附属病院歯科診療センターにおける口臭診療の標準的な流れは以下のようにまとめられる。

1）メディカルインタビュー（口臭に対する解釈モデルの把握）

2）検査（口臭測定，う蝕・歯周疾患検査，細菌検査，唾液検査，QOL測定）

3）口腔清掃指導，舌清掃指導，歯疾の治療，PMTC

4）対話による援助（ガイダンス，コンサルテーション，カウンセリング）

5）治療効果の評価（口臭測定，自覚的口臭強度，QOL測定）

6）フォローアップリコール

しかし，患者によってこれらの内容は大きく異なる。実際，口臭を主訴としながら，断じて口臭測定を望まない患者もいるのである。はじめに述べたように，現在のところ口臭診療に標準的な方法はないといえる。目の前の患者が自己の口臭に対してどのような思いを抱き，我々に何を期待しているのかを理解しようとすることだけが今のところ我々の診療の指標である。そしてそれは，個人の歯科診療所で口臭診療を行う場合にも共通する事柄であろうと考える。

本稿が，口臭診療を行っている，あるいはこれから取組もうとしている諸先生方に少しでも参考になれば幸いである。

謝　辞

稿を終えるにあたり，執筆機会を与えてくださいました岩手医科大学歯学会雑誌編集委員長加藤裕久先生に感謝の意を表します。

文　献

1）Tonzetich, J., Coil, J. M. and Ng, W. : Gas chromatographic method for trapping and detection of volatile organic compounds from human mouth air. *J. Cin. Dent.* 2 : 79-82, 1992.

2）Persson, S., Edlund, M-B. and Clarsson, J. : The formation of hydrogen sulfide and methyl mercaptan by oral bacteria. *Oral Microbiol. Immunol.* 5 : 195-201. (1990).

3）Yaegaki, K. and Sanada, K. : Biochemical and clinical factors influencing oral malodor in periodontal patients. *J. Periodontol.* 63 : 783-789, 1992.

4）Yaegaki, K. and Sanada, K. : Volatile sulfur compounds in mouth air from clinically healthy subjects and patients with disease. *J. Periodontol Res,* 27 : 233-238, 1992.

5）Kozlovsky, A., Gordon, D., Gelernter, I., Loesche, W. J. and Rosenberg, M. : Correlation between the BANA test and oral malodor parameters. *J. Dent.* Res. 73 : 1036-1042, 1994.

6）Ratcliff, P. A. and Johnson, P. W. : The relationship between oral malodor, gingivitis, and periodontitis. A review. *J. Periodontol.* 70 : 485-489, 1999.

7）Umeda, M., Chen, C., Bakker, I., Contreras, A., Morrison, J. L. and Slots, J. : Risk indicators for harboring periodontal pathogens. *J. Periodontol.* 69 : 1111-1118, 1998.

8）Morita, M. and Wang, H-L. : Relationship between sulcular sulfide level and oral malodor in subjects with periodontal disease. *J. Periodontol.* 72 : 79-84, 2001.

9）星　佳芳：全身由来の口臭，歯界展望95；790-795, 2000.

10）宮崎秀夫，荒尾宗孝，岡村和彦，川口陽子，豊福明，星　佳芳，八重垣　健：口臭症分類の試みとその治療必要性，新潟歯誌29；11-15, 1999.

11）Rosenberg, M., Kulkarni, G. V., Bosy, A. and MeCulloch, C. A. G. : Reproducibility and sensitivity of oral malodor measurements with a portable sulphide monitor. *J. Dent. Res.* 70 : 1436-1440, 1991.

12）Preti, G., Clark, L., Cowart, B. J., Feldman, R. S., Lowry, L. D., Weber, E. and Young, I. M. : Non-oral etiologies of oral malodor and altered chemosensation. *J. Periodontol.* 63 : 790-796, 1992.

13）Miyazaki, H., Sakao, S., Katoh, Y. and Takehara, T. : Correlation between volatile sulphur

compounds and certain oral health measurements in the general population. *J. Periodontol.* 66 : 679-684, 1995.
14) Kishi, M., Kimura, S., Ohara-Nemoto, Y., Kishi, K., Toshiki M. and Yonemitsu, M. : Oral malodor and periodontopathic microorganisms in tongue coat of periodontally healthy subjects. *Dentistry in Japan* 38 : 24-28, 2002.
15) 八重垣　健：生理的口臭，歯界展望95；777-782, 2000.
16) 川口陽子，志村則夫，中村千賀子，米満正美，岡田昭五郎：歯科領域における心身症患者に関する研究，口病誌52 : 44-54, 1985.
17) 川口陽子，品田佳世子，志村則夫：「口臭についての質問票」の臨床統計的検討，日歯心身　7 : 134-140, 1992.
18) Iwakura, M., Yasuno, Y., Shimura, M. and Sakamoto, S. : Clinical characteristics of halitosis : differences in two patient groups with primary and secondary complaints of halitosis. *J. Dent. Res.* 79 : 1568-1574, 1994.
19) 相澤文恵，岸　光男，南　健太郎，米満正美：高校生の口臭に対する意識からみる自臭症リスク要因の行動科学的検討，口腔衛生会誌，50 : 189-196, 2000.
20) 相澤文恵，岸　光男，森谷俊樹，南　健太郎，米満正美：高校生を対象とした口臭の主観的評価とVSCレベルの関連性の分析，口腔衛生会誌，53 : 535-543, 2003.
21) Tanaka, M., Anguri, H., Nonaka, A., Kataoka, K., Nagata, H., Kita, J., and Shizukuishi, S. : Clinical assessment of oral malodor by the electronic nose system. *J. Dent. Res.* 83 : 317-321, 2004.
22) Coil, J. M., Yaegaki, K., Matsuo, T. and Miyazaki, H : Treatment needs (TN) and practical remedies for halitosis. *Int. Dent. J.* 52 Suppl. 3 : 187-91, 2002.
23) Eisenberg, L. : Disease and illness. Distinctions between professional and popular ideas of sickness. *Cult. Med. Psychiatry* 1 : 9-23, 1977.
24) Cole, S. A. and Bird, J. ; 飯島克巳，佐々木將人訳，メディカルインタビュー　三つの機能モデルによるアプローチ，第2版，メディカル・サイエンス・インターナショナル，東京，173-185ページ，2003 : The Medical Interview. The Three-Function Approach ; 2nd ed., Mosby Inc., St. Louis, 2000.
25) 小森まり子，鈴木清美，橋本佐由理：カウンセリングマインドを使った栄養指導のための面接技法，第1版，株式会社チーム医療，東京，12-15ページ，2002.
26) 岸　光男：大学病院のユニーク外来　口臭外来，デンタルハイジーン　22 : 228-231, 2002.
27) Kishi, M., Abe, A. and Yonemitsu, M. : Relationship between the SF-36 questionnaire and patient's satisfaction following halitosis therapy. *Oral Diseases* 11 suppl. 1 : 89-91, 2005.
28) Fukuhara, S., Bito, S., Green, J., Hsiao, a. and Kurokawa, A. : Translation, adaptation, and validation of the SF-36 health survey for use in Japan. *J. Clin. Epidemiol.* 51 : 1037-1044, 1998.
29) Fukuhara S, Ware J E, Kosinski M, et al : Psychometric and clinical tests of validity of the Japanese SF-36 Health Survey. Journal of Clinical Epidemiology, *J. Clin. Epidemiol.* 51 : 1045-1053., 1998.
30) 福原俊一，鈴鴨よしみ編・著，黒川　清監修：SF-36日本語版マニュアル（ver. 1.2）健康関連QOL尺度，第1版，（財）パブリックヘルスリサーチセンター，東京，2001.

Eur J Oral Sci 2005; 113: 34–40
Printed in UK. All rights reserved

European Journal of Oral Sciences

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

Mitsuru Shimonishi[1], Juta Sato[1], Nobuhiro Takahashi[2], Masashi Komatsu[1]

[1]Division of Operative Dentistry, Department of Restorative Dentistry, and [2]Division of Oral Ecology and Biochemistry, Department of Oral Biology, Tohoku University Graduate School of Dentistry, Japan

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.

Mitsuru Shimonishi, Division of Operative Dentistry, Department of Restorative Dentistry, Tohoku University Graduate School of Dentistry, 4–1, Seiryo-machi, Aoba-ku, Sendai, 980-8575, Japan

Telefax: +81–22–7178344
E-mail: shimo@ddh.tohoku.ac.jp

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

of Oral Surgery, Tohoku University Graduate School of Dentistry. Informed consent was obtained from the patients before extractions. After washing the teeth several times with alpha-minimum essential medium (α-MEM; Cosmo Bio, Tokyo, Japan) supplemented with 10% fetal bovine serum and antibiotics (60 $\mu g\ ml^{-1}$ kanamycin, 20 units ml^{-1} penicillin G, 10 $\mu g\ ml^{-1}$ fungison, Sigma, St Louis, MO, USA), PDL explants were carefully removed from the root with a scalpel. They were then plated in 35-mm culture dishes in α-MEM. The explants produced outgrowths that were primarily composed of fibroblasts. After 1 wk, α-MEM was replaced by a modified serum-free medium (3 : 1 (v/v) MCDB153 medium; Sigma) supplemented with 5 $\mu g\ ml^{-1}$ insulin (Sigma), 0.5 $\mu g\ ml^{-1}$ hydrocortisone (Sigma), 10 $\mu g\ ml^{-1}$ transferrin (Sigma), 14.1 $\mu g\ ml^{-1}$ phosphorylethanolamine (Sigma), 10 $ng\ ml^{-1}$ epidermal growth factor (EGF; Sigma) and antibiotics (24–26): α-MEM) including 40 $\mu g\ ml^{-1}$ bovine pituitary extract (Kyokuto, Tokyo, Japan) (27–29). This resulted in outgrowths of epithelial cells next to fibroblast-like cells. Cultures were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Differential adhesion to the surface in the presence of 0.25% trypsin/0.02% ethylenediaminetetraacetic acid (EDTA) solution was used to produce cultures that were predominantly of one cell type (i.e. either fibroblasts (5–10 min) or epithelial cells (15–20 min)) (30).

Gingival tissues were removed from clinically healthy patients at the time of the third molar extraction. After mincing they were cultured as described above. Cultures of PDL epithelial cells only or PDL fibroblasts only were used as controls.

Growth curves

Epithelial cells from PDLs and gingival tissues (second subculture) were seeded at 2×10^4 cells cm^{-1} in 12-well culture plates (Falcon; Nippon Becton Dickinson, Tokyo, Japan). The number of cells per dish on successive days was determined using a hemocytometer after trypsinizing the cells from the dish.

Statistical analysis

Statistical analysis of data was performed using Student's *t*-test ($P < 0.05$).

Scanning electron microscopy

Outgrowths of both epithelial cells and fibroblasts from human PDL cultured on the dishes were fixed with 2.5% glutaraldehyde in PBS at 4°C for 1 h and processed according to the tannic acid technique of KATSUMOTO *et al.* (31). Specimens were dried in a critical point dryer (HCP-2; Hitachi, Tokyo, Japan), ion-sputtered with Au–Pd in a coating unit (Hitachi 01–101), and examined with a scanning electron microscope (Hitachi S-510).

Immunohistochemistry

Cells were fixed with 4% paraformaldehyde at room temperature for 10 min. Alternatively, cells cultured on plastic discs (Sumilon; Sumitomo Bakelite, Tokyo, Japan) were dehydrated using a graded series of ethanol treatments, and then embedded in paraffin. Serial sections of 10-μm thickness were cut on a microtome. Both the cells on the dish and the vertical sections were processed for immunohistochemical examination. To inhibit endogenous peroxidase, 3% hydrogen peroxide was added. The cells and the vertical sections were then incubated with 10% normal goat serum for 30 min to block non-specific binding. They were then treated at room temperature for 2 h with a primary antibody to monoclonal mouse antihuman cytokeratin AE1/AE3 (1 : 50) (Dako, Carpinteria, CA, USA) to detect epithelial cells. The cells and vertical sections were then incubated overnight at 4°C with primary antiserum to rabbit antiporcine 25 kDa amelogenin (0.1 $\mu g\ ml^{-1}$) (courtesy of Dr T. Uchida, Second Department of Oral Anatomy, Hiroshima University School of Dentistry, Japan), which recognizes both human and rat amelogenin (6, 32), and with primary antibodies to monoclonal mouse antihuman type IV collagen (1 : 500) (Sigma) or polyclonal rabbit antihuman laminin (1 : 50) (Rockland, Gilbertsville, PA, USA). After rinsing in PBS, the cells were incubated with biotinylated immunoglobulin at room temperature for 30 min and stained by the avidin-biotinylated peroxidase complex (ABC) method, using an ExtrAvidin peroxidase staining kit (Sigma) and an AEC (3-amino-9-ethylcarbazole) chromogen kit (Sigma).

