するものではなく，診療の場面に応じて，自分が今どの援助方法をとっているかを認識しながら行うことが大切である。

たとえば，口臭の測定は原則的に，食後3時間程度，飲食などの口腔の活動を制限し，口臭が強くなる条件で行う。つまり，その人において最大の口臭強度を測定する。強い口臭が測定されれば，患者は当然不安に陥る。この場合，口臭が強くなる条件などの情報を与えるガイダンスが必要である。次いで口腔清掃や定期的な飲食物の摂取など，口臭を低減させるための方法を提示（コンサルテーション）する。それでも口臭への不安が消えない場合は，飲食や口腔清掃を行ってもらった後，再度口臭測定を行って，どれだけ口臭が低下するかを確認してもらい，ある条件下では口臭が強くとも，それをコントロールできるのだと気づかせる。それは患者が問題を克服できるよう支援（カウンセリング）することである。

患者との信頼関係の構築はカウンセリングにおいて不可欠である。しかし，患者が自分で問題を解決するための支援をするのがカウンセリングの目的であり，最終的には患者は歯科医師から自立しなければならない。我々はカウンセリングを必要とする口臭患者の診療過程における認知状態を便宜的に以下のように考えている。

1）口臭があるための不都合の自覚：患者が身体的問題として口臭を認知し，受診する段階。

2）身体的問題と精神・社会的問題の錯誤の認識：自己を悩ませている本来の問題が，身体的な問題ではなく，精神・社会的問題であると気づき始める段階。

3）精神・社会的問題の自己受容：気づいた問題に直面し，受容していく段階。

4）精神・社会的問題に関する歯科医師への依存：頻繁に相談に訪れるなど，気づき，受容した問題を解決するために，歯科医師に依存する段階。

5）問題解決のための自助努力：自ら問題を解決しようと行動し始める段階。

6）問題消退への自覚：自助努力により，問題が消退していくことを自覚する段階。

7）歯科医師からの自立：歯科医師への依存がなくなり，自分で問題解決できるようになる段階。

口臭への拘泥が強い者がすべてこのような経過をたどるわけではない。しかし，ある場面で患者が口臭をどのように認知，解釈しているかを把握しようとすることは大切である。患者の口臭に対する解釈モデルは診療の段階に応じて大きく変化する。我々は患者に繰り返しその時の解釈モデルを語ってもらい，それを理解しようとする。そのプロセスは，口臭診療におけるカウンセリングの重要な部分を占めていると考えている。

5．口臭診療の評価

一般に，口臭診療の効果は，客観的には口臭強度が低下したか否かで評価される。しかし，初診時に口臭強度が低い場合，診療の効果は数値として現れにくい。また，口臭強度が初診時に比べて大幅に低下したにもかかわらず，患者の自覚状態に変化がない例も多くみられる。反対に，著明な口臭強度の低下がなくとも主訴が改善する例もある。いわゆる自臭症患者に限らず，口臭患者の主訴の改善は，主観的評価によるところが大きい。そこで我々は主観的健康感をモニターすることで口臭診療の効果を判定し，患者と医療者の評価を一致させることを試みてきた[26,27]。機器によって口臭強度を測定して口臭診療の指標にするのと同様に，患者の主観的な状態を評価するためには数量的尺度が求められる。我々はその尺度をSF-36[28-30]という健康関連QOL尺度に求めた。SF-36は8つのサブスケールから構成され（表3），それぞれについてスコアが算出できる自己回答形式の質問調査票である。当口臭外来受診者に対して，初診時にこの調査を行ったところ，日本人の平均に比べて，多くの評価項目でQOLスコアが有意に低く，とくに社会生活機能（SF）が低値であった（図8）。また，初診時の口臭測定値が嗅