Proliferating, DNA-synthesizing cells in co-cultures were identified by nuclear incorporation of 100 $\mu mol\ l^{-1}$ 5-bromo-2′-deoxy-uridine (BrdU; Boehringer Mannheim Biochemica, Germany) for 24 h. Labeled nuclei were detected with monoclonal mouse anti-BrdU antibodies for 30 min at 37°C. After washing three times in PBS, the cells were incubated with HRP-conjugated goat antimouse immunoglobulin (Chemicon International, Temecula, CA, USA) for 30 min at 37°C and stained with 3,3-diaminobenzidine (DAB) and H_2O_2. Mayer's hematoxylin solution was applied for counterstaining. For control staining, PBS was used instead of the primary antibody.

In situ hybridization

The oligonucleotide probes used for *in situ* hybridization were synthesized by Nihon Gene Research Laboratories, Sendai, Japan. The sequences were as follows:

Probe 1: 5′-TCC AGG GTA GCC CCT CTC TCC TTT TTC TCC CAA AGG TCC TGT GCC-3′ for type IV collagen α-1 mRNA (33);
Probe 2: 5′-GCC ACC AGG TGG TGT CGG CCT GGT TGT TGT AGT CGG TCA GGA AGG-3′ for laminin γ-1 mRNA (34).

A biotin label was added at the 3′ end.

A computer-assisted search (GenBank) using the above antisense sequences, as well as the corresponding sense sequences, revealed no significant homology with any known sequences, other than that of the type IV collagen α-1 chain and the laminin γ-1 chain, respectively.

In situ hybridization was carried out using the *In Situ* Hybridization Detection Kit for Biotin Labeled Probes (Sigma). Briefly, the cells were fixed with 4% paraformaldehyde at room temperature for 10 min. They were then immersed in PBS including RNase inhibitor and then digested with proteinase K (0.1 $\mu g\ ml^{-1}$) at room temperature. To inhibit endogenous peroxidase, 3% hydrogen peroxide was added. The specimens were hybridized with biotin-labeled probes in the hybridization solution in a humid incubation chamber overnight at 37°C. After washing

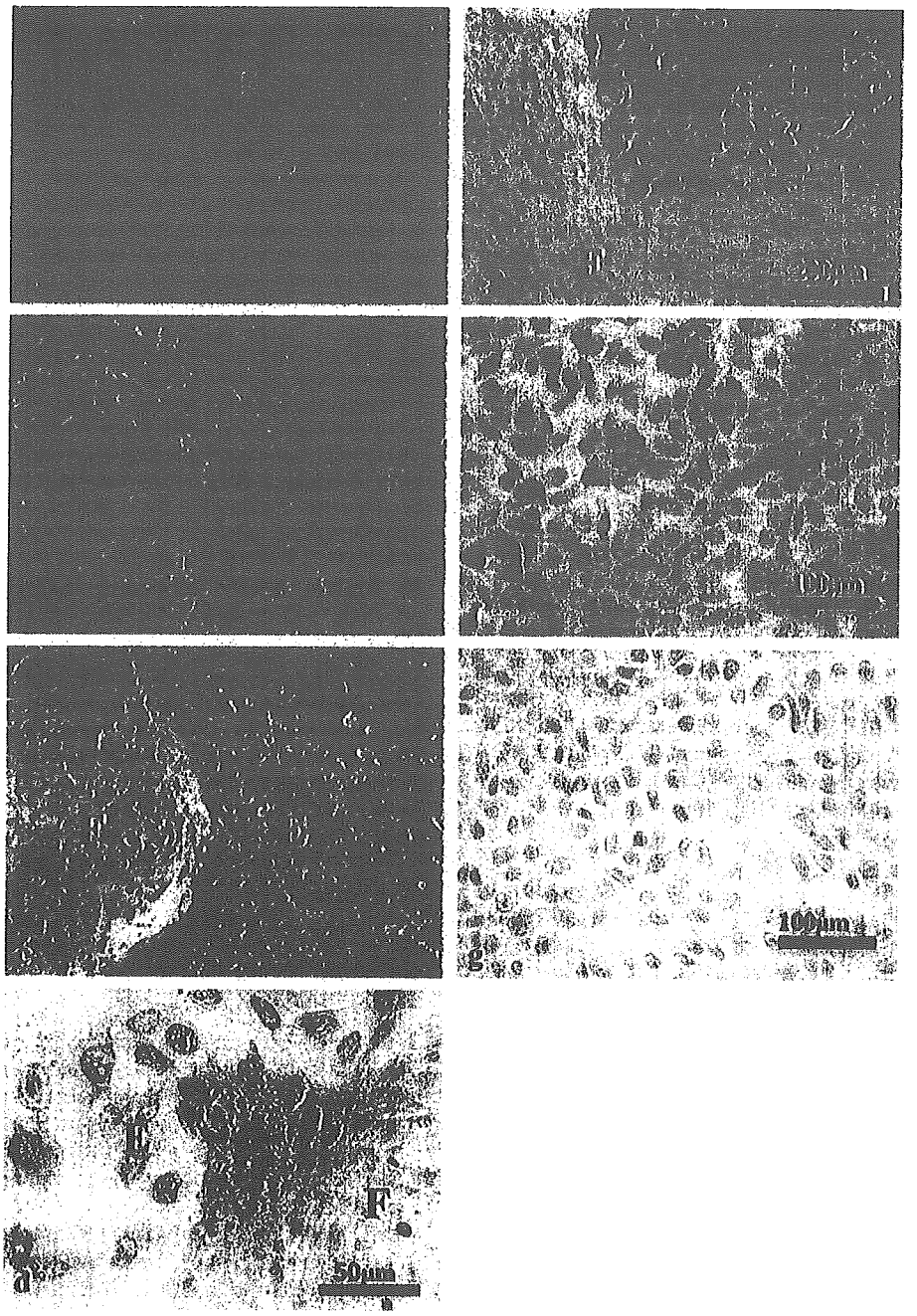

Fig. 1. (a) Phase-contrast photomicrograph of a primary coculture from human periodontal ligament. A sheet of epithelial cells (E) is covered by a ribbon (R) of fibroblasts (F). (b,c) Scanning electron micrographs of a primary cultures: (b) in the mixed multilayer, the epithelial cells (E) are seen to move under the fibroblasts (F); (c) epithelial cells (E) tightly attach to each other and push out the fibroblasts (F). (d) Photomicrograph showing assessment of proliferation by BrdU-labeling in epithelial cells (E) and fibroblasts (F) from human periodontal ligament. (e) Photomicrograph showing intense immunoreactivity for cytokeratin AE1/AE3 in epithelial cells (E) from human periodontal ligament. Immunostaining for cytokeratin in fibroblasts (F) is not seen. (f) Photomicrograph showing intense immunoreactivity for amelogenin in epithelial cells from human periodontal ligament; (g) immunostaining for amelogenin is not seen in epithelial cells from human gingival tissue.

in PBS, the specimens were reacted with blocking solution (5% BSA, 500 μg ml^{-1} normal sheep IgG, 100 μg ml^{-1} salmon testicular DNA, and 100 μg ml^{-1} yeast tRNA in PBS) at room temperature for 15 min. They were then incubated with ExtrAvidin peroxidase solution (Sigma) at 37°C for 20 min, reacted with biotin-conjugated anti-avidin antibody (Sigma) in a humid chamber at 37°C for 30 min, and re-reacted with ExtrAvidin peroxidase solution. Following this, they were washed three times in PBS and the peroxidase sites were visualized using a solution containing

DAB and H_2O_2, with counterstaining using Mayer's hematoxylin solution.

Results

Characterization of cells in mixed cultures

Explant cultures from human PDL showed initial outgrowth of fibroblasts from the explant edges when maintained in α-MEM for 1 week. After replacing α-MEM with the modified serum-free medium, outgrowth of epithelial cells was induced in 50 out of 225 explant cultures (22.2%), and multilayers of both epithelial cells and fibroblasts were seen within 2–3 wk. All the co-cultures showed similar epithelial–mesenchymal interaction phenomena. When both epithelial cells and fibroblasts were cultured together in the modified serum-free medium, they were distinguishable from each other morphologically (Fig. 1a). Furthermore, scanning electron microscopy revealed that the epithelial colonies grew under fibroblasts (Fig. 1b), or attached adjacent to them (Fig. 1c).

Figure 2 shows typical growth curves of epithelial cells from PDLs and gingival tissues. The PDL epithelial cells had a lower growth rate than gingival epithelial cells under our culture conditions. In addition, PDL epithelial cells incorporated BrdU more extensively than PDL fibroblasts derived from the same PDL explant, indicating that PDL epithelial cells have a higher proliferation rate than PDL fibroblasts (Fig. 1d).

Immunohistochemistry

Human PDL epithelial cells in mixed cultures stained positive for broad-spectrum antibodies to cytokeratins (AE1/AE3), indicating their epithelial origin, while fibroblasts in the same culture did not show cytokeratin expression (Fig. 1e). Moreover, immunoreactivity for amelogenin was observed in human PDL epithelial cells, indicating their odontogenic epithelial origin, while human gingival epithelial cells did not show amelogenin expression (Fig. 1f,g).

In co-cultures (Fig. 3a) and vertical sections (Fig. 3c,d), the intensity of immunostaining for type IV collagen in human PDL fibroblasts immediately adjacent to human PDL epithelial cells was markedly high. Similarly, the intensity of immunostaining for laminin was increased at the interface (Fig. 3b). Immunostaining for both proteins showed little if any staining in areas further away from this interface. Control epithelial cells cultured alone showed only weak staining for type IV collagen (Fig. 3e) and laminin (data not shown). Control fibroblasts cultured alone showed little if any staining for type IV collagen (Fig. 3f) and laminin (data not shown).

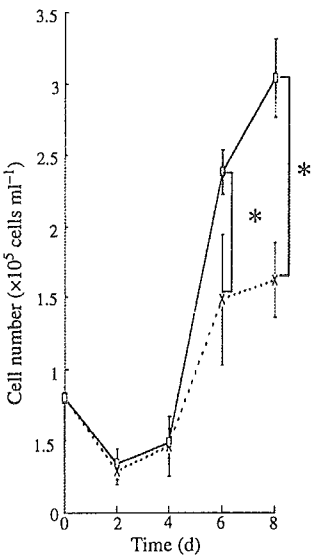

Fig. 2. Growth curves of epithelial cells from human periodontal ligament (dashed line with Xs) and gingival tissue (unbroken line with boxes). Values are the mean ± S.D. of five samples. $*P < 0.05$.

In situ hybridization

To localize mRNA expression of type IV collagen α-1 chain and laminin γ-1 chain, the cells were hybridized *in situ* with biotin-labeled antisense oligo-DNA probes. The PDL fibroblasts immediately adjacent to PDL epithelial cells showed higher positive signals for both type IV collagen α-1 mRNA and laminin γ-1 mRNA (Fig. 4a,b). These results were consistent with the immunohistochemical observations (Fig. 3a,b). In the epithelial cells or the fibroblasts cultured alone, signals for type IV collagen α-1 mRNA and laminin γ-1 mRNA (data not shown) were weak compared with the co-culture.

Discussion

Both fibroblasts and epithelial cells were successfully cultured from human PDL tissue, using the explant technique in a modified serum-free medium. There were two steps in our co-culture system: in the first step, outgrowth of fibroblasts was induced from explants in α-MEM; and in the second step, outgrowth of epithelial cells was induced from the same explants, after replacing α-MEM with a modified serum-free medium. In this case, two different cell types were derived from the same tissue, but these cells did not mix, and instead formed fibroblasts as multilayers around epithelial cells. Keratin expression clearly distinguished epithelial cells from fibroblasts, since only the former cell type expressed this protein (Fig. 1e). In addition, amelogenin was expressed only in the cultured human PDL epithelial cells, and was not detectable in the human gingival epithelial cells (Fig. 1f,g). In a previous *in vitro* study, amelogenin synthesis has been reported in rat ameloblast-lineage cells (35), and these results suggest that our cultured human PDL epithelial cells were derived from the odontogenic epithelium, the epithelial cells of Malassez.

The morphological properties observed in the present study were similar to those found in an *in vitro* culture of monkey PDL fibroblasts and epithelial cells (22), and

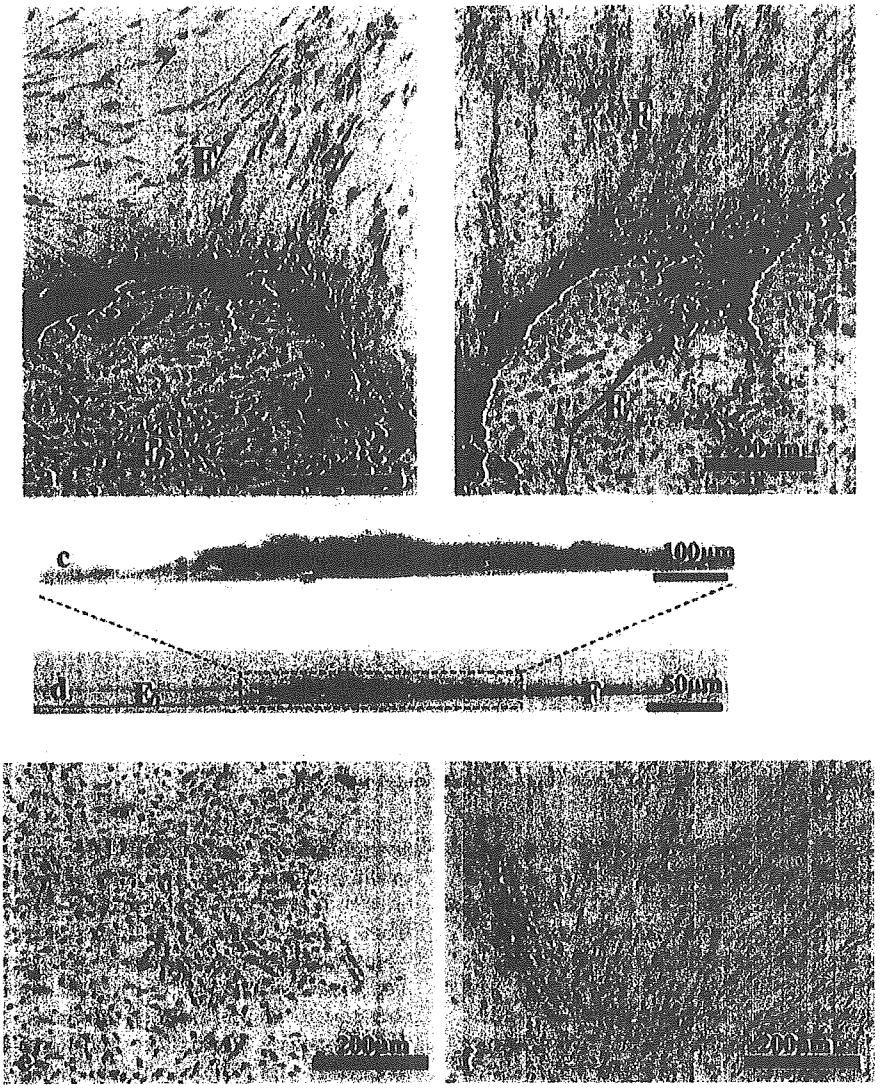

Fig. 3. (a,b) Photomicrographs showing intense immunoreactivity for type IV collagen (a) and laminin (b) between epithelial cells (E) and fibroblasts (F) from human periodontal ligament. (c,d) Photomicrographs showing intense immunoreactivity for type IV collagen at the interface between epithelial cells (E) and fibroblasts (F) from human periodontal ligament in vertical section. (e) Photomicrograph showing weak immunoreactivity for type IV collagen in control epithelial cells from human periodontal ligament. (f) Photomicrograph showing little immunoreactivity for type IV collagen in control fibroblasts from human periodontal ligament.

those of human PDL fibroblasts and mouse PDL epithelial cells (23). Ultrastructural changes of fibroblasts adjacent to the epithelial cells have also been reported for intracellular organelles, and have been related to protein synthesis, processing and transport (23). Additional features of interest in the ultrastructural studies were the presence of extracellular matrix between epithelial cells and fibroblasts under co-culture conditions (22, 23).

It is well established that extracellular matrix molecules regulate various physiological activities, such as cell growth, migration and differentiation (14–19). The basement membrane, a part of the extracellular matrix, separates the connective tissue from the epithelium and contains collagen (mainly type IV) and non-collagenous glycoproteins such as laminin. Under *in vitro* culture conditions, many cells secrete the components of the basement membrane (14, 15, 34, 36–41). In our study, enhanced expression and distribution of type IV collagen and laminin were found at the interface between epithelial cells and fibroblasts, while little staining for these proteins was apparent in areas further away from the interface of the mixed culture. These results suggest that interactions between epithelial cells and fibroblasts stimulate type IV collagen and laminin expression in epithelial cells *in vitro*. The modulation of the expression of extracellular molecules in fibroblasts by epithelial cells has been documented in previous cell culture studies (23), which showed that, under co-culture conditions, the synthesis of type I and III collagen and fibronectin was stimulated in PDL fibroblasts adjacent to PDL epithelial

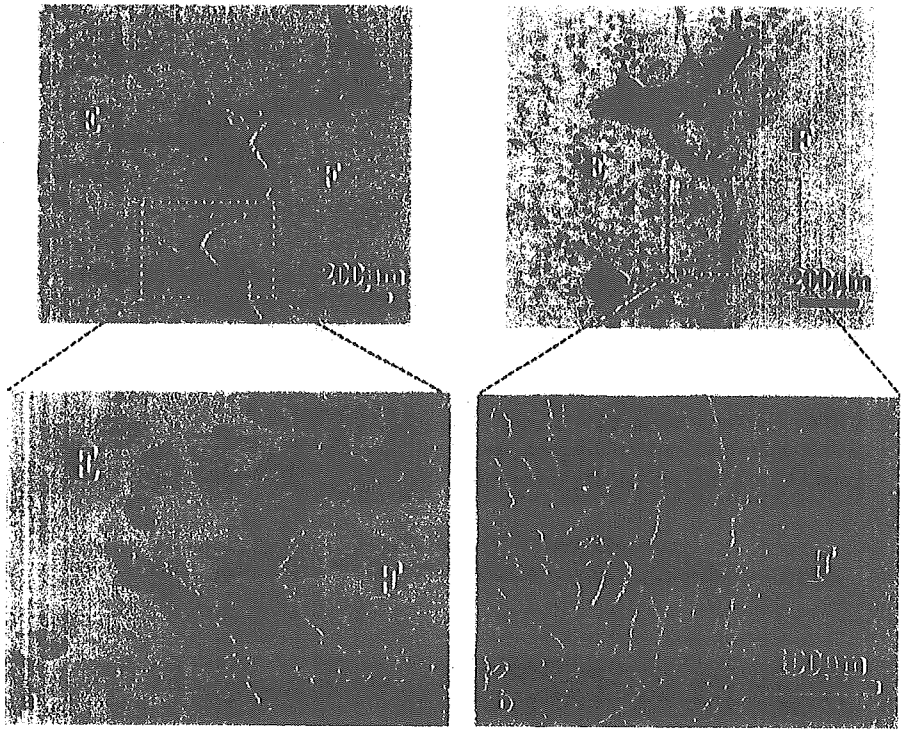

Fig. 4. Photomicrographs showing intense immunoreactivity for type IV collagen mRNA (a) and laminin mRNA (b) at the interface between epithelial cells (E) and fibroblasts (F) from human periodontal ligament.

cells. These observations are concordant with our observations, but the matrix proteins (type I and III collagen and fibronectin) detected are not components specific to the basement membrane. In another study in organotypic co-cultures of epithelial cells and fibroblasts derived from gingival tissue (41), type IV collagen and laminin were observed immunohistochemically underneath the basal epithelial cells. However, to our knowledge, our study is the first to show the synthesis of basement membrane constituents at the interface between epithelial cells and fibroblasts derived from PDL.

The effects of cell density and cell-cell contacts on protein syntheses appear very interesting. In our study, fibroblasts were clearly distinguishable from epithelial cells (Fig. 1a), and type IV collagen and laminin were strongly expressed only at the interface (Fig. 3a,b) where direct cell–cell contacts occurred, suggesting the involvement of such contacts in protein expression. During cell culture, an area of high cell density is sometimes produced, and in our study this area was observed in the culture of fibroblasts alone and epithelial cells alone (Fig. 3e,f), but significant protein expression was not observed in either of these cultures. Hence, these observations do not support the hypothesis that cell density itself affects protein expression. However, it is still unclear whether increased protein expression requires cell–cell contacts, synthesis of other proteins, or an appropriate cell density, and this remains to be elucidated.

The role of PDL epithelial cells in periodontal physiology is unclear. However, it is known that, when stimulated by endodontal infection, these cells can proliferate and give rise to periapical cyst formation (1, 42). It has been reported that epithelial cells derived from porcine rests of Malassez secrete prostaglandin, which possibly contributes to cyst growth (43). It has also been speculated that PDL epithelial cells may participate in periodontal diseases by proliferating during pocket formation and development of new junctional epithelium during postoperative gingival healing (2).

Acknowledgements – We gratefully acknowledge Dr Yasutaka Nitta (Department of Oral Surgery, Tohoku University Graduate School of Dentistry) for kindly supplying the extracted human third molars. This work was supported by a Grant-in-Aid for Encouragement of Young Scientists (B) (No. 15791088) from the Japan Society for the Promotion of Science.

References

1. TEN CATE AR. The epithelial cell rests of Malassez and the genesis of the dental cyst. *Oral Surg Oral Med Oral Pathol* 1972; **34**: 956–964.
2. SPOGE JD. Rests of Malassez and chronic marginal periodontal disease. *J Can Dent Assoc* 1980; **11**: 712–716.
3. MELCHER AH. Repair of wounds in the periodontium of the rat. Influence of periodontal ligament on osteogenesis. *Arch Oral Biol* 1970; **15**: 1183–1204.
4. SLAVKIN HC, BOYDE A. Cementum: an epithelial secretory product? *J Dent Res* 1975; **53**: 157.
5. SLAVKIN HC. Towards a cellular and molecular understanding of periodontics: cementogenesis revisited. *J Periodontol* 1976; **47**: 249–255.