表3：SF-36のサブスケールとその意義

評価項目	略称	評価項目の解釈
身体機能 Physical functioning	PF	全般的身体能力
日常役割機能（身体） Role physical	RP	仕事や普段の生活上の身体的な理由で生じる問題
身体の痛み Bodily pain	BP	体の痛みの自覚とそれにより生じる日常生活上の問題
社会生活機能 Social functioning	SF	身体的，心理的理由で家族，友人とのつきあいに生じた問題
全体的健康感 General health	GH	自覚される全体的健康状態
活力 Vitality	VT	自分が元気かどうか
日常役割機能（精神） Role emotional	RE	仕事や普段の生活上の心理的な理由で生じる問題
こころの健康 Mental health	MH	憂鬱か，穏やかな気分か

参考文献30より，一部改変。

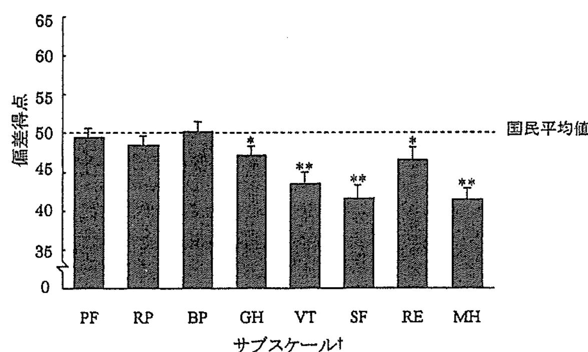

図8：口臭外来受診者の初診時 QOL スコア
$^{*}p<0.05$, $^{**}p<0.01$；t 検定。
†サブスケールの略称については表3を参照。
偏差得点は国民平均値を50，国民標準偏差を10として調整した値。

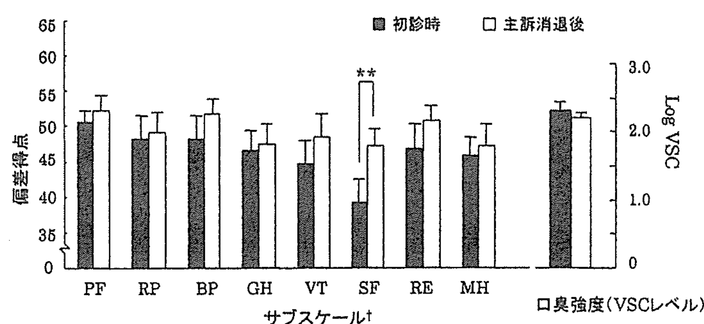

図9：口臭への訴えが改善された患者の QOL スコアと口臭強度
$^{**}p<0.01$；対応のある t 検定。
†サブスケールの略称は図8と同様
偏差得点については図8と同様。
VSC レベルは対数変換すると正規的な分布になるため，対数変換値で表記および検定した。

覚閾値（VSC で150ppb）以上の者と以下の者でのスコアの比較では有意な差は認められず，口臭による QOL の低下は客観的口臭の有無と関連しないことが示唆された。さらに，我々の診療で口臭に関する主訴が消失した者の QOL を測定すると，初診時に比べて社会生活機能（SF）のみが有意に上昇しており，口臭測定値は低下傾向にはあったものの明確な差ではなかった（図9）。この結果は，口臭測定ではとらえられない主観的変化を QOL 測定がとらえる可能性を示している。また，前項で述べたような診療を行っても，患者が明らかな主訴の改善

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。

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

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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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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