6. Hamamoto Y, Nakajima T, Ozawa H, Uchida T. Production of amelogenin by enamel epithelium of Hertwig's root sheath. *Oral Surg Oral Med Oral Pathol* 1996; **81**: 703–709.
7. Hammarstrom L. Enamel matrix, cementum development and regeneration. *J Clin Periodontol* 1997; **24**: 658–668.
8. Grupe AE jr, ten Cate AR, Zander HA. A histochemical and radiobiological study of *in vitro* and *in vivo* human epithelial cell rest proliferation. *Arch Oral Biol* 1967; **12**: 1321–1329.
9. Johansen JR. Incorporation of tritiated thymidine by the epithelial rests of Malassez after attempted extraction of rat molars. *Acta Odont Scand* 1969; **19**: 463–470.
10. Timpl R, Dziadek M. Structure, development, and molecular pathology of basement membranes. *Int Rev Exp Pathol* 1986; **29**: 1–112.
11. Timpl R, Rohde H, Robey PG, Rennard SI, Foidart JM, Martin GR. Laminin – a glycoprotein from basement membranes. *J Biol Chem* 1979; **254**: 9933–9937.
12. Engel J, Odermatt E, Engel A, Madri JA, Furthmayr H, Rohde H, Timpl R. Shapes, domain organizations and flexibility of laminin and fibronectin, two multifunctional proteins of the extracellular matrix. *J Mol Biol* 1981; **150**: 97–120.
13. Carlin BE, Jaffe R, Bender B, Chung AE. Entactin, a novel basal-lamina associated sulfated glycoprotein. *J Biol Chem* 1981; **256**: 5209–5214.
14. Uitto VJ, Larjava H, Peltonen J, Brunette DM. Expression of fibronectin and integrins in cultured periodontal ligament epithelial cells. *J Dent Res* 1992; **71**: 1203–1211.
15. Yamada KM. Cell surface interactions with extracellular materials. *Annu Rev Biochem* 1983; **52**: 76–795.
16. Pital S, Hekmati M, Metzger Z, Savion N. Epithelial–connective tissue interaction on the tooth surface: an *in vitro* model. *J Periodont Res* 1991; **26**: 461–467.
17. Terranova VP, Martin GR. Molecular factors determining gingival tissue interaction with tooth structure. *J Periodont Res* 1986; **57**: 311–317.
18. Lin CQ, Bissell MJ. Multi-faceted regulation of cell differentiation by extracellular matrix. *FASEB J* 1993; **7**: 737–743.
19. Pital S, Hekmati M, Metzger Z, Savion N. The effect of partial demineralization and fibronectin on migration and growth of gingival epithelial cells *in vitro*. *J Periodontol* 1989; **60**: 293–301.
20. Hamamoto Y, Nakajima T, Ozawa H. Ultrastructure of epithelial rests of Malassez in human periodontal ligament. *Archs Oral Biol* 1989; **34**: 179–185.
21. Hamamoto Y, Suzuki I, Nakajima T, Ozawa H. Immunocytochemical localization of laminin in the epithelial rests of Malassez of immature rat molars. *Arch Oral Biol* 1991; **36**: 623–626.
22. Brunette DM, Kanoza RJ, Marmary Y, Chan J, Melcher AH. Interactions between epithelial and fibroblast-like cells in cultures derived from monkey periodontal ligament. *J Cell Sci* 1977; **27**: 127–140.
23. Hou LT, Kollar EJ, Yaeger JA. Epithelial cell–fibroblast interactions: modulation of extracellular matrix protein in cultured oral cells. *J Periodont Res*, 1993; **28**: 102–114.
24. Rheinwald JG, Green H. Serial cultivation of strains of human epidermal keratinocytes. *Cell* 1975; **6**: 331–334.
25. Rheinwald JG, Green H. Epidermal growth factor and the multiplication of cultured human epidermal keratinocytes. *Nature* 1977; **265**: 421–424.
26. Boyce ST, Ham RG. Calcium-regulated differentiation of normal human epidermal keratinocytes in chemically defined clonal cultures and serum-free serial cultures. *J Invest Dermatol* 1983; **81**: 33–40.
27. Yamanaka T, Sakamoto A, Tanaka Y, Zhang Y, Hayashido Y, Toratani S, Akagawa Y, Okamoto T. Isolation and serum-free culture of epithelial cells derived from epithelial rests of Malassez in human periodontal ligament. *In Vitro Cell Dev Biol* 2000; **36**: 548–553.
28. Pittelkow MR, Scott RE. New techniques for the *in vitro* culture of human skin keratinocytes and perspectives on their use for grafting of patients with extensive burns. *Mayo Clin Proc* 1986; **61**: 771–777.
29. Coffey RJ jr, Derynck R, Wilcox JN, Bringman TS, Goustin AS, Moses HL, Pittelkow MR. Production and auto-induction of transforming growth factor-α human keratinocytes. *Nature* 1987; **328**: 817–820.
30. Brunette DM, Melcher AH, Moe HK. Culture and origin of epithelium-like and fibroblast-like cells from porcine periodontal ligament explants and cell suspensions. *Arch Oral Biol* 1976; **21**: 393–400.
31. Katsumoto T, Naguro T, Iino A, Takagi A. The effect of tannic acid on the preservation of tissue culture cells for scanning electron microscopy. *J Electron Microsc* 1981; **30**: 177–182.
32. Uchida T, Tanabe T, Fukae M. Immunocytochemical localization of amelogenins in the deciduous tooth germs of the human fetus. *Arch Histol Cytol* 1989; **52**: 543–552.
33. Brazel D, Oberbaumer I, Dieringer H, Babel W, Glanville RW, Deutzmann R, Kuhn K. Completion of the amino acid sequence of the α1 chain of human basement membrane collagen (type IV) reveals 21 non-triplet interruptions located within the collagenous domain. *Eur J Biochem* 1987; **168**: 529–536.
34. Hann CR, Springett MJ, Wang X, Johnson DH. Ultrastructural localization of collagen IV, fibronectin, and laminin in the trabecular meshwork of normal and glaucomatous eyes. *Ophthalmic Res* 2001; **33**: 314–324.
35. Tabata MJ, Matsumura T, Liu J-G, Wakisaka S, Kurisu K. Expression of cytokeratin 14 in ameloblast-lineage cells of the developing tooth of rat, both *in vivo* and *in vitro*. *Arch Oral Biol* 1996; **41**: 1019–1027.
36. de Arcangelis A, Lefebvre O, Mechine-Neuville A, Arnold C, Klein A, Remy L, Kedinger M, Simon-Assmann P. Overexpression of laminin α1 chain in colonic cancer cells induces an increase in tumor growth. *Int J Cancer* 2001; **94**: 44–53.
37. Tokida Y, Aratani Y, Morita A, Kitagawa Y. Production of two variant laminin forms by endothelial cells and shift of their relative levels by angiostatic steroids. *J Biol Chem* 1990; **265**: 18123–18129.
38. Zhang X-H, Sun H-M, Yuan J-Q. Extracellular matrix production of lens epithelial cells. *J Cataract Refract Surg* 2001; **27**: 1303–1309.
39. Baker SE, Dipasqualeip AP, Stock EL, Quaranta V, Fitchmun M, Jones JC. Morphogenetic effects of soluble laminin-5 on cultured epithelial cells and tissue explants. *Exp Cell Res* 1996; **228**: 262–270.
40. Odioso LL, Doyle MJ, Quinn KW, Bartel RL, Zimber MP, Stevens-Burns D. Development and characterization of an *in vitro* gingival epithelial model. *J Periodont Res* 1995; **30**: 210–219.
41. Tomakidi P, Fusenig NE, Kohl A, Komposch G. Histomorphological and biochemical differentiation capacity in organotypic co-cultures gingival cells. *J Periodont Res* 1997; **32**: 388–400.
42. Gao Z, MacKenzie IC, Williams DW, Cruchley AW, Leigh I, Lane B. Patterns of keratin-expression in rest of Malassez and periapical lesions. *J Oral Pathol* 1988; **17**: 178–185.
43. Brunette DM, Heersche JNM, Purdon AD, Sodek J, Moe HK, Assuras JN. *In vitro* cultural parameters and protein and prostaglandin secretion of epithelial cells derived from porcine rests of Malassez. *Archs Oral Biol* 1979; **24**: 199–203.

Journal of Medical Microbiology (2005), 54, 889–895 DOI 10.1099/jmm.0.46118-0

Hydrogen sulfide-producing bacteria in tongue biofilm and their relationship with oral malodour

Jumpei Washio,[1,2] Takuichi Sato,[2] Takeyoshi Koseki[1] and Nobuhiro Takahashi[2]

[1,2]Division of Preventive Dentistry[1], and Division of Oral Ecology and Biochemistry[2], Tohoku University Graduate School of Dentistry, 4-1 Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan

Correspondence
Nobuhiro Takahashi
nobu-t@mail.tains.tohoku.ac.jp

Received 12 April 2005
Accepted 1 June 2005

The aims of this study were to identify hydrogen sulfide (H_2S)-producing bacteria among tongue biofilm microflora and to investigate the relationship between bacterial flora and H_2S levels in mouth air. Oral malodour levels in 10 subjects (age 21–56 years) were assessed by gas chromatography, and Breathtron and organoleptic scores. Based on these assessments, subjects were divided into two groups: an odour group and a no/low odour group. Tongue coatings were sampled and spread onto Fastidious Anaerobe Agar plates containing 0·05 % cysteine, 0·12 % glutathione and 0·02 % lead acetate, and were then incubated anaerobically at 37 °C for 2 weeks. Bacteria forming black or grey colonies were selected as H_2S-producing phenotypes. The numbers of total bacteria ($P < 0·005$) and H_2S-producing bacteria ($P < 0·05$) in the odour group were significantly larger than those in the no/low odour group. Bacteria forming black or grey colonies (126 isolates from the odour group; 242 isolates from the no/low odour group) were subcultured, confirmed as producing H_2S and identified according to 16S rRNA gene sequencing. Species of *Veillonella* (38·1 % in odour group; 46·3 % in no/low odour group), *Actinomyces* (25·4 %; 17·7 %) and *Prevotella* (10·3 %; 7·8 %) were the predominant H_2S-producing bacteria in both the odour and no/low odour groups. These results suggest that an increase in the number of H_2S-producing bacteria in the tongue biofilm is responsible for oral malodour, although the bacterial composition of tongue biofilm was similar between the two groups.

INTRODUCTION

Oral malodour is foul-smelling breath exhaled from the oral cavity and is due to metabolic products of bacteria in the oral cavity but can also be caused by systemic diseases, such as gastrointestinal disorders, hepatic diseases and diabetes, ingestion of certain foods and smoking (Greenman, 1999). Approximately 90 % of oral malodour is believed to originate from foul-smelling gases, such as volatile sulfur compounds (VSCs), produced by oral bacteria in the oral cavity (Ayers *et al.*, 1998; Scully *et al.*, 1994). The major components of VSCs in oral malodour are hydrogen sulfide (H_2S), methyl mercaptan and dimethyl sulfide (Kleinberg & Westbay, 1990). These VSCs are produced through bacterial metabolism of sulfur amino acids such as cysteine and methionine (Persson *et al.*, 1990).

VSC-producing bacteria are present at various sites in the oral cavity, particularly on the dorsum of the tongue, where they have easy access to nutrients, such as saliva, desquamated epithelium and food debris (Roldan *et al.*, 2003). Therefore, the coating on the dorsum of the tongue is widely recognized as a major source of VSCs (De Boever & Loesche, 1995; Nakano *et al.*, 2002; Rosenberg, 1996; Yaegaki & Sunada, 1992a, b).

Most previous studies have focused on the relationship between oral malodour and salivary or dental plaque bacteria (Awano *et al.*, 2002; Paryavi-Gholami *et al.*, 1999; Persson *et al.*, 1990). Following work by Gordon *et al.* (1966), studies have been conducted to analyse bacteria in the tongue biofilm, but most have targeted a limited number of bacterial species (Frisken *et al.*, 1990; Miyake *et al.*, 1991; van Winkelhoff *et al.*, 1986). Comprehensive analyses of tongue biofilm microflora using culture methods or molecular biological methods have recently been reported (Hartley *et al.*, 1996, 1999; Kazor *et al.*, 2003; Milnes *et al.*, 1993). Due to its complexity, however, the characteristics of tongue biofilm microflora and its relationship with oral malodour remain unclear (Hartley *et al.*, 1996, 1999; Kazor *et al.*, 2003).

Paryavi-Gholami *et al.* (1999) reported the isolation and identification of H_2S-producing bacteria from the saliva of children, using agar plates including lead acetate, and discussed the relationship between salivary H_2S-producing bacteria and oral malodour. Applying their methods, the aims of this study were to isolate and identify H_2S-producing

Abbreviation: VSC, volatile sulfur compound.

bacteria from the tongue biofilm using molecular biological methods, such as PCR and DNA sequencing, and to determine any relationships between the number or type of H_2S-producing bacteria and oral malodour.

METHODS

Subjects. Ten subjects (five females and five males; age, mean ± SD, 36·3 ± 11·1 years; range, 21–56 years) were selected for this study. Informed consent was obtained from each subject. All subjects were patients who visited Tohoku University Dental Hospital complaining of halitosis. They had no systemic disease and received no antibiotic therapy for at least 3 months. On the first visit, an assessment of oral malodour and observable tongue coating, a clinical oral examination and sampling of tongue biofilm were performed as described below.

Oral malodour assessment. Level of oral malodour was assessed by gas chromatography (GC; Shimadzu GC-7A, Kyoto), and Breathtron (New Cosmos Electric) and organoleptic scoring. Breathtron is a portable monitor with a zinc-oxide thin film semiconductor sensor specific to VSCs (Shimura *et al.*, 1996). All subjects were asked not to brush, rinse or smoke immediately prior to the assessment, and not to eat and drink for at least 2 h before assessment. GC analysis was carried out in duplicate. After closing the lips for 1 min, 5 ml of mouth air was obtained with a gastight syringe and immediately injected into the GC equipment. Standard samples of H_2S and methyl mercaptan (Sumitomo Seika Chemicals) were used as controls. Breathtron analysis was also performed in duplicate. Organoleptic scores were assessed by three judges immediately after closing the lips for 30 s. Scores were given as follows: 0, no malodour; 1, slight malodour; 2, clearly noticeable malodour; 3, strong malodour; and 4, extremely strong malodour.

Clinical oral examination. All subjects were examined for dental caries, plaque accumulation by O'Leary plaque control record index (O'Leary *et al.*, 1972) and probing depth using a periodontal pocket probe. No subjects lacked numerous teeth, wore dentures or exhibited severe caries, severe gingivitis, periodontitis or any other oral disease associated with oral malodour.

Observable tongue coating assessment. Thickness and extent of tongue coating were estimated by the naked eye according to the method of Nara (1977). Both thickness and extent of tongue coating were scored as 0, 1, 2 or 3, and then the thickness score and the extent score were multiplied.

Sampling of tongue biofilm. In order to collect tongue biofilm, an area of 1 cm^2, predetermined by a window made of sterilized plain paper on the rear dorsal surface of the tongue, was firmly scraped 10 times with sterilized toothpicks. All samples were immediately introduced into an anaerobic chamber containing 80 % N_2, 10 % CO_2 and 10 % H_2 (model AZ-Hard, Hirasawa) and were suspended in 1 ml of distilled 40 mM potassium phosphate buffer (PPB, pH 7·0) solution. After homogenization for 5 min, decimal dilutions from 10^{-3} to 10^{-6} were prepared in 40 mM PPB solution.

Culture conditions. One hundred microlitres from each dilution sample was dispersed and spread either onto Fastidious Anaerobe Agar (FAA, Lab M) plates containing 0·05 % L-cysteine, supplemented with 5 % rabbit blood (Nippon Bio-Test Laboratories), 0·12 % glutathione and 0·02 % lead acetate, according to the method of Paryavi-Gholami *et al.* (1999) with minor modifications, or onto FAA plates without 0·02 % lead acetate as a control. Plates were incubated at 37 °C for 2 weeks in an anaerobic chamber. To ensure strictly anaerobic conditions in the chamber, reduction of methylviologen (−446 mV) was carefully confirmed whenever experiment procedures were carried out.

After 2 weeks of incubation, bacteria forming black or grey colonies were regarded as H_2S-producing. All of the black or grey colonies on plates with less than 100 colonies were picked up using sterilized plastic loops or toothpicks and subcultured on FAA agar plates. These bacterial isolates were confirmed as producing H_2S in test tubes of Fastidious Anaerobe Broth (Lab M) liquid media. Bacterial isolates were grown anaerobically, and the presence of H_2S in the headspace of the test tubes was determined from the blackening of filter paper strips immersed in lead acetate.

DNA extraction and 16S rRNA gene sequencing. Colonies subcultured from four malodourous and four nonodourous subjects were harvested by centrifugation at 7700 *g* for 5 min and the supernatant was removed. Genomic DNA was then extracted from the pellets using the InstaGene Matrix Kit (Bio-Rad) according to the manufacturer's instructions.

The 16S rRNA gene sequences were amplified by PCR using universal primers 27F and 1492R (Lane, 1991) and *Taq* DNA polymerase (HotStarTaq Master Mix, Qiagen) according to the manufacturer's instructions. The primer sequences were: 27F, 5′-AGAGTTT GATCMTGGCTCAG-3′; and 1492R, 5′-TACGGYTACCTTGTTAC GACTT-3′. Amplification proceeded using a PCR Thermal Cycler MP (TaKaRa Biomedicals) programmed as follows: 15 min at 95 °C for initial heat activation and 35 cycles of 1 min at 94 °C for denaturation, 1 min at 52 °C for annealing and 1·5 min at 72 °C for extension, followed by 10 min at 72 °C for final extension. PCR products were sequenced at Hokkaido System Science using the BigDye Terminator Cycle Sequencing Kit and an automated DNA sequencer (PRISM-3100, Applied Biosystem). Primers 27F and 1492R were used to sequence both strands (at least 1000 bp), and DNA data were analysed using the DNASIS program (Hitachi Software Engineering). BLAST searches were performed through the website of the National Center for Biotechnology Information. Bacterial species were determined by percentage sequence similarity (>97 %).

Data analysis. An unpaired *t*-test was used to analyse significance. *P* values of < 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Relationship between oral malodour level and clinical indicators

Based on the results of GC, the 10 subjects were divided into the H_2S-undetected group (below the detection limit) and the H_2S-detected group (mean ± SD, 1·05 ± 0·97 p.p.m.) (Table 1). In addition, there were significant differences between the two groups in Breathtron and organoleptic scores, which were also used to assess oral malodour (Table 1). Therefore, the H_2S-detected group was designated the odour group, and the H_2S-undetected group was designated the no/low odour group. Methyl mercaptan was detected only in two subjects belonging to the odour group and no dimethyl sulfide was detected.

With regard to clinical parameters, there were no significant differences in age, number of present teeth, number of teeth with untreated caries, number of teeth with probing depth >4 mm, largest probing depth or O'Leary plaque control record score between the two groups (Table 1). There were also no significant differences in tongue coating score between the two groups (Table 1). Considering that the maximum observable tongue coating score is 9, the mean

Table 1. Clinical assessment of no/low odour and odour groups in this study

Data are presented as mean ± SD.

Assessment	No/low odour group ($n = 5$)	Odour group ($n = 5$)
Age	41·8 ± 13·9	30·8 ± 2·4
No. of teeth present	24·0 ± 3·5	27·2 ± 1·3
No. of teeth with untreated caries	0·2 ± 0·48	0·2 ± 0·49
No. of teeth with probing depth >4mm	1·6 ± 2·5	1·6 ± 3·6
Largest probing depth (mm)	3·8 ± 0·8	4·0 ± 2·2
Plaque control record score	38·9 ± 22·9	47·5 ± 23·2
Tongue coating score	1·2 ± 1·1	2·0 ± 1·2
H_2S concentration* (p.p.m.)	Not detected†	1·05 ± 0·97‡
Breathtron score (p.p.b.)	41·50 ± 17·76	1129 ± 903‡
Organoleptic score	0·30 ± 0·30	1·29 ± 0·40‡

*Determined by gas chromatography.

†Below the detection limit.

‡Significantly different ($P < 0·05$) from the no/low odour group.

scores in this study were relatively low (1·2 and 2·0 in the no/low odour and odour groups, respectively; Table 1). In addition, there were no significant differences in thickness score of observable tongue coating between the two groups (data not shown).

Relationship between oral malodour level and densities of total bacteria and H_2S-producing bacteria in tongue biofilm

After 2 weeks of anaerobic incubation, black or grey colonies were observed on plates containing lead acetate and these were designated H_2S-producing bacteria. Few black or grey colonies appeared on plates when the same samples were cultured without lead acetate (data not shown). Total numbers of colonies on plates with and without lead acetate were almost equal, thus indicating that lead acetate did not inhibit bacterial growth. Black or grey isolates were subcultured and confirmed to produce H_2S.

The total number of bacteria (total c.f.u.) in the odour group (mean, $1·4 \times 10^8$) was significantly higher than that in the no/low odour group (mean, $1·3 \times 10^7$; $P < 0·005$) (Fig. 1). This is consistent with previous studies by Hartley *et al.* (1996, 1999). In addition, the number of black or grey colonies in the odour group (mean, $6·4 \times 10^7$) was significantly higher (approximately six-fold) than that in the no/low odour group (mean, $8·1 \times 10^6$; $P < 0·05$). This suggests that H_2S-producing bacteria in the tongue biofilm are the source of oral malodour.

On the other hand, there was no significant difference in the percentage of black or grey colony-forming units among the total colony-forming units between the two groups, although this percentage varied among individuals (20–89 %) (Fig. 2). In this study, tongue biofilm samples were obtained from the same part of the tongue using a standardized method, and no significant differences were noted in observable tongue coating and thickness scores between the two groups (Table 1). This indicates that the amounts of observable tongue coating were similar among the subjects in this study. These results suggest that the number of bacteria per unit of tongue

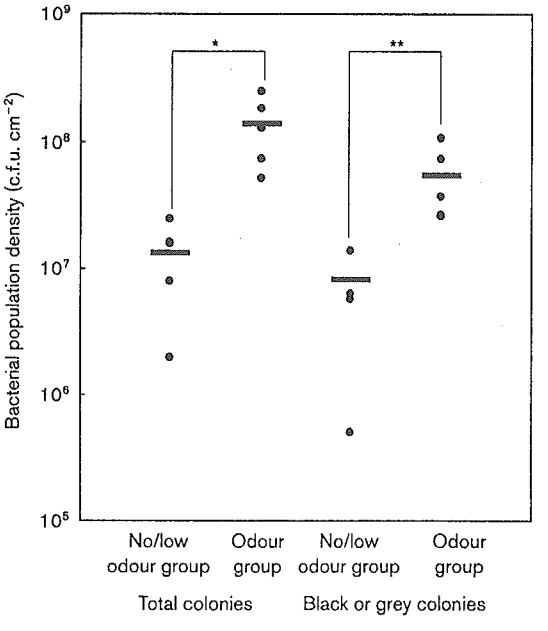

Fig. 1. Numbers of total and black or grey colonies in no/low odour and odour groups. *$P = 0·002$; **$P = 0·012$. Horizontal bars represent means.

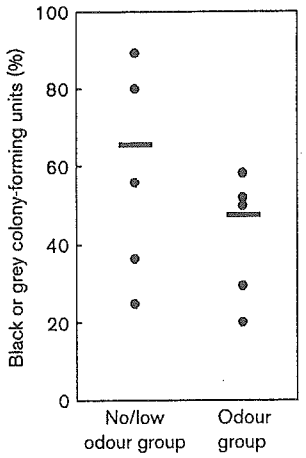

Fig. 2. Proportion of black or grey colony-forming units among total colony-forming units in no/low odour and odour groups. Horizontal bars represent means.

biofilm, i.e. bacterial density in the tongue biofilm, is higher in subjects with oral malodour than in those without oral malodour, and that H_2S-producing bacteria in tongue biofilm are responsible for oral malodour.

Yaegaki & Sanada (1992a, b) and Miyazaki *et al.* (1995) reported correlations between the degree of oral malodour, the amount of observable tongue coating and/or periodontal conditions. In addition, it is suggested that periodontal disease can induce observable tongue coating accumulation (Yaegaki & Sunada, 1992a). The tongue biofilm comprises not only micro-organisms but also epithelial cells released from the oral mucosa and leukocytes from periodontal pockets. Salivary levels of the latter two components could be elevated in patients with periodontal disease, thus leading to an increase in the amount of observable tongue coating. This indicates that the amounts of observable tongue coating bear little relationship to the microbial population density on the tongue coating and it is only the latter (microbial density) that relates to hydrogen sulfide levels or oral malodour.

Hartley *et al.* (1996) reported that the percentage of H_2S-producing bacteria in subjects with strong oral malodour (organoleptic scores >3 on a 0–5 scale) was higher than that in the no/low odour group. In our study, however, a significant correlation was observed with the number rather than the percentage of H_2S-producing bacteria. Organoleptic scores of the subjects with oral malodour in our study were lower (mean 1·29 on a 0–4 scale) (Table 1) than those in the study by Hartley *et al.* (1996) (mean 3·84 on a 0–5 scale). The discrepancy could thus be explained as follows: oral malodour increases with the number of both total and H_2S-producing bacteria in the tongue biofilm, and then becomes more severe as the percentage of H_2S-producing bacteria increases.