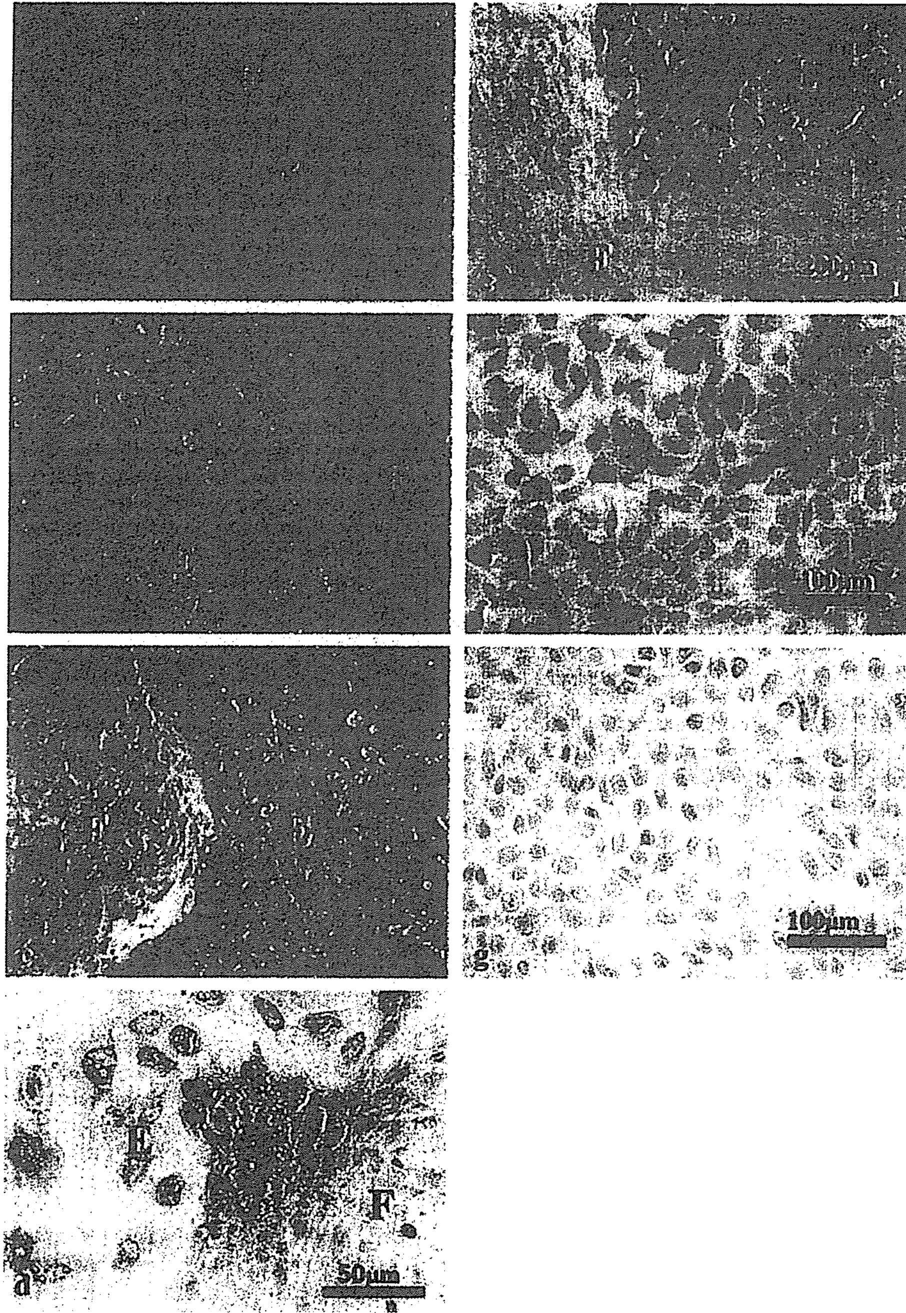

Fig. 1. (a) Phase-contrast photomicrograph of a primary coculture from human periodontal ligament. A sheet of epithelial cells (E) is covered by a ribbon (R) of fibroblasts (F). (b,c) Scanning electron micrographs of a primary cultures: (b) in the mixed multilayer, the epithelial cells (E) are seen to 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).

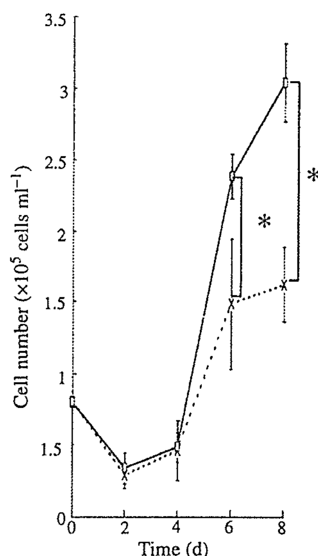

Fig. 2. Growth curves of epithelial cells from human periodontal ligament (dashed line with Xs) and gingival tissue (unbroken line with boxes). Values are the mean ± S.D. of five samples. $*P < 0.05$.