Identification of H_2S-producing bacteria in tongue biofilm

The H_2S-producing bacteria isolated in this study were identified using molecular biological methods. *Veillonella*, *Actinomyces* and *Prevotella* species were the predominant H_2S-producing bacteria, followed by *Streptococcus* species, in the odour and no/low odour groups (Table 2). *Veillonella dispar* accounted for over 15 % of total H_2S-producing bacteria in each sample. However, there were no significant differences in the profiles of H_2S-producing bacteria between the two groups.

Hartley *et al.* (1996) also frequently identified these bacterial species in both odour and no/low odour groups, and Donaldson *et al.* (2005) reported that *Veillonella*, *Prevotella* and *Fusobacterium* species were found in both odour and no/low odour groups, and that *Vibrio* species and unidentifiable Gram-negative and Gram-positive anaerobes were more commonly found in the odour group. Loesche & Kazor (2002) reported that 74 % of total cultivable bacteria of the tongue biofilm could be *Veillonella parvula*, *Actinomyces odontolyticus*, *Streptococcus intermedius* and *Clostridium innocuum*, and Mager *et al.* (2003) reported that a *Veillonella* species was one of the prominent bacteria in the tongue biofilm. However, in all these studies, the H_2S-productivity of the bacteria was not assessed. Thus, our study is the first report to show that *Veillonella*, *Actinomyces* and *Prevotella* are predominant as H_2S-producing bacteria in tongue biofilm and are responsible for oral malodour when they increase in number.

Actinomyces species are saccharolytic bacteria that produce lactic acids from carbohydrates, while *Veillonella* species utilize lactic acids as a carbon and energy source instead of carbohydrates. In a mixed culture where carbohydrate is supplied, *Veillonella* species are able to grow together with *Actinomyces* species (Distler & Kröncke, 1981), indicating that *Actinomyces* supply lactic acids to *Veillonella* species. This suggests that, in the tongue coating, *Actinomyces* and *Veillonella* species create a food chain and subsequently establish a stable microbial ecosystem.

In the tongue coating, cysteine and proteins/peptides containing cysteine are thought to be supplied by saliva and desquamated tongue epithelia, and are degraded into H_2S through bacterial metabolism. Some isolates of *Actinomyces* and *Veillonella* have been reported to produce H_2S during growth (Persson *et al.*, 1990; Schaal, 1986; Shibuya, 2001), as shown in this study (Table 2). This indicates that members of *Actinomyces* and *Veillonella* possess an enzyme responsible for the breakdown of cysteine into H_2S, although no information is available regarding cysteine-degrading enzymes such as cysteine desulfhydrase (Claesson *et al.*, 1990; Pianotti *et al.*, 1986) in these bacteria. *Prevotella* species including *Prevotella veroralis* ferment amino acids and some

Table 2. H_2S-producing bacterial species in tongue biofilm of eight subjects

Species	No. (%)* isolated from each subject									
	No/low odour group					Odour group				
	1	2	3	4	Total	5	6	7	8	Total
Total H_2S-producing isolates	58 (50·9)†	63 (39·4)†	64 (80·0)†	57 (72·2)†	242 (55·9)†	10 (55·5)†	24 (46·2)†	39 (52·7)†	53 (42·4)†	126 (46·8)†
Gram-positive cocci										
Atopobium parvulum	1 (1·7)	0 (0·0)	1 (1·6)	4 (7·0)	6 (2·5)	0 (0·0)	0 (0·0)	0 (0·0)	4 (7·5)	4 (3·2)
Gemella sanguinis	1 (1·7)	0 (0·0)	0 (0·0)	0 (0·0)	1 (0·4)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)
Micromonas micros	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)	1 (4·2)	0 (0·0)	0 (0·0)	1 (0·8)
Streptococcus mitis	0 (0·0)	0 (0·0)	1 (1·6)	0 (0·0)	1 (0·4)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)
Streptococcus parasanguinis	0 (0·0)	3 (4·8)	0 (0·0)	0 (0·0)	3 (1·2)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)
Streptococcus vestibularis	0 (0·0)	11 (17·5)	2 (3·1)	0 (0·0)	13 (5·4)	0 (0·0)	0 (0·0)	0 (0·0)	8 (15·1)	8 (6·3)
Gram-positive rods										
Actinomyces graevenitzii	0 (0·0)	0 (0·0)	0 (0·0)	3 (5·3)	3 (1·2)	0 (0·0)	0 (0·0)	0 (0·0)	1 (1·9)	1 (0·8)
Actinomyces odontolyticus	0 (0·0)	8 (12·7)	32 (50·0)	0 (0·0)	40 (16·5)	2 (20·0)	5 (20·8)	13 (33·3)	11 (20·8)	31 (24·6)
Eubacterium saburreum	0 (0·0)	0 (0·0)	1 (1·6)	0 (0·0)	1 (0·4)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)
Eubacterium species	0 (0·0)	0 (0·0)	1 (1·6)	0 (0·0)	1 (0·4)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)
Mogibacterium species	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)	1 (2·6)	0 (0·0)	1 (0·8)
Gram-negative cocci										
Megasphaera micronuciformis	1 (1·7)	4 (6·3)	0 (0·0)	1 (1·8)	6 (2·5)	0 (0·0)	0 (0·0)	4 (10·3)	2 (3·8)	6 (4·8)
Veillonella dispar	27 (46·6)	26 (41·3)	10 (15·6)	48 (84·2)	111 (45·9)	8 (80·0)	6 (25·0)	16 (41·0)	16 (30·2)	46 (36·5)
Veillonella parvula	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)	2 (8·3)	0 (0·0)	0 (0·0)	2 (1·6)
Veillonella species	0 (0·0)	0 (0·0)	1 (1·6)	0 (0·0)	1 (0·4)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)
Gram-negative rods										
Bulleidia moorei	1 (1·7)	3 (4·8)	2 (3·1)	0 (0·0)	6 (2·5)	0 (0·0)	1 (4·2)	0 (0·0)	0 (0·0)	1 (0·8)
Campylobacter concisus	1 (1·7)	1 (1·6)	0 (0·0)	0 (0·0)	2 (0·8)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)
Campylobacter showae	6 (10·3)	0 (0·0)	0 (0·0)	0 (0·0)	6 (2·5)	0 (0·0)	1 (4·2)	0 (0·0)	0 (0·0)	1 (0·8)
Capnocytophaga gingivalis	3 (5·2)	0 (0·0)	0 (0·0)	0 (0·0)	3 (1·2)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)
Fusobacterium nucleatum	0 (0·0)	0 (0·0)	6 (9·4)	0 (0·0)	6 (2·5)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)
Prevotella veroralis	10 (17·2)	5 (7·9)	0 (0·0)	0 (0·0)	15 (6·2)	0 (0·0)	0 (0·0)	4 (10·3)	7 (13·2)	11 (8·7)
Prevotella melaninogenica	1 (1·7)	0 (0·0)	1 (1·6)	0 (0·0)	2 (0·8)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)
Prevotella species	0 (0·0)	0 (0·0)	2 (3·1)	0 (0·0)	2 (0·8)	0 (0·0)	2 (8·3)	0 (0·0)	0 (0·0)	2 (1·6)
Selenomonas dianae	0 (0·0)	0 (0·0)	1 (1·6)	0 (0·0)	1 (0·4)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)
Selenomonas species	1 (1·7)	0 (0·0)	0 (0·0)	0 (0·0)	1 (0·4)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)
Unidentified	5 (8·6)	2 (3·2)	3 (4·7)	1 (1·8)	11 (4·5)	0 (0·0)	5 (20·8)	1 (2·6)	4 (7·5)	10 (7·9)

*Percentage among H_2S-producing isolates from each subject except where indicated.

†Percentage among total bacterial isolates from each subject.

species possess proteolytic activity (Shah & Collins, 1990), thus suggesting that these species can degrade proteins/peptides and ferment the resultant cysteine into H_2S as detected in our study.

Periodontal disease-associated bacteria such as *Porphyromonas gingivalis* and *Prevotella intermedia*, which produce VSCs (Loesche & Kazor, 2002; Persson *et al.*, 1990), were not detected in the present study (Table 2), in which no periodontal disease patients were included (Table 1). *Fusobacterium* species, known to be VSC-producing periodontal inhabitants (Claesson *et al.*, 1990), were scarcely detected (Table 2). These results suggest that periodontal disease-associated bacteria are not associated with oral malodour in patients without periodontal disease or with low to intermediate levels of oral malodour.

Conclusions

H_2S-producing bacteria in the tongue biofilm appear to cause low to intermediate levels of oral malodour in patients without periodontitis, and the predominant H_2S-producing bacteria are mainly commensal species of the oral cavity, such as *Veillonella* and *Actinomyces* species. Furthermore, the numbers of both H_2S-producing bacteria and total bacteria in the tongue biofilm were higher in the odour group, suggesting that for subjects with low to intermediate levels of malodour an increase in bacterial density in the tongue biofilm is associated with oral malodour.

ACKNOWLEDGEMENTS

This study was supported in part by Grants-in-Aid for Scientific Research (16390601, 17659659, 17591985) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

REFERENCES

Awano, S., Gohara, K., Kurihara, E., Ansai, T. & Takehara, T. (2002). The relationship between the presence of periodontopathogenic bacteria in saliva and halitosis. *Int Dent J* **52**, 212–216.

Ayers, K. M. & Colquhoun, A. N. (1998). Halitosis: causes, diagnosis, and treatment. *N Z Dent J* **94**, 156–160.

Claesson, R., Edlund, M. B., Persson, S. & Carlsson, J. (1990). Production of volatile sulfur compounds by various *Fusobacterium* species. *Oral Microbiol Immunol* **5**, 137–142.

De Boever, E. H. & Loesche, W. J. (1995). Assessing the contribution of anaerobic microflora of the tongue to oral malodor. *J Am Dent Assoc* **126**, 1384–1393.

Distler, W. & Kröncke, A. (1981). Acid formation by mixed cultures of dental plaque bacteria *Actinomyces* and *Veillonella*. *Arch Oral Biol* **26**, 123–126.

Donaldson, A. C., McKenzie, D., Riggio, M. P., Hodge, P. J., Rolph, H., Flanagan, A. & Bagg, J. (2005). Microbiological culture analysis of the tongue anaerobic microflora in subjects with and without halitosis. *Oral Dis* **11** (Suppl. 1), 61–63.

Frisken, K. W., Higgins, T. & Palmer, J. M. (1990). The incidence of periodontopathic microorganisms in young children. *Oral Microbiol Immunol* **5**, 43–45.

Gordon, D. F., Jr & Gibbons, R. J. (1966). Studies of the predominant cultivable microorganisms from the human tongue. *Arch Oral Biol* **11**, 627–632.

Greenman, J. (1999). Microbial aetiology of halitosis. In *Dental Plaque Revisited. Oral Biofilms in Health and Disease*, pp. 419–442. Edited by H. N. Newman & M. Wilson. Cardiff: BioLine.

Hartley, M. G., El-Maaytah, M. A., McKenzie, C. & Greenman, J. (1996). The tongue microbiota of low odour and malodorous individuals. *Microb Ecol Health Dis* **9**, 215–223.

Hartley, G., McKenzie, C., Greenman, J., El-Maaytah, M. A., Scully, C. & Porter, S. (1999). Tongue microbiota and malodour. Effects of metronidazole mouthrinse on tongue microbiota and breath odour. *Microb Ecol Health Dis* **11**, 226–233.

Kazor, C. E., Mitchell, P. M., Lee, A. M., Stokes, L. N., Loesche, W. J., Dewhirst, F. E. & Paster, B. J. (2003). Diversity of bacterial populations on the tongue dorsa of patients with halitosis and healthy patients. *J Clin Microbiol* **41**, 558–563.

Kleinberg, I. & Westbay, G. (1990). Oral malodor. *Crit Rev Oral Biol Med* **1**, 247–259.

Lane, D. J. (1991). 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*, pp. 115–175. Edited by E. Stackebrandt & M. Goodfellow. Chichester: Wiley.

Loesche, W. J. & Kazor, C. (2002). Microbiology and treatment of halitosis. *Periodontol 2000* **28**, 256–279.

Mager, D. L., Ximenez-Fyvie, L. A., Haffajee, A. D. & Socransky, S. S. (2003). Distribution of selected bacterial species on intraoral surfaces. *J Clin Periodontol* **30**, 644–654.

Milnes, A. R., Bowden, G. H., Gates, D. & Tate, R. (1993). Predominant cultivable microorganisms on the tongue of preschool children. *Microb Ecol Health Dis* **6**, 229–235.

Miyake, Y., Iwai, T., Sugai, M., Miura, M., Suginaka, H. & Nagasaka, N. (1991). Incidence and characterization of *Staphylococcus aureus* from the tongues of children. *J Dent Res* **70**, 1045–1047.

Miyazaki, H., Sakao, S., Katoh, Y. & Takehara, T. (1995). Correlation between volatile sulphur compounds and certain oral health measurements in the general population. *J Periodontol* **66**, 679–684.

Nakano, Y., Yoshimura, M. & Koga, T. (2002). Correlation between oral malodor and periodontal bacteria. *Microbes Infect* **4**, 679–683.

Nara, F. (1977). The relationship between the halitosis and oral conditions of the periodontal patients. *J Jpn Assoc Periodontol* **19**, 100–108.

O'Leary, T. J., Drake, R. B. & Naylor, J. E. (1972). The plaque control record. *J Periodontol* **43**, 38–39.

Paryavi-Gholami, F., Minah, G. E. & Turng, B. F. (1999). Oral malodor in children and volatile sulfur compound-producing bacteria in saliva: preliminary microbiological investigation. *Pediatr Dent* **21**, 320–324.

Persson, S., Edlund, M. B., Claesson, R. & Carlsson, J. (1990). The formation of hydrogen sulfide and methyl mercaptan by oral bacteria. *Oral Microbiol Immunol* **5**, 195–201.

Pianotti, R., Lachette, S. & Dills, S. (1986). Desulfuration of cysteine and methionine by *Fusobacterium nucleatum*. *J Dent Res* **65**, 913–917.

Roldan, S., Herrera, D. & Sanz, M. (2003). Biofilms and the tongue: therapeutical approaches for the control of halitosis. *Clin Oral Investig* **7**, 189–197.

Rosenberg, M. (1996). Clinical assessment of bad breath: current concepts. *J Am Dent Assoc* **127**, 475–482.

Schaal, K. P. (1986). Genus *Actinomyces*. In *Bergey's Manual of Systematic Bacteriology*, vol 2, pp. 1383–1418. Edited by P. H. A. Sneath, N. S. Mair, M. E. Sharpe & J. G. Holt. Baltimore: Williams & Wilkins.

Scully, C., Porter, S. & Greenman, J. (1994). What to do about halitosis. *BMJ* **308**, 217–218.

Shah, H. N. & Collins, D. M. (1990). *Prevotella*, a new genus to include *Bacteroides melaninogenicus* and related species formerly classified in the genus *Bacteroides*. *Int J Syst Bacteriol* **40**, 205–208.

Shibuya, K. (2001). Constituents and origins of physiological malodor. *J Dent Health* **51**, 778–792.

Shimura, M., Yasuno, Y., Iwakura, M., Shimada, Y., Sakai, S., Suzuki, K. & Sakamoto, S. (1996). A new monitor with a zinc-oxide thin film semiconductor sensor for the measurement of volatile sulfur compounds in mouth air. *J Periodontol* **67**, 396–402.

van Winkelhoff, A. J., van der Velden, U., Winkel, E. G. & de Graaff, J. (1986). Black-pigmenting *Bacteroides* and motile organisms on oral mucosal surfaces in individuals with and without periodontal breakdown. *J Periodontal Res* **21**, 434–439.

Yaegaki, K. & Sanada, K. (1992a). Biochemical and clinical factors influencing oral malodor in periodontal patients. *J Periodontol* **63**, 783–789.

Yaegaki, K. & Sanada, K. (1992b). Volatile sulfur compounds in mouth air from clinically healthy subjects and patients with periodontal disease. *J Periodontal Res* **27**, 233–238.

Oral Microbiology Immunology 2005: 20: 333–338
Printed in Singapore. All rights reserved

Oral Microbiology and Immunology

Quantification and detection of bacteria from postoperative maxillary cyst by polymerase chain reaction

M. Yamaura[1,2], T. Sato[2], S. Echigo[1], N. Takahashi[2]
[1]Division of Oral Surgery and [2]Division of Oral Ecology and Biochemistry, Tohoku University Graduate School of Dentistry, Sendai, Japan

Yamaura M, Sato T, Echigo S, Takahashi N. Quantification and detection of bacteria from postoperative maxillary cyst by polymerase chain reaction.
Oral Microbiol Immunol 2005: 20: 333–338. © Blackwell Munksgaard, 2005.

Background/aims: Postoperative maxillary cyst (POMC) is known to occur as a delayed complication of radical maxillary sinus surgery, such as Caldwell-Luc surgery. The cyst gradually expands with no symptoms over a period of years, and then occasionally causes swelling and pain in the buccal region and/or the mucogingival fold. It is probable that bacterial infection affects the progression of POMC symptoms. The aims of this study were to determine the bacterial density and to examine the presence of 20 oral bacteria in POMC fluids.
Methods: POMC fluids (4 purulent, 2 mucous and 4 serous) were sampled from 10 subjects (aged 43–77 years). Bacterial quantification and detection were performed by real-time polymerase chain reaction (PCR) and nested PCR based on bacterial 16S rRNA genes, respectively.
Results: Bacterial DNA was detected in all samples and the average concentrations of bacterial DNA were 5.9 (purulent), 0.5 (mucous), and 0.7 (serous) ng/mg of sample. Twelve bacterial species, including anginosus streptococci, known to be associated with abscess formation, were detected in the purulent fluids, while two and five species were detected in the mucous and serous fluids, respectively.
Conclusion: Purulent fluids contained numerous bacteria of various types, thus suggesting that oral bacteria may cause symptoms such as pain in POMC with purulent fluids. Mucous and serous fluids also contained bacteria, although their numbers were small, thus suggesting an association between bacteria and progression of POMC.

Key words: 16S ribosomal RNA; cyst fluid; maxillary sinus; polymerase chain reaction; postoperative complications

Professor Nobuhiro Takahashi, Division of Oral Ecology and Biochemistry, Tohoku University Graduate School of Dentistry, 4–1 Seiryo-machi, Aoba-ku, Sendai 980–8575, Japan
Tel.: +81 22 7178294;
fax: +81 22 7178297;
e-mail: nobu-t@mail.tains.tohoku.ac.jp
Accepted for publication April 5, 2005

Postoperative maxillary cyst (POMC) is known to occur as a delayed complication of radical maxillary sinus surgery, such as Caldwell-Luc surgery. It is believed that POMC develops when the residual mucosa of maxillary sinus and mucous glands is trapped in the wound and the sinus ostia is obstructed (16, 22). The cyst gradually expands as a result of increased fluid retention and bone remodeling over a period of years, and occasionally causes swelling and pain in the buccal region and/or the mucogingival fold (11, 12). Although the cyst cavity is isolated from the nasal cavity by the cyst wall, which is covered with lining epithelium, it is probable that oral bacteria invade the obstructed cyst cavity through the teeth or periodontal pockets and cause the symptoms of POMC (34). Because the bottom of the sinus antrum is thin across the oral cavity and the cyst tends to expand toward the oral cavity by gravitation (10, 30), bone resorption of the bottom of sinus antrum of POMC sometimes occurs, resulting in vulnerability to bacterial invasion.

It has been estimated by culturing and molecular biological methods that more than 600 bacterial species inhabit the human oral cavity (21, 31, 35, 40, 49). Few bacteriological studies on POMC have been performed (17, 42), and thus the relationship between the presence of bacteria and the progression of POMC symptoms remains unclear.

The aims of this study were to determine the bacterial density of the cyst fluids by real-time polymerase chain reaction (PCR) and to examine the presence of 20 oral bacteria (Table 1) by nested PCR based on bacterial 16S rRNA genes. These bacteria were selected in this study since these species have frequently been isolated as putative pathogens from various oral diseases such as odontogenic infections, alveolar abscesses, periodontitis, and dental caries lesions (14, 23, 24, 37).

Material and methods

Subject population

Informed consent was obtained from each subject, and POMC fluids were sampled from 10 subjects (mean age, 57.2 ± 10.3 years; range, 43–77 years) (Table 2). Three of these subjects received antimicrobial therapy less than 2 weeks before sampling.

Collection of samples

Retained POMC fluids were collected using disposable sterilized 19-gauge needles attached to syringes and poured into sterilized vials. At the time of cystectomy or marsupialization, fluids were aspirated directly from the cysts. Samples were punctured from a disinfected area in the most prominent or fluctuant mucosal point overlaying the cyst. All samples were immediately transferred and stored at −20°C before extraction of genomic DNA.

DNA extraction

Each sample (5.0–9.9 mg) was immersed in 1 ml of sterilized distilled water and genomic DNA was extracted using a GFX Genomic Blood DNA Purification Kit (Amersham Bioscience, Piscataway, NJ) according to the manufacturer's instructions.

Real-time PCR

Quantitative real-time PCR amplification was performed with universal primers 357F and 907R (27), and iQ SYBR Green Supermix (Bio-Rad Laboratories, Richmond, CA), according to the manufacturer's instructions. The primer sequences were: 357F, 5′-CTC CTA CGG GAG

Table 1. Target bacteria and their species-specific primers

Species	Sequence (5′–3′)	Size (bp)	References
Anaerococcus sp.	GCG TGA TTT AGA AGG C ACG GGC GGT GTG TAC	980	(41)
Campylobacter rectus[a]	TTT CGG AGC GTA AAC TCC TTT TC TTT CTG CAA GCA GAC ACT CTT	598	(1)
Dialister pneumosintes	TTC TAA GCA TCG CAT GGT GC GAT TTC GCT TCT CTT TGT TG	1105	(6)
Eikenella corrodens	CGA TTA GCT GTT GGG CAA CTT ACC CTC TGT ACC GAC CAT TGT AT	410	(9)
Eubacterium saphenum	TCT ACT AAG CGC GGG GTG A ATA CCC GAT TAA GGG TAC	430	(13)
Finegordia magna	GCA TAA AAT CGT AGA AAC AC ACG GGC GGT GTG TAC	1200	(41)
Fusobacterium nucleatum	GAA GAA ACA AAT GAC GGT AAC AAC GTC ATC CCC ACC TTC CTC CT	705	(39)
Mogibacterium timidum	AAG CTT GGA AAT GAC GC CCT TGC GCT TAG GTA A	524	(13)
Peptoniphilus asaccharolyticus	ATG AAA ATC AAA CAG AAC C ACG GGC GGT GTG TAC	300	(41)
Peptostreptococcus anaerobius	GTA GTT AGC CTC CGA AA ACG GGC GGT GTG TAC	780	(41)
Peptostreptococcus micros	TCG GGA CAA CTA TAC AG ACG GGC GGT GTG TAC	380	(41)
Porphyromonas gingivalis[b]	GCG TAT GCA ACT TGC CTT AC GTT TCA ACG GCA GGC TGA AC	518	(46)
Prevotella intermedia	CGT GGA CCA AAG ATT CAT CGG TGG A CCG CTT TAC TCC CCA ACA AA	259	(33)
Prevotella nigrescens	GTG TTT CAT TGA CGG CAT CCG ATA TGA AAC CCA CGT CTC TGT GGG CTG CGA	828	(33)
Propionibacterium acnes[c]	AAG GCC CTG CTT TTG TGG ACT CAC GCT TCG TCA CAG	388	(15)
Slackia exigua	GCC AAG CGG CCT CGT CGA AG GCC GGC TTT AAG GGA TTC GCT CG	697	(13)
Streptococcus anginosus	ATG CAA TTG CAT CGC TAG T GCA GGC TTT GGA AAC TGT TTA ACT	445	(18)
Streptococcus constellatus	GTG CAA GAG CAT CAC TAC C GCA GGC TTT GGA AAC TGT TTA ACT	445	(18)
Streptococcus intermedius[d]	GTG CAA ATG CAT CAC TAC C GCA GGC TTT GGA AAC TGT TTA ACT	445	(18)
Streptococcus mutans	GGT CAG GAA AGT CTG GAG TAA AAG GCT A GCG GTA GCT CCG GCA CTA AGC C	282	(39)

[a] *In-silico* determination of the specificity, the primers may cross-react to *Campylobacter curvus*, *Campylobacter showae*, and *Campylobacter sputorum*.
[b] *In-silico* determination of the specificity, the primers may cross-react with *Porphyromonas gulae*.
[c] *In-silico* determination of the specificity, the primers may cross-react with *Propionibacterium* sp. LG and uncultured phylotypes (clones), i.e. lw29, Tc134–108, PE40, PE36, PE34, PE33PE30, PE27, PE22, PE21, PE20, PE15, PE13, PE12, PE06, 47 mm60, ACTINO8A, AT425_EubE10, DZ_D6, 1519, and PH-B24N.
[d] *In-silico* determination of the specificity, the primers may cross-react with *S. pneumoniae*, *S. pseudopneumoniae*, and *Streptococcus* sp. MGH.