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

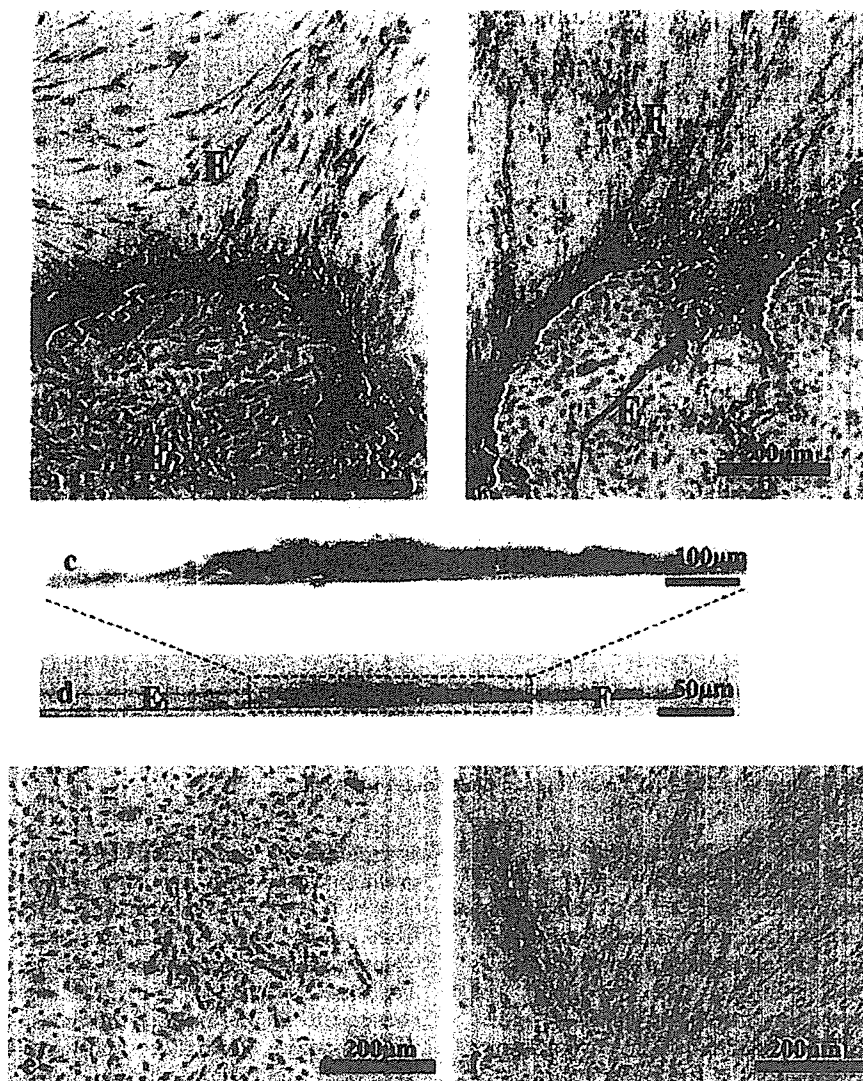

Fig. 3. (a,b) Photomicrographs showing intense immunoreactivity for type IV collagen (a) and laminin (b) between epithelial cells (E) and fibroblasts (F) from human periodontal ligament. (c,d) Photomicrographs showing intense immunoreactivity for type IV collagen at the interface between epithelial cells (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