Table 2. Clinical signs and conditions of subjects in this study

	Subjects									
	1	2	3	4	5	6	7	8	9	10
Age	50	50	48	43	66	58	53	62	65	77
Gender	M	M	M	M	F	M	M	F	M	F
Swelling	+	+	+	+	+	+	+	+	+	+
Pain	+	+	–	+	–	+	–	+	–	–
Antibiotic therapy[a]	–	–	–	+	–	+	–	–	+	–
Fluid state	Purulent	Purulent	Purulent	Purulent	Mucous	Mucous	Serous	Serous	Serous	Serous

[a]Application of antibiotic therapy (2 weeks before sampling) is designated as positive.

GCA GCA G-3′; and 907R, 5′-CCG TCA ATT CMT TTR AGT TT-3′. Real-time PCR amplification was performed in an iCycler (Bio-Rad Laboratories) programmed for 3 min at 95°C for initial heat activation, followed by 40 cycles of 15 s at 95°C for denaturation, 30 s at 55°C for annealing and 30 s at 72°C for extension. During the extension step, fluorescence emissions were monitored, and data were analyzed using iCycler iQ Software (Bio-Rad Laboratories). The genomic DNA of *Propionibacterium acnes* ATCC 6919 was used as a standard for quantitative analysis.

Nested PCR

In the first amplification, the 16S rRNA genes were amplified by PCR with universal primers 27F and 1492R (27) 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 TCM TGG CTC AG-3′; and 1492R, 5′-TAC GGY TAC CTT GTT ACG ACT T-3′. 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, followed by 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 PCR product with the universal primers was 1505 bp in length. In the second amplification (nested PCR), the first PCR products were used as templates and 20 bacteria were identified using species-specific primers based on 16S rRNA gene sequences (Table 1). In order to determine the specificity of the primers *in-silico*, blast searches were performed through the web site of the National Center for Biotechnology Information. PCR amplification was performed in a PCR Thermal Cycler MP (TaKaRa Biomedicals) programmed for 15 min at 95°C for initial heat activation, followed by 35 cycles of 1 min at 94°C for denaturation, 1 min at 58.5°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.

PCR products were separated on 1% agarose gels (Certified Low Range Ultra Agarose, Bio-Rad Laboratories) stained with ethidium bromide and photographed under ultraviolet light. The sizes of PCR products were compared with a molecular size marker (Ready-Load 100-bp DNA Ladder, Invitrogen Corp., Carlsbad, CA), and confirmed to correspond to those listed in Table 1.

Data analysis

Fisher's exact probability tests and Tukey's tests were used to analyze significance. *P*-values of <0.05 were considered statistically significant.

Results

Clinical signs of subjects and the state of cyst fluids are shown in Table 2. All patients had swelling and half had pain in the buccal region and/or mucogingival fold. Three patients received antibiotic therapy less than 2 weeks before sampling. Four of the fluids samples were purulent, two mucous and four serous.

The results of quantitative real-time PCR and nested PCR are shown in Table 3. Bacterial DNA was detected in all samples, and thus bacteria were proven to be present in all samples. The average quantity of bacterial DNA was 5.9 ± 2.1 (purulent), 0.5 ± 0.2 (mucous), and 0.7 ± 0.1 (serous) ng/mg of sample. Purulent fluids contained approximately 8–12 times more bacteria than mucous and serous fluids. Bacterial DNA was detected in the cyst fluids of the patients who received antibiotic therapy less than 2 weeks before sampling (Tables 2 and 3).

P. acnes was detected in nine samples, and its detection frequency was the highest among the bacterial species tested in this study. *Porphyromonas gingivalis*, *Streptococcus constellatus*, and *S. intermedius* were detected in five samples, Anaerococcus sp., *Fusobacterium nucleatum* and *Streptococcus anginosus* in three samples, *Campylobacter rectus*, *Dialister pneumosintes*, *Eikenella corrodens*, *Mogibacterium timidum*, and *Peptostreptococcus micros* in two samples, and *Slackia exigua* in one sample. Anaerobic gram-positive cocci such as *Peptostreptococcus* species, which is frequently detected in oral maxillofacial infections (24), were detected in small numbers in this study. The other seven species tested for were not detected in this study.

In the purulent fluids, 12 bacterial species including anginosus streptococci, which is reported to be associated with abscess formation (25, 26), were detected, while two and five species were detected in the mucous and serous fluids, respectively. *P. gingivalis* was detected with a high frequency (three of four cases) in the serous fluids. In eight cases, two species and/or more than two species were detected. In two cases (one mucous and one serous), *P. acnes* was the only species detected in this study.

In *in-silico* determination of the specificity of the primers used in this study, the primers of *P. gingivalis* were found to cross-react with *Porphyromonas gulae*; those of *P. acnes* to cross-react with Propionibacterium sp. LG and uncultured phylotypes (clones), i.e. lw29, Tc134–108, PE40, PE36, PE34, PE33PE30, PE27, PE22, PE21, PE20, PE15, PE13, PE12, PE06, 47 mm60, ACTINO8A, AT425_EubE10, DZ_D6, 1519, and PH-B24N; those of *C. rectus* to cross-react with *Campylobacter curvus*, *Campylobacter showae*, and *Campylobacter sputorum*; and those of *S. intermedius* to cross-react with *Streptococcus pneumoniae*, *Streptococcus pseudopneumoniae*, and *Streptococcus* sp. MGH.

Discussion

In this study, bacterial DNA was detected by quantitative real-time PCR in all samples (Table 3). A low bacterial detection frequency (< 50%) has been reported in POMC fluids (17, 42), and the discrepancy

Table 3. Total bacterial DNA and detected bacterial species

	Purulent				Mucous		Serous				
	1	2	3	4	5	6	7	8	9	10	
	7.9 [b]	7.5	4.7	3.5	0.4	0.6	0.6	0.7	0.9	0.8	No. of positive samples
Universal[a]	(5.9 ± 2.1[b, c, d])				(0.5 ± 0.2)		(0.7 ± 0.1)				10
Propionibacterium acnes	+	+	+	–	+	+	+	+	+	+	9
Porphyromonas gingivalis	–	–	+	–	+	–	+	+	+	–	5
Streptococcus constellatus	+	+	+	+	–	–	+	–	–	–	5
Streptococcus intermedius	+	+	+	+	–	–	+	–	–	–	5
Anaerococcus sp.	+	+	+	–	–	–	–	–	–	–	3
Fusobacterium nucleatum	+	+	+	–	–	–	–	–	–	–	3
Streptococcus anginosus	+	+	+	–	–	–	–	–	–	–	3
Campylobacter rectus	+	+	–	–	–	–	–	–	–	–	2
Dialister pneumosintes	+	+	–	–	–	–	–	–	–	–	2
Eikenella corrodens	+	+	–	–	–	–	–	–	–	–	2
Peptostreptococcus micros	+	+	–	–	–	–	–	–	–	–	2
Mogibacterium timidum	+	–	–	+	–	–	–	–	–	–	2
Slackia exigua	–	–	–	–	–	–	–	+	–	–	1
Eubacterium saphenum	–	–	–	–	–	–	–	–	–	–	0
Finegordia magna	–	–	–	–	–	–	–	–	–	–	0
Peptostreptococcus anaerobius	–	–	–	–	–	–	–	–	–	–	0
Peptoniphilus asaccharolyticus	–	–	–	–	–	–	–	–	–	–	0
Prevotella intermedia	–	–	–	–	–	–	–	–	–	–	0
Prevotella nigrescens	–	–	–	–	–	–	–	–	–	–	0
Streptococcus mutans	–	–	–	–	–	–	–	–	–	–	0
No. of positive bacterial species	11	10	7	3	2	1	4	3	2	1	

[a] Quantification of total bacterial DNA by real-time PCR with universal primers of 16S ribosomal RNA genes.
[b] ng per mg of sample.
[c] Mean values ± standard deviations.
[d] Significantly different ($P < 0.05$) from mucous and serous sample.

could be because previous reports depended on culturing methods and fastidious bacteria were dominant in POMC fluids. In our study, however, the use of molecular biology made it possible to detect bacteria from mucous and serous cyst fluids despite their small numbers.

P. acnes was detected in nine samples and was the most frequently detected bacterium (Table 3). *P. acnes* is an anaerobic gram-positive rod that is considered to be an indigenous bacteria of skin as well as oral and intestinal mucosa. However, the species has been reported to have pathogenicity based on superantigenicity and mitogen activity of T cells (2, 8, 19). *P. acnes* has been recently detected in various diseases, such as alveolar abscess, sinusitis, osteomyelitis, meningitis, noma, endocarditis, septicemia, hepatitis granuloma, facial acne, and abscess of orbit (5, 28, 29) as well as in opportunistic infection (5).

P. gingivalis, detected in five samples in this study (Table 3), is an obligate anaerobic gram-negative rod, and is reportedly associated with the pathogenicity of periodontitis, alveolar abscesses, and oral infections (7, 23). *P. gingivalis* possesses high proteolytic activity to degrade proteins in periodontal tissues and gingival fluids due to expression of various peptidases, such as gingipain (36, 45). *P. gingivalis* also produces lipopolysacccharide and cytotoxic metabolites such as ammonia, butyrate, and propionate (32, 44). Therefore, *P. gingivalis*, detected in POMC fluids, may be associated with POMC inflammation. *P. gingivalis* was detected with a high frequency in the serous fluids in this study, thus suggesting that the properties of serous fluids resembling those of serum, which is rich in protein and neutral in pH (3), may provide a suitable environment for *P. gingivalis* to survive (43).

S. constellatus and *S. intermedius*, detected in five samples in this study (Table 3), and *S. anginosus,* detected in three samples in this study (Table 3), are designated as the anginosus group of streptococci. The anginosus groups are facultative anaerobic gram-positive cocci and are indigenous bacteria in humans. It has been reported that they possess pathogenicity to form abscesses (4, 24, 48) based on tolerance to polymorphonuclear leukocytes (47), cellular components such as capsules (20), and extracellular enzymes such as hyaluronidase (38). It has also been reported that abscess formation by the anginosus group is enhanced by interaction with other bacteria, such as *F. nucleatum*, because its heat-resistant extracellular materials may enhance the pathogenicity of the anginosus group (25, 26). The growth of *S. constellatus* was enhanced by the presence of *E. corrodens* (4). In this study, particularly in the purulent samples, the anginosus group of streptococci was frequently detected with *F. nucleatum* and *E. corrodens* (Table 3). These results support previous reports, and suggest that bacterial interaction may be a symptom of POMC.

In *in-silico* determination of the specificity of the primers used in this study, the primers of *P. gingivalis, P. acnes, C. rectus,* and *S. intermedius* were found to cross-react with other established species and uncultured phylotypes. Thus, it is possible that some uncultured phylotypes which resemble established species, such as *P. acnes,* were frequently detected by PCR in this study, and that these phylotypes may relate to the pathogenicity of POMC, although further studies on the taxonomy of these phylotypes and their pathogenic roles are required.

Purulent fluids of POMC contained numerous bacteria of various types in this study (Table 3), suggesting that the bacteria in the purulent fluids are associated with POMC symptoms such as pain. Pain was the most common clinical manifestation in purulent cases in this study (Table 2). On the other hand, mucous and serous fluids also contained bacteria, although their numbers were small (Table 3), suggesting that the increase in internal pressure of POMC resulting from increases in the amount of fluid is closely related to POMC symptoms, although bacteria may be related to the progression of POMC symptoms.