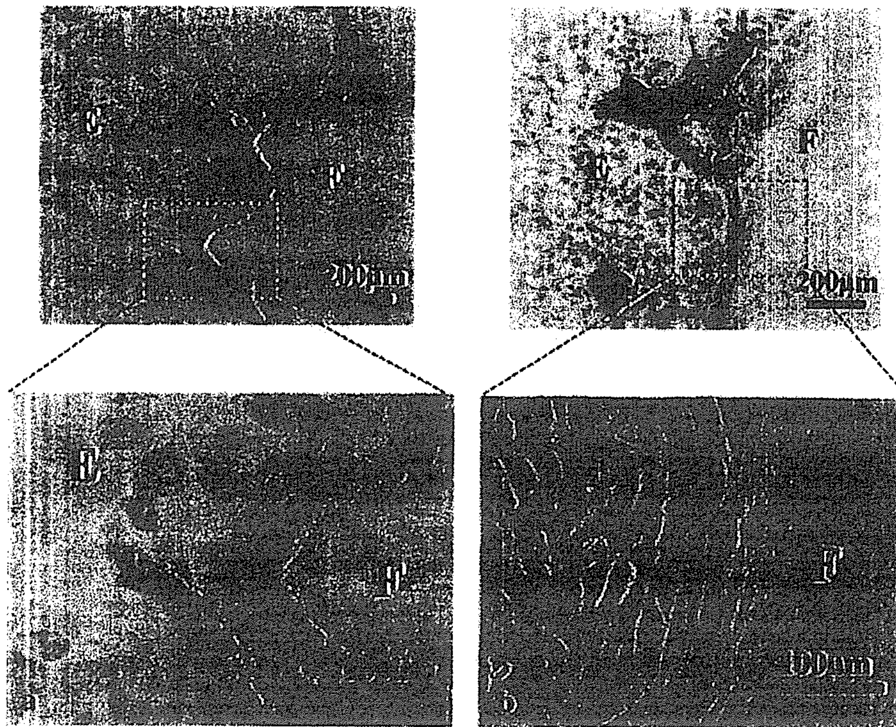

Fig. 4. Photomicrographs showing intense immunoreactivity for type IV collagen mRNA (a) and laminin mRNA (b) at the interface between epithelial cells (E) and fibroblasts (F) from human periodontal ligament.

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).

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

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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 ($P < 0{\cdot}005$) and H_2S-producing bacteria ($P < 0{\cdot}05$) 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

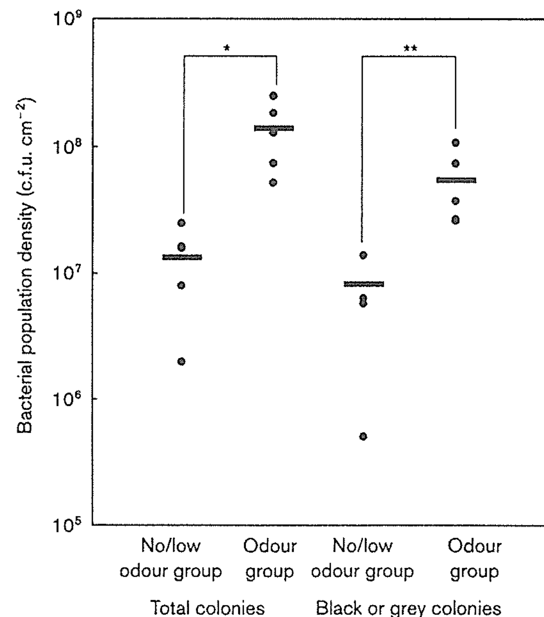

Fig. 1. Numbers of total and black or grey colonies in no/low odour and odour groups. *$P = 0·002$; **$P = 0·012$. Horizontal bars represent means.

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

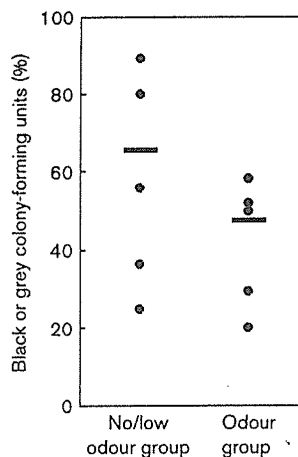

Fig. 2. Proportion of black or grey colony-forming units among total colony-forming units in no/low odour and odour groups. Horizontal bars represent means.

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.

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Oral Microbiology and Immunology

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

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Yamaura M, Sato T, Echigo S, Takahashi N. Quantification and detection of bacteria from postoperative maxillary cyst by polymerase chain reaction.
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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

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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